I.I. Generalov

MEDICAL MICROBIOLOGY, VIROLOGY & IMMUNOLOGY

Part 1.
General Microbiology & Medical Immunology

Lecture Course
for Students of Medical Universities

VITEBSK STATE MEDICAL UNIVERSITY

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The Lecture Course on Medical Microbiology, Virology and Immunology accumulates a broad scope of data covering the most essential areas of medical microbiology. The textbook is composed according to the educational standard, plan and program, approved by Ministry of Education and Ministry of Health Care of Republic of Belarus. This edition encompasses all basic sections of the subject – General Microbiology, Medical Immunology, Medical Bacteriology and Virology. Part 1 of the Lecture Course comprises General Microbiology and Medical Immunology sections.
This book is directed for students of General Medicine faculties and Dentistry faculties of higher educational establishments.

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CONTENTS

ABBREVIATION LIST

Ab – antibody
ADCC – antibody-dependent cell-mediated cytotoxicity
AFM – atomic force microscopy
Ag – antigen
APC – antigen-presenting cell
ATP – adenosine triphosphate
BALT – bronchial-associated lymphoid tissue
BCR – B cell receptor
CALT – conjunctival-associated lymphoid tissue
cAMP – 3',5'-cyclic AMP
CAP – cyclic AMP-binding protein
Cas proteins – CRISPR-associated proteins
CD – clusters of differentiation
CDRs – complementarity-determining regions
CFU – colony-forming units
CLSI – Clinical and Laboratory Standards Institute
CRISPR – clustered regularly interspaced short palindromic repeat
CRP – C-reactive protein
DAF – decay-accelerating factor
DAMPs – damage-associated molecular patterns
DAP – diaminopimelic acid
DC – dendritic cell
Dcl – dosis certa letalis
ddNTP – dideoxy-nucleotides
DLM – dosis letalis minima
DNA – deoxyribonucleic acid
EGF – endothelial growth factor
ELISA – enzyme-linked immunosorbent assay
EPS – extracellular polymeric substance
EUCAST – European Committee on Antimicrobial Susceptibility Testing
FACS – fluorescence-activated cell sorter
FAE cells – follicule-associated epithelial cells
FITC – fluorescein isothiocyanate
FRET – fluorescence resonance energy transfer
GALT – gut-associated lymphoid tissue
G-CSF – granulocyte-colony stimulating factor
GM-CSF – granulocyte-macrophage-colony stimulating factor
GTP – guanosine triphosphate
HA – hemagglutinin
Hfr – high frequency of recombination
HIV – human immunodeficiency virus

HLA – human leukocyte antigen
IFN – interferon
IL – interleukin
ICSP – International Committee on Systematics of Prokaryotes
ICTV – International Committee on Taxonomy of Viruses
IU – International Unit
IUPAC – International Union of Pure and Applied Chemistry
LATS-factor – long acting thyroid stimulator
LPS – lipopolysaccharide
LSCM – laser scanning confocal microscopy
M cells – microfold cells
mAbs – monoclonal antibodies
MALDI-TOF analysis – matrix-assisted laser desorption ionization – time-of-flight detection analysis
MALT – mucosal-associated lymphoid tissue
MAC – membrane attack complex
MBC – minimum bactericidal concentration
MBL – mannose-binding lectin
MHC – major histocompatibility complex
MIC – minimum inhibitory concentration
MPA – meat-peptone agar
MPB – meat-peptone broth
MPS – mononuclear phagocyte system
mRNA – messenger RNA
MRSA – methicillin resistant Staphylococcus aureus
NA – neuraminidase
NALT – nasal-associated lymphoid tissue
NBT-test – nitroblue tetrazolium reduction test
NETs – neutrophil extracellular traps
NGS – next-generation sequencing
NK cell – natural killer cell
PABA – p-aminobenzoic acid
PAF – platelet-activating factor
PAGE – polyacrylamide gel electrophoresis
PAMP – pathogen-associated molecular patterns
PBP – penicillin-binding protein
PCR – polymerase chain reaction
PDGF – platelet-derived growth factor
PTFE – polytetrafluoroethylene
R genes – resistance genes
RA – rheumathoid arthritis
Rag – recombination-activating genes
Rh – rhesus factor

RIA – radioimmunoassay
RNA – ribonucleic acid
ROS – reactive oxygen species
RTF – resistance transfer factor
SARS – severe acquired respiratory syndrome
SDS – sodium dodecyle sulfate
SEM – scanning electron microscopy
SLE – systemic lupus erythematosus
SMRT sequencing – single molecule real time sequencing
SRBC – sheep red blood cell
SRID – single radial immunodiffusion
SS – secretion system
STED-microscopy – stimulated emission-depletion fluorescent microscopy
Tc – T cytotoxic cell
TCA – tricarboxylic acid
TCR – T cell receptor
TEM – transmission electron microscopy
T_{FH} – follicular T helper cell
TGF – transforming growth factor
Th – T helper cell
TLR – Toll-like receptor
TNF – tumor necrosis factor
TSST – toxic shock syndrome toxin
TST – tuberculin skin test
UV – ultraviolet
VRE – vancomycin-resistant enterococci

GENERAL MICROBIOLOGY

Chapter 1

THE SUBJECT AND BASIC FIELDS OF MODERN MICROBIOLOGY. A BRIEF HISTORICAL OVERVIEW ON MICROBIOLOGY

The Subject and Main Tasks of Microbiology

The microbiology has grown up into independent scientific discipline from the more aged science, biology. Any branch of knowledge is regarded as a science, if it operates with the own subject of study and has its intrinsic methods of research.

The subject of microbiology *is the special world of living beings invisible by naked eye which sizes are within the range from 1-10 nm up to 0.1-1 mm.*

Microorganisms comprise several diverse groups of agents – ***bacteria***, ***viruses***, ***protozoans***, ***fungi***, and ***prions*** (the latter are known as "proteinaceus infectious particles" or infectious proteins).

The title of the science "*microbiology*" originates from combination of Greek words – "*micros*" – minute, "*bios*" – the life, "*logos*" – the science.

Thus, ***microbiology*** *is the science that studies the life and development of smallest living creatures – microorganisms – together with their complex environmental relationships.*

Taking into account the tremendous role the microorganisms play in nature, the problems of microbiology are rather diverse. Microbiology steadily differentiates into various scientific parts and disciplines.

Modern microbiology includes ***general*** microbiology (investigates common principles of structural organization and general functions of microorganisms), ***special*** microbiology (performs the detailed study of certain microbial agents and groups); ***industrial*** microbiology that is the major part of modern ***biotechnology***; ***agricultural*** microbiology; ***space*** microbiology; ***sanitary*** microbiology; ***veterinary*** microbiology; and ***medical*** microbiology.

The ***subject of medical microbiology*** encompasses *pathogenic microorganisms that cause the diseases in humans and those non-pathogenic microorganisms – inhabitants of living beings or external environment – that can influence human health*.

The ***main goals of medical microbiology***:

1. *Laboratory diagnosis of diseases caused by microorganisms* by means of versatile microbiological methods; the detection of pathogenic microbial agents in living organisms and external environment.
2. *Sanitary control of microbial pollution* of water, air, soil, house, foodstuffs, drugs, etc.
3. *The development of biological products for medicine* (antibiotics, vaccines, immune sera, polyclonal and monoclonal antibodies, cytokines and others) that are used for prophylaxis and treatment of bacterial, viral, fungal and protozoan diseases; autoimmune and inflammatory disorders.

A Brief Historical Overview on Microbiology

At previous times many greatest minds of the world tried to solve the mystery of infectious diseases origin. Outstanding scientists of antiquity Hippocrates, Lucretius, Galen and their followers supposed the minute living organisms as possible causative agents of contagious diseases. Later this idea was shared by investigators of Middle Ages (G. Fracastoro, T. Sidenham) but it remained to be out of direct evidences.

Nevertheless, industry development promoted the progress of science and technique. At the beginning of XVII century famous physicist Galileo Galilei improved the previously invented magnifying glasses and constructed the first simple microscope. Then C. Drebbel devised the first double-lens microscope with optical system, composed of the convex objective and eyepiece. And finally Dutch drapery merchant Antony van Leeuwenhoek, the person without university education but with a great taste for knowledge, discovered the microbial world.

A. van Leeuwenhoek made the microscopes with magnification up to 300 times and investigated a great number of different objects and substances, including living tissues. He revealed tissue cell structure, described blood capillaries, erythrocytes, spermatozoids, plant germs, but the discovery of microbes – *animalculi viva* – added his name to the row of the most prominent minds in the history of natural science.

Leeuwenhoek sent more than 150 letters to the Royal Society in London, Great Britain, where he described all basic morphological forms of bacteria. In 1695 he published the work “The Secrets of Nature Discovered by Antony Leeuwenhoek” with the main results of his experiments.

From Leeuwenhoek's discoveries the ***first scientific period*** in microbiology (sometimes termed as *morphologic*) has started. His findings stimulated further studies of microorganisms. Unfortunately, other scientists for a long period of time were not able to reproduce completely Leeuwenhoek's results mainly due to microscopy technique imperfection. Nevertheless, in 1839 J. Shoenlein has found the causative agent of favus, in 1849 A. Pollender, C. Davaine and F. Brauell revealed the anthrax bacillus.

Another discovery of tremendous importance was made by E. Jenner (1749-1823), who proved the principle of specific prophylaxis of infectious diseases. He has found that inoculation of cowpox material to humans protected them from the smallpox. E. Jenner termed this manipulation as ***vaccination*** (Lat. "*vacca*" – cow). Vaccination appeared to be the most universal procedure to protect humans and animals against infectious diseases of different origin.

The rise of the ***second period of microbiology*** (also known as *physiological* and *immunological*) is related with the investigations of outstanding researchers Louis Pasteur and Robert Koch.

Great French scientist Louis Pasteur (1822-1895) is known in the history of science as "the father of modern microbiology". He has been trained as a chemist, thus he applied chemical approach to microbiology problems.

Many works of L. Pasteur have been started from the requests of French manufacturers. They applied for his help to overcome numerous difficulties appeared in various industrial processes.

L. Pasteur thoroughly investigated the diseases of wine, beer, silkworms and revealed that many processes of unknown origin or supposed to be of chemical nature are caused by various microorganisms. He successfully proved the microbial origin of putrefaction, alcoholic, lactic and butyric acid fermentations. He discovered a new anaerobic type of respiration in bacteria.

About 1860 he demonstrated that heating could inactivate microorganisms in wine and beer. This process later termed as ***pasteurization*** (heat killing of microbes at moderate temperatures that reduce the total number of live microorganisms) now is used everywhere for dairy products decontamination.

With the aid of a specially designed "swan-necked" flask L. Pasteur has proved the *impossibility of spontaneous generation of living matter*. After boiling the liquid content in the flask remained sterile being prevented from direct contact with atmosphere air that contains live

microbial bodies. As the result of this elegant and persuasive experiment L. Pasteur established the ***germ theory of diseases*** postulating microorganisms as essential causes of infectious disorders.

Pasteur improved significantly the development of vaccines. He demonstrated that the inoculation of bacteria with decreased virulence (***attenuated microorganisms***) is highly effective for specific prophylaxis of infectious diseases. L. Pasteur successfully created the vaccine against chicken cholera. The same approach he used to develop the attenuated vaccine against anthrax. And finally Pasteur achieved the great success creating the vaccine against rabies. Since that time the vaccine prophylaxis became a powerful weapon against many threatening infectious diseases.

L. Pasteur founded the prominent scientific school of investigators in the field of microbiology and immunology. The most outstanding scientists of XIX-XX century were the followers of Pasteur: J. Bordet, A. Yersin, E. Roux, F. d'Herelle, A. Calmette and C. Guerin, G. Ramon, Ch. Nicolle, and many others. Famous Russian scientists E. Metchnikoff and S. Vinogradsky also worked at the Pasteur Institute in Paris.

Great German scientist Robert Koch (1843-1910) created another world-famous school in microbiology.

First of all, Robert Koch and co-workers designed a large number of new methods and introduced them into microbiology practice. R. Koch obtained bacteria in pure culture. The scientists used solid nutrient media, e.g. meat-peptone agar, gelatin or coagulated serum for bacterial cultivation. Also they improved microscopy technique. E. Abbe invented special microscope lenses that corrected the aberrations in magnifying lenses. Then the oil immersion lens was applied which allowed the improved resolution in light microscopy.

Paul Ehrlich proposed the staining of bacteria with aniline dyes (e.g., methylene blue) that enhanced the discriminative power of light microscopy. Further in 1884 Hans Christian Gram developed a differential staining method for bacteria. The Gram stain technique revealed the difference between two basic variations in bacterial cell wall structure. The latter is used in microbial classification.

Also the microbiologists began to exploit microphotography for bacterial visualization.

Finally in 1876 R. Koch confirmed microbial etiology of anthrax, investigated thoroughly the purulent wound infections, discovered and isolated the causative agents of tuberculosis (1882) and cholera (1883). He derived tuberculin from tubercle bacilli and tried to use it for tuberculosis

treatment. Unfortunately, these first attempts appeared to be unsuccessful, but tuberculin is applied in medicine now for tuberculosis diagnostics.

R. Koch and his mentor and friend F. J. Genle proposed the basic postulates to determine the infectious origin of the diseases. These principles became known as "***Genle-Koch's triad***". They were derived from Koch's work on infectious diseases such as anthrax and tuberculosis. Koch's postulates were presented in Berlin in 1890 as follows:

– the parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease;

– the parasite occurs in no other disease as a fortuitous and nonpathogenic parasite;

– after being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

If these three conditions were confirmed, Koch asserted, ''…the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered".

Later a fourth postulate was added, consisting of a requirement to re-isolate the causative microbe from the experimentally inoculated host.

The limitations of Koch's postulates were evident even at the beginning of XX century (e.g. viruses, or some protozoa as *Plasmodium falciparum* cannot be grown on nutrient media; several microbial agents have a host range that is restricted only to humans, etc). These discrepancies became more pronounced today after discovery of almost non-cultivable microorganisms (e.g. herpes virus type 8 – causative agent of Kaposi's sarcoma, or *Tropheryma whipplei*).

Nevertheless, Koch's principles were undoubtedly useful, because they emphasized a specific association of the microbe with particular infectious disease.

R.Koch founded an outstanding school in microbiology. Among his disciples were F. Loeffler, S. Kitasato, E. Klebs, K. Eberth, G. Gaffky, and many others.

The discoveries made by Pasteur and Koch opened the new horizons in microbiology and indisputably determined the causative role of bacteria in many human diseases.

Almost at the same time the investigation of intrinsic mechanisms of host defensive reactions against various pathogens has started. The works of great Russian scientist Ilya Metchnikoff (1845-1916) and outstanding

German researcher Paul Ehrlich (1854-1915) elucidated two basic routes of body self-defense – cellular and humoral immune response.

I.I. Metchnikoff, who started his scientific carrier as zoologist, discovered and investigated the process of foreign agents digestion by specialized host cells. In 1882 he revealed that mesodermal cells of starfish larvae could migrate to the area of inflammation, and then engulf and digest the invaded agents. I.I. Metchnikoff called these mobile cells "***phagocytes***". It was demonstrated further that other types of cells, including blood leukocytes, possessed the same activity. Also he has found these cells to participate in inflammatory reactions against the infectious agents. Thus he proved the inflammation as an active defensive reaction of the host.

As the result of these experiments, I.I. Metchnikoff created the *phagocytic theory of immunity* in 1883-1884. It has become the basis for the field of cellular immunology for many years.

In 1890 Robert Koch's co-workers, E. von Behring and S. Kitasato, discovered the specific substances of blood sera which were able to neutralize microbial toxins. The scientists called them "***antitoxins***". Small doses injections of laboratory animals with tetanus or diphtheria toxins stimulated serum antitoxin production that ultimately protected the animals against toxin action.

E. Behring and S. Kitasato, as well as E. Roux in the Pasteur Institute obtained anti-diphtheric and anti-tetanus sera and applied them for successful treatment of these severe diseases.

On the basis of these results in 1891 Paul Ehrlich propounded the *theory of humoral immunity*. He differentiated the active and passive humoral immune reactions. And finally he suggested that antitoxins and other specific defensive substances are the receptor products of some cells, specific to foreign agent. This fruitful idea was extremely important for the development of *cell receptor theory*.

Numerous experiments followed by fierce discussions of the supporters of cellular and humoral theories of immunity proved the rightness of both scientific trends. In 1908 Metchnikoff and Ehrlich were awarded the Nobel Prize for their contribution to the science of immunity.

Remarkable achievements in the field of microbiology and immunology clarified complex host-pathogen interactions appeared in the course of infection. These results made possible to develop versatile antimicrobial measures for successful control of infectious diseases.

As long far as in the 1840s Hungarian physician Ignaz Semmelweis proposed first efficient measures (hand wash, clear linen use, isolation of

patients, etc.) to prevent infection spread. This approach is called now ***asepsis***. Later in 1860s, an English surgeon Joseph Lister worked out the principles of ***antisepsis***. He used chemicals to kill microorganisms, thus preventing the microbial contamination of surgical wounds. At the first time he introduced chemical antiseptics (e.g. phenol or carbolic acid) to the medical practice.

First successful attempt to synthesize specific antimicrobial drug was performed by P. Ehrlich, who obtained arsenic-containing compound salvarsan. Salvarsan was shown to be effective against syphilis.

Broad class of potent antimicrobials was discovered by Gerhardt Domagk and co-workers in 1935. They synthesized sulphonamides and proved them to possess the high activity against various groups of bacteria.

In 1928 English scientist Alexander Fleming made another discovery of tremendous importance in the field of microbiology. He revealed the remarkable ability of a *Penicillium* species moulds to produce substance that killed pathogenic bacteria with the greatest efficacy. He called this substance ***penicillin*** and the substances with the same activity were further called ***antibiotics***. Fleming was not able to obtain the pure and stable penicillin preparation. It was done further by H. Florey and E. Chain during the years of the World War II. Since that time the modern era of chemotherapy has started. Very soon A. Schatz and S. Waksman found the first effective antibiotic streptomycin for tuberculosis treatment.

Thus antibiotics became the extremely powerful tool to combat against infectious diseases. The search for new effective antibiotics is in great progress now.

Further advance of microbiology has required new deep investigations of microbial metabolism and microbial ecology.

Russian scientist S. Vinogradsky and Dutch researcher M. Beijerinck has revealed and definitively proved the tremendous role of bacteria in the continuous turnover of chemical elements that takes place on the Earth. S. Vinogradsky discovered the ***nitrifying bacteria*** which convert inorganic ammonium ions to nitrite and nitrate anions. Also he proved the ability of some bacteria to gain carbon from carbon dioxide, thus converting it into organic form. M. Beijerinck described the ***nitrogen fixating bacteria*** that can utilize atmospheric nitrogen. Bacterial fixing of atmospheric nitrogen provides its further use by other living organisms (plants, animals etc.)

Numerous aspects of bacterial cellular metabolism were investigated as well. Since the middle of XX century the burst growth of research in this field opened the new era in microbiology. Sometimes it is termed as

molecular genetic period of microbiology, taking into account the great role of genetics and molecular biology in the extraordinarily remarkable progress of modern microbiology.

Bacteria and viruses appeared to be the most suitable objects for genetic manipulations due to their haploid genome, relatively simple structure, high reproduction rate, low cost and convenience of cultivation. Thus it is not surprising that most outstanding discoveries in genetic science were made on bacterial and viral models.

First of all, the cell DNA was proven to be the hereditary molecule that encodes genetic information. It has been received from the experiments of English scientist F. Griffith in 1928. F. Griffith described the transformation of non-pathogenic non-capsulated *S. pneumoniae* strain into the pathogenic encapsulated variant. He regarded pneumococcal capsule as putative transforming agent. However, correct interpretation of these experiments was made by O. Avery, M. MacLeod and C. McCarty in 1941. They discovered the DNA to be the only possible hereditary molecule.

At the same year G. Beadle and E. Tatum have found that separate DNA parts (called as ***genes***) govern biochemical activities of bacteria and fungi, coding for the proteins with specific functions.

These results stimulated further DNA investigation, and in 1953 J. Watson and F. Crick established the double-helical structure of DNA molecule. Then F. Crick proposed the triplet organization of protein-coding function of DNA, where three bases in DNA sequence encode one amino acid in corresponding protein.

In 1961 F. Jacob and J. Monod discovered messenger RNA first in bacteria and then in eukaryotic organisms. And finally they determined the basic functional unit of bacterial genomic organization and called it **"*operon*"**. Using *E. coli* model they discovered that operon comprises *regulatory gene*, *operator gene*, and *structural genes*. These gene assemblage works in concerted manner, governing the seria of metabolic reactions.

Deep studies of molecular events of bacterial metabolism resulted in the discoveries of basic biochemical pathways common to most bacteria. H. Krebs in 1953 revealed citric acid cycle, M. Calvin in 1961 clarified carbon dioxide fixation in bacteria during photosynthesis, and finally P. Mitchell worked out membrane chemiosmotic theory of energy gain in living organisms.

Significant achievements of molecular methods in microbiology created new promising perspectives for laboratory diagnosis, prophylaxis

and treatment of infectious diseases. ***Nucleic acid-based assays*** (e.g., nucleic acid hybridization) appeared to be extremely sensitive and specific ensuring rapid high-sensitive diagnosis of infections.

Invention of ***polymerase chain reaction*** (***PCR***) by K. Mullis in early 80^{th} of XX century revolutionized molecular diagnostics. PCR raised the sensitivity of genetic methods up to detection of several molecules of nucleic acid in tested sample. This method made possible the investigation of viable but non-culturable bacteria and viruses.

In the same vein, current advances in biology and medicine are closely related with remarkable progress of ***genetic engineering***. It uses methods of molecular chemistry and microbiology to create recombinant DNA molecules that encode protein products with predicted activities. Nowadays recombinant technologies are applied intensively into design of new vaccines and many other biological substances for disease prophylaxis and treatment (cytokines, humanized monoclonal antibodies, etc.) First positive results in ***gene therapy*** evoked the great expectations of successful treatment of the diseases considered to be incurable.

Finally, the least but not last radical changes happening now in modern microbiology were stimulated greatly by new technologies of ***DNA microarray*** analysis and ***nucleic acid sequencing***. For instance, practical implementation of ***next-generation sequencing methods*** (known as "***deep***" and "***ultra-deep sequencing***") allowed to trace numerous individual variations within large microbial communities. Taken together they generated the ways to control the state of ***microbiome*** – total number of microbial cells (and their genes) that inhabit human body.

Outstanding progress of microbiology, immunology and genetics, the impressing achievements of antimicrobial chemotherapy substantially decreased the mortality rate in infectious diseases and slowed down their global spread.

Nevertheless, infectious diseases remain to be the leading cause of people death especially in developing countries. Wide spread of multi-drug microbial resistance, appearance of novel extremely dangerous infections (e.g. HIV-infection or severe acquired respiratory syndrome – SARS), the return of some life-threatening diseases earlier supposed to be under the tight control, such as tuberculosis, rapid spread of hospital-acquired infections are considered to be the most evident menaces that will challenge the humans in XXI century. Only united efforts of public authorities, scientists and physicians all over the world can help to solve these serious problems.

Chapter 2

PRINCIPLES OF MICROBIAL CLASSIFICATION. MORPHOLOGY AND ULTRASTRUCTURE OF BACTERIA. BASIC METHODS FOR EXAMINATION OF MICROBIAL MORPHOLOGY

Principles of Microbial Classification

Modern nomenclature and ***classification schemes for bacteria*** are elaborated under the guidance of *International Committee on Systematics of Prokaryotes* (ICSP). The ICSP summarizes all the data of current bacterial classification within *International Code of Nomenclature of Bacteria* and publishes International Journal of Systematic and Evolutionary Microbiology, where the last changes of bacterial taxonomy are indicated.

The existing principles of bacterial classification as well as the detailed descriptions of all bacterial taxa are also given in second edition of *Bergey's Manual of Systematic Bacteriology* (published in 2001-2012). It is worthy to note that first publication of the manual of determinative bacteriology was prepared by US bacteriologist D.H. Bergey as far as 1923. Current version of Bergey's Manual comprises an immense scope of data of all known bacterial representatives.

Modern ***classification of viruses*** is performed by *International Committee on Taxonomy of Viruses* (ICTV). In contrast to any other biological objects, the ICTV states that "nomenclature of viruses is independent of other biological nomenclature".

Several basic principles are employed for microbial taxonomy.

Numerical taxonomy (also known as *computer taxonomy*, or phenetics) was introduced into microbiological practice from late 1950s. Numerical classification schemes use a large number of taxonomically useful phenotypic characteristics (usually 100-200 or even more). Among them are morphological, cultural, biochemical, antigenic, and many other microbial features.

The process of identification discriminates bacterial strains at defined levels of their overall similarity that results from the frequency of their common traits (for instance, more than 80% of similarity at the species level)

Following the advances of molecular genetics, *molecular-based methods*, especially ***genotyping***, created new opportunities for bacterial taxonomy.

Genetic-based taxonomy plays now a pivotal role in the process of identification of unknown microbial representative.

According to genetic-based scheme, *bacterial identification at species level* is made by *molecular hybridization* analysis. Genomic DNA of tested bacterial strain undergoes hybridization with DNA of bacteria that are typical for certain species (species-specific strains). If the level of DNA similarity between the bacterial strains (*DNA relatedness*) exceeds >70%, the tested bacteria can be accounted as members of the same species.

The ranks of bacterial classification *from genus and above* (family, order, etc.) are established on the base of ***sequence of 16S ribosomal RNA genes***.

It has been found that genes encoding ribosomal RNAs and ribosomal proteins are highly conserved throughout evolution and they diverged more slowly than other chromosomal genes. Comparison of the nucleotide sequence of *16S ribosomal RNA* from various microbial groups demonstrates evolutionary relationships among broadly divergent microorganisms (***phylogenetic taxonomy***). As an example, it has led to separation of two distinct domains *Bacteria* and *Archaea* from primary domain *Prokaryota*.

Nevertheless, despite outstanding achievements of genetic-based taxonomy, a lot of questionable situations in microbial classification cannot be resolved solely on the ground of genetic methods. By fact, this is clearly evident for closely related bacterial species. Many of them are of great medical relevance. For instance, *Bordetella pertussis* and *Bordetella parapertussis*; *E. coli* and shigellae; *Yersinia pestis* and other yersiniae; bacterial species from genus *Brucella* share DNA similarity >80-90%. However, these bacteria are distinct by many phenotypic traits especially in their virulence for humans. Thus, they remain placed into separate bacterial species.

In order to make numerical and genetic-based taxonomy consistent with existing laboratory and clinical data the *concept of* ***polyphasic taxonomy*** is generally adopted for current microbial classification. Here the identification of bacterial species is performed on the base of genetic analysis but in combination with the most important phenotypic characteristics.

The value of this universal approach becomes evident in the light of recent inventions of high-throughput one-step tests for bacterial species

identification. Among them are the methods based on mass spectrometry of bacterial chemical components (eg, matrix-assisted laser desorption ionization–time-of-flight detection or *MALDI-TOF analysis*), whole cell fatty acid analysis and others. Such tests generate the huge array of data about chemical composition of investigated bacterial culture. These individual chemical patterns are the unique characteristics of any microbial representative. Being compared with the known data from microbiological computer databases the results of these tests provide rapid and precise identification of bacterial isolates, strains and species.

Modern Classification Scheme of Microorganisms

Now all cellular forms of living world are grouped into major clusters known as *domains*. ***Domain*** *is the highest taxon* of current biological classification.

There are ***3 domains***: *Bacteria, Archaea, Eukarya*.

Domains *Bacteria* and *Archaea* comprise microbial agents *without cell nucleus* (***prokaryotic*** domains).

Domains ***Bacteria*** and ***Archaea*** are divided into ***phyla***. Domain *Bacteria* now includes 23 bacterial phyla. Further division of main bacterial phyla is summarized in Table 1.

Domain ***Eukarya*** (or ***Eukaryota***) contains biological organisms *with cellular nucleus*. Until quite recently this domain was divided into ***kingdoms***: *Fungi, Protista, Cromista, Plantae,* and *Animalia*.

Kingdom *Fungi* encompasses 7 ***phyla***. The phyla *Ascomycota* and *Basidiomycota* harbor the certain fungal agents that cause the disorders in humans. The members of phyla *Ascomycota* and *Basidiomycota* are separately placed into ***sub-kingdom*** *Dikarya* as the fungi with sexual reproduction and *dicaryon* formation.

The most entangled is the situation with protozoan taxonomy. Protozoa were primarily placed into separate kingdom *Protista*. In 2005 International Society of Protistologists proposed to re-classify all the domain *Eukarya* on monophyletic principle. ***Monophyletic principle*** presumes hierarchic division of biological objects originated from *common ancestor* (*phylogenetic tree*). Phylogenetic relationships (*distances*) between various groups of organisms are determined by molecular genetic methods. Last revision of new classification was published in 2012.

On this ground, the whole domain of *Eukarya* was re-exposed as 5 great genetic clusters termed as ***super-groups*** (or genetic ***clades***).

Former kingdom *Protista* was proven to be *polyphyletic*. Thus, various groups of protozoans were spread throughout clades *Alveolata* (with phyla *Apicomplexa* and *Ciliophora*), *Excavata* (phylum *Metamonada*), and *Amoebozoa*.

As the result of rapid progress of molecular genetic analysis, this division is not stable yet and can be regarded as provisional. Following the gain of new experimental data, this classification should be re-ascertained.

Subsequent more low taxonomy units for bacteria are: ***class***, ***order***, ***family***, ***genus***, and ***species***.

Species is the most common unit in microbial taxonomy. In contrast with higher organisms, bacterial species is *unstable category*. It is the subject of permanent evolution resulting in rapid environmental adaptation of bacteria. Species ability of easy alterations is largely based on *lateral gene transfer* that occurs not only among related bacterial species but also between distant microbial groups (genera or families).

The correct definition of a species is always difficult problem in microbial classification, thereby many variations of species definition were proposed.

In medical microbiology ***species*** presumes *the populations of microorganisms of the common origin with closely related genotypes, properties and area of habitation, which possess genetically fixed ability to induce similar reactions in the affected organism or in the external environment.*

For microbial designation the ***binominal nomenclature*** is accepted where each species has a generic and a specific name. The generic name is written in italicized upper case, and the specific name – in lower case. For instance, pathogenic staphylococcus of golden color is called *Staphylococcus aureus*, the anthrax bacillus – *Bacillus anthracis*, the diphtheria causative agent – *Corynebacterium diphtheriae*, etc.

If the differences with the typical species characteristics are found on examination of isolated bacteria, the culture is regarded as *subspecies*. Subspecies is the lowest taxonomic rank in official nomenclature.

The term ***strain*** designates *a microbial population of the same species isolated from different sources, or even from the same source, but in different period of time*.

Strains are identified by all properties, thoroughly described, acquire legend with number, date and site of isolation and placed into strain collection. Usually the members of the same strain demonstrate homology of genomic DNA more than 95%.

Table 1. Modern taxonomy of Bacteria

Phylum B XII. Proteobacteria

Class I. Alphaproteobacteria

Order II. Rickettsiales
Family I. Rickettsiaceae
Genus I. Rickettsia
Genus II. Orientia
Genus III. Wolbachia
Family II. Ehrlichiaceae
Genus I. Ehrlichia
Order VI. Rhizobiales
Family II. Bartonellaceae
Genus I. Bartonella
Family III. Brucellaceae
Genus I. Brucella

Class II. Betaproteobacteria Order

I. Burkholderiales
Family I. Burkholderiaceae
Genus I. Burkholderia
Family IV. Alcaligenaceae
Genus I. Alcaligenes
Род III.Bordetella
Order IV. Neisseriales
Family I. Neisseriaceae
Genus I. Neisseria
Genus VI. Eikenella
Genus Kingella
Order V. Nitrozomonadales
Family II. Spirillaceae
Genus I. Spirillum

Class III. Gammaproteobacteria

Order V. Thiotrichales
Family III. Francisellaceae
Genus I. Francisella
Order VI. Legionellales
Family I. Legionellaceae
Genus I. Legionella
Family II. Coxiellaceae
Genus I. Coxiella
Order IX. Pseudomonadales
Family I. Pseudomonadaceae
Genus I. Pseudomonas
Family II. Moraxellaceae
Genus I. Moraxella
Genus II. Acinetobacter
Order XI. Vibrionales
Family I. Vibrionaceae

Genus I. Vibrio
Order XII. Aeromonadales
Family I. Aeromonadaceae
Genus I. Aeromonas
Order XIII. Enterobacteriales ***Family*** I. Enterobacteriaceae
Genus I. Enterobacter
Genus VIII. Calymmatobacterium
Genus X. Citrobacter
Genus XI. Edwardsiella
Genus XII. Erwinia
Genus XIII Escherichia
Genus XV. Hafnia
Genus XVI. Klebsiella
Genus XVII. Kluyvera
Genus XXI. Morganella
Genus XXVI. Plesiomonas
Genus XXVIII. Proteus
Genus XXIX. Providencia
Genus XXXII. Salmonella
Genus XXXIII. Serratia
Genus XXXIV. Shigella
Genus XL. Yersinia
Order IV. Pasteurellales
Family I. Pasteurellaceae
Genus I. Pasteurella
Genus II. Actinobacillus
Genus III. Haemophilus

Class V Epsilonproteobacteria

Order I. Campylobacteriales
Family I. Campylobacteriaceae
Genus I. Campylobacter
Family II. Helicobacteriaceae
Genus I. Helicobacter
Genus II Wolinella

Phylum B XIII. Firmicutes

Class I Clostridia

Order I. Clostridiales
Family I. Clostridiaceae
Genus I. Clostridium

Genus IX. Sarcina
Family III. Peptostreptococcaceae
Genus I. Peptostreptococcus
Family V. Peptococcaceae
Genus I. Peptococcus
Family VII. Acidaminococcaceae
Genus XIV. Veillonella

Class II. Mollicutes

Order I. Mycoplasmatales
Family I. Mycoplasmataceae
Genus I. Mycoplasma
Genus IV. Ureaplasma

Class III. Bacilli

Order I. Bacillales
Family I. Bacillaceae
Genus I. Bacillus
Family II. Planococcaceae
Genus I. Planococcus
Genus IV. Sporosarcina
Family IV. Listeriaceae
Genus I. Listeria
Family V. Staphylococcaceae
Genus I. Staphylococcus
Genus II. Gemella
Order II. Lactobacillales
Family I. Lactobacillaceae
Genus I. Lactobacillus
Genus III. Pediococcus
Family II. Aerococcaceae
Genus I. Aerococcus
Family IV. Enterococcaceae
Genus I. Enterococcus
Family V. Leuconostocaceae
Genus I. Leuconostoc
Family VI. Streptococcaceae
Genus I. Streptococcus
Genus II. Lactococcus

Phylum B XIV. Actinobacteria

Class I. Actinobacteria

Subclass V. Actinobacteridae **Order** I. Actinomycetales
Sub-order V. Actinomycineae
Family I. Actinomycetaceae
Genus I. Actinomyces
Genus IV. Mobiluncus
Sub-order VI. Micrococcineae
Family I. Micrococcaceae
Genus I. Micrococcus
Genus VII. Stomatococcus
Sub-order VII. Corynebacterineae
Family I. Corynebacteriaceae
Genus I. Corynebacterium
Family II. Mycobacteriaceae
Genus IV. Mycobacterium
Family V. Nocardiaceae
Genus I. Nocardia
Sub-order VIII. Propionibacterineae
Family I. Propionibacteriaceae
Genus I. Propionibacterium
Order II. Bifidobacteriales
Family I. Bifidobacteriaceae
Genus I. Bifidobacterium
Genus III. Gardnerella

Phylum B XVI. Chlamydiae

Class I. Chlamydiae

Order I. Chlamydiales
Family I. Chlamydiaceae
Genus I. Chlamydia
Genus II. Chlamydophila

Phylum B XVII. Spirochaetes

Class I. Spirochairtes

Order I. Spirochaetales
Family I. Spirochaetaceae
Genus I. Spirochaeta
Genus II. Borrelia
Genus IX. Treponema
Family III. Leptospiraceae
Genus II. Leptospira

Phylum B XX. Bacteroidetes

Class I. Bacteroidetes

Order I. Bacteroidales
Family I. Bacteroidaceae
Genus I. Bacteroides
Family III. Porphyromonadaceae
Genus I. Porphyromonas
Family IV. Prevotellaceae
Genus I. Prevotella

If the description is not completed, the strain is termed as bacterial ***isolate*** (clinical or environmental).

Other *infra-species subdivisions* in bacteria are known as *variants* (suffix ***–var*** for short). Usually they are beyond the lines of official classification indicating some differences in microbial properties: antigenic – *serovar (syn. serotype)*, morphological – *morphovar*, chemical – *chemovar*, biochemical or physiological – *biovar*, pathogenic – *pathovar*, bacteriophage susceptibility – *phagovar*, etc.

Morphology of Bacteria

Bacteria (Gk. *bakterion* – small stick) are predominantly unicellular organisms. The size of bacteria varies from 0.1 to 20 μm or more. Most of pathogenic bacteria are within the range 0.2 to 10 μm.

The shapes and sizes of microbes are not strictly stable. They get adaptation to the surroundings and environmental conditions. But in constant situations bacteria maintain their sizes and shapes that are specific characteristics for certain microbial groups.

Bacteria demonstrate several *basic morphological forms*. Among them are ***spherical*** round-shaped cells (or ***cocci***); ***rod-shaped*** (*bacteria*, *bacilli*, and *clostridia*); ***coiled*** or spiral forms (*vibrios*, *spirilla*, *spirochetes*); ***filamentous*** and branched bacteria.

Spherical Bacteria or Cocci

Cocci (Gk. *kokkos* – berry) are round-shaped bacterial forms. They can be spherical, ellipse-, bean- or lancet-like. Cocci are further arranged into six main groups according to number of cells in clusters, planes of cellular division, their common biological features, etc.

1. ***Micrococci***. These round cells are placed separately, singly and irregularly.

2. ***Diplococci*** (Gk. *diplos* – double) are divided within one plane being attached in *pairs*. Among them are pathogenic *Neisseriae*: *meningococci* – causative agents of cerebrospinal meningitis; and *gonococci* – causative agents of gonorrhoea and ophthalmia neonatorum (or blennorrhoea).

3. ***Streptococci*** (Gk. *streptos* – curved) are divided in one plane making long or short *chains*. Many of them are pathogenic for humans causing suppurative infections, pneumonia, caries.

4. ***Tetracocci*** (Gk. *tetra* – four) are reproduced within two planes at right angles making *clusters of four* cocci. They are non-pathogenic.

5. ***Sarcinae*** are divided in three perpendicular planes at right angles producing *packages of 8-16* or more cells. They can be present as normal habitants in air.

6. ***Staphylococci*** (Gk. *staphyle* – grape) are irregularly divided within several planes producing variable cell clusters; the latter in most cases resemble *grapes*. Various species of staphylococci cause suppurative diseases in humans.

Rod-shaped Bacteria

Rod-like bacterial forms comprise ***bacteria***, ***bacilli***, and ***clostridia***.

Bacteria are rod-like microorganisms that don't produce spores. Among them are all enterobacteria, corynebacteria, bacteroids, fusobacteria and many others.

Bacilli and ***clostridia*** are the bacteria that produce spores.

Bacilli cells contain spore that don't exceed the width of microbial cell (e.g., causative agent of anthrax), whereas ***clostridiae*** spores protrude out of microbial body (causative agents of tetanus, botulism, gas gangrene, etc.)

Rod-shaped bacteria vary by their forms and sizes. There are short or middle-size rods (eg, enterobacteria, bordetella or whooping cough agent and others); some bacteria are long (such as anthrax bacilli). Most of bacteria demonstrate blunt ends, certain species (eg, fusobacteria) carry tapered ends.

Bacteria that occur in pairs are ***diplobacteria*** (eg, *Klebsiella pneumoniae*) or ***diplobacilli*** (if they contain spore).

Streptobacteria and ***streptobacilli*** make chains of various length (*B. anthracis*).

Nevertheless, vast number of bacteria, bacilli or clostridia are separated one from another without regular pattern.

Coiled or Spiral Bacterial Forms

Vibrios, spirilla and spirochetes pertain to this group of bacteria

1. ***Vibrios*** (Lat. *vibrio* – to vibrate) are the curved rods that make half a coil and look like *comma*.

Typical representative here is *Vibrio cholerae* – causitive agent of cholera.

2. ***Spirilla*** (Lat. *spira* – coil) are cork-screw-like coiled bacteria with twists of one or more turns. Pathogenic *Helicobacter pylori* belongs to spirilla.

3. ***Spirochetes*** are twisted forms of bacteria exhibiting multiple compacted twists with many turns. Pathogenic *Treponemas*, *Borreliae*, and *Leptospirae* pertain to spirochetes.

Filamentous Bacteria

Filamentous and branched bacteria exhibit long and thin thread-like microbial cells. Typical representatives of thread-like bacteria are actinomycetes. Tangled mixture of their cells produce complex interwoven structure known as *mycelium*.

Ultra-Structure of Bacteria

Bacteria as prokaryotes are greatly different from eukaryotic cells in their structure.

All bacterial cells possess *obligate* and *optional* (non-obligate) structural components.

Minimal essential number of ***bacterial obligate components*** encompasses nucleoid, cytoplasm, ribosomes and cytoplasmic membrane as part of bacterial envelope.

Non-obligate ***optional*** components display remarkable variability. They comprise other parts of bacterial envelope (cell wall and capsule or slime layer), cytoplasmic inclusions, endospore, pili and fimbriae, flagella, injectisome or needle complex, plasmids and episomes.

Nucleoid

The ***nucleoid*** in bacteria is a complex structure containing *double helix of DNA* that is covalently closed into circle. It also has RNA and polyamine proteins but without histones. Unlike ekaryotes, bacteria are devoid of the membrane that separates nucleoid from cytoplasm. Nucleoid DNA is the major carrier of genetic information in bacterial cell.

DNA of nucleoid is tremendously super-coiled making numerous fibrils and loops 300-500 nm in diameter. Usually nucleoid is located in cytoplasm of central part of bacterial cell. It is linked to cytoplasmic membrane and mesosomes. In most cases single bacterial cell harbors one nucleoid; before cellular division the number of nucleoids per cell can be also 2 or 4. Nucleoid actively participate in bacterial reproduction.

Commonly, nucleoid can be detected in bacterial cell by fluorescent microscopy with specific dyes for DNA – ethydium bromide, Sytox Green and many others. The fluorescent dye acridine orange stains cellular DNA green whereas cellular RNA becomes red. The details of nuleoid structure can be determined by electron microscopy.

Bacterial Cytoplasm

The ***cytoplasm*** of bacteria is the colloidal matter containing water, mineral compounds, proteins, lipids, carbohydrates, etc.

Multiple ribosomes are present in cytoplasm being the sites of protein synthesis. Bacterial ribosomes have 70S sedimentation constant in ultra-centrifugation (30S for small subunit and 50S for large one). A cluster of 50 to 55 closely related ribosomes is known as *polysome*. The ribosomes and polysomes are linked to the cellular membrane.

The cytoplasm also contains autonomous genetic structures – small circular DNA molecules of *plasmids* and *episomes*. They encode the synthesis of variety of substances (toxins, microbial enzymes and many others).

Cytoplasmic Inclusions

Various kinds of ***inclusions*** are stored in the cytoplasm. Among them are volutin and starch granules, lipoprotein bodies, pigments, accumulations of sulfur, microcristals of calcium, etc.

Volutin granules are of sizes to 0.1-0.5 μm. They are located in cytoplasm on the ends of *Corynebacterium diphtheriae* rods; also they can be found in the yeasts and other microorganisms.

Volutin granules contain metaphosphates. According to ***Neisser's staining*** method volutin granules stain blue, vegetative part of bacterial cell stains brown-yellow.

Lipoprotein droplets are accumulated in cytoplasm, when bacteria grow on rich nutrient media. They are actively used by microbial cells in starvation conditions. Microscopical methods for their detection include *Sudan* stain.

Similar manner starch granules can be stored and next consumed by microbial cell in case of unfavourable situations. They are detected by stain with *Lugol's iodine* solution.

Bacterial Envelope

Combination of external layers that cover the bacterial cell is known as ***bacterial envelope***. The structure of envelope is greatly different in gram-positive and gram-negative bacteria; in fact, it is this difference that defines these two major sets of bacterial species.

The ***bacterial envelope*** usually consists of ***cytoplasmic membrane***, ***cell wall*** and ***slime layer***. Many bacterial species are surrounded by ***capsule*** as the external layer of cellular envelope.

Cytoplasmic Membrane

The cytoplasmic membrane encases the whole contents of bacterial cell. It is the innermost layer of bacterial envelope usually of 5-10 nm in thickness. The membrane is generally organized like phospholipid bilayer with embedded membrane proteins. Bacterial membranes are lack of sterols except mycoplasma cells.

Cytoplasmic membrane produces a selectively permeable barrier that provides controlled transportation of water, electrolytes, and various nutrients into bacterial cell for its vital activity. The transfer of water and ions creates osmotic pressure within bacterial cell.

The membrane carries the vast number of receptors with diverse functions. They recognize the variety of signals from the environment; signal transmission results in changes of bacterial metabolism.

Multiple membrane enzymes perform biological oxidation in bacteria. Also they support biosynthetic reactions for building of bacterial components (proteins, nucleic acids, etc.) and actively govern bacterial trans-membrane transportation.

Membrane invaginations or ***mesosomes*** are the nearest mitochondria analogs of bacterial cells. They harbor oxidative enzymes of electron

transport chain thus providing energy for bacteria. Mesosomes actively participate in the growth of bacterial cells and their reproduction.

The best method for study of bacterial cytoplasmic membrane is electron microscopy.

Bacterial Cell Wall

The part of the cell envelope located between the cytoplasmic membrane and the slime layer or capsule is termed as the ***cell wall***.

One of the major functions of the cell wall is cellular *protection*. Inner osmotic pressure within bacteria is as high as 5-20 atm being created by active transportation of ions inside the cell. Rigid and tough cell wall maintains the integrity of bacterial envelope. Also it protects bacteria from various environmental challenges.

Bacteria *maintain their sizes and shapes* that are typical for certain bacterial species, owing to the structure of their cell wall.

The cell wall is generally characterized by marked non-selective permeability. Nevertheless, in gram-negative bacteria the outer membrane of the cell wall can block the entry of molecules from outside.

In addition, the cell wall actively *participates in cell division*. It plays a role of primary template for its own synthesis .

The bacterial cell wall is composed predominantly of a substance termed as ***murein***, or ***peptidoglycan***.

Peptidoglycan is multi-component polymeric structure. It is composed of three main structural units.

Long polymeric chains of peptidoglycan ***backbone*** are formed by two alternating carbohydrate residues of N-acetyl-D-glucosamine and N-acetylmuramic acid.

Multiple ***tetrapeptide side chains*** of equal structure are attached to N-acetylmuramic acid.

Tetrapeptide side chains are linked together by uniform ***peptide cross-bridges***.

The backbone of the cell wall is standard in all bacterial species.

By contrast, peptide cross-bridges and tetrapeptide side chains are different depending on bacterial species. In large amount of gram-negative bacteria tetrapeptide side chains are directly linked by peptide bond between terminal D-alanine carboxyl group in one side chain and the amino group of *diaminopimelic acid* (***DAP***) of opposite side chain.

Vast number of antigenic determinants (*epitopes*) are present within bacterial cell wall.

The *glycan part* of cell wall peptidoglycan can be destroyed under the action of hydrolytic enzyme ***lysozyme***. Lysozyme *hydrolyzes glycosidic bonds* between N-acetylmuramic acid and N-acetyl-D-glucosamine.

The *synthesis of peptidoglycan* of the cell wall *is inhibited* by highly efficient group of antibiotics bearing beta-lactam ring in their structure (***beta-lactam*** drugs). The group includes *penicillins*, *cephalosporins*, *monobactams* and *carbapenems*. These bactericidal antibiotics irreversibly block bacterial ***transpeptidase*** enzymes (also called as *penicillin-binding proteins* or ***PBPs***) that catalyze the formation of peptide cross-bridges within cell wall.

All bacterial representatives were separated into two major groups depending on thickness and unique details of composition of their cell walls. The method for their differential stain was adapted from technique proposed by Danish bacteriologist H. C. Gram as far back as in 1884.

As the result of Gram staining the bacteria are divided into ***gram-positive*** or ***gram-negative***.

Gram-positive bacteria were shown to stain by Gram in ***dark-violet*** owing to their thick cell wall whereas ***gram-negative bacteria*** with thin cell wall appear to be ***red***.

In gram-negative bacteria the part of their cell wall known as ***lipopolysaccharide*** (or ***LPS***) displays ***endotoxin*** activity. In gram-positive microbes non-specific endotoxic activity is related with *lipoteichoic acids*.

Gram-Positive Cell Envelope

The envelope of gram-positive bacteria is relatively simple, consisting of two to three main layers: the ***cytoplasmic membrane***, a ***thick peptidoglycan layer***; and sometimes ***capsule*** or ***slime layer***.

In the cell wall of gram-positive bacteria there are many sheets of peptidoglycan (40-50 or even more) that embrace about 50% of the cell wall substance. On the contrary, gram-negative bacteria have only one or two sheets of murein that include about 5-10% of the wall contents. In addition, gram-positive microbial cells carry negatively charged ***teichoic acids*** that possess toxic activity. Precursors of teichoic acids, ***lipoteichoic acids***, are anchored within the cell membrane of gram-positive bacteria.

Gram-Negative Cell Envelope

The ***cytoplasmic membrane*** or the ***inner membrane*** in gram-negative bacteria is surrounded by a single planar sheet of ***peptidoglycan***. Peptidoglycan is linked to a complex layer called the ***outer membrane***. An outermost ***capsule*** or ***slime layer*** may also be present. The space between the inner and outer membrane is termed as ***periplasmic space***.

Gram-negative cell walls include three components that are located outside of the peptidoglycan layer: ***lipoprotein***, and ***outer membrane*** with its external leaflet ***lipopolysaccharide***.

Numerous ***lipoprotein*** molecules cross-link peptidoglycan and the outer membrane of gram-negative bacteria. They stabilize the joining of outer membrane with peptidoglycan layer.

Lipoprotein is the most exuberant molecule present in gram-negative cells (about 700,000 molecules per 1 cell).

The ***outer membrane*** has its *inner* and *outer* leaflets.

Inner leaflet is organized similarly to the cytoplasmic membrane. The *outer leaflet* is composed of ***lipopolysaccharide*** (***LPS***) molecules. Therefore, these leaflets are asymmetrical in structure and their activity is substantially different from standard cytoplasmic membrane.

Unlike typical biologic membranes, outer membrane demonstrates the evident capacity of exclusion of hydrophobic molecules. It is non-ordinary trait for the membrane and helps to protect microbial cells such as enterobacteria from surface-active agents (bile salts and others).

Lipid part of outer membrane is also poorly permeable for hydrophilic molecules. Nevertheless, it has a large set of channels made of special proteins ***porins*** that foster passive diffusion of hydrophilic substances with low molecular weight (ions, carbohydrates, amino acids, etc.) In the same vein, the outer membrane is a serious barrier for entry of antibiotic molecules; the latter supports enhanced antibiotic resistance of gram-negative microbial cells.

The level of permeability of the outer membrane strongly depends on species of gram-negative bacteria. For instance, outstanding antibiotic resistance of *Pseudomonas aeruginosa* is maintained in part by very low permeability of the outer membrane, about 100 times less than membrane permeability of *E. coli*.

The ***lipopolysaccharide*** or ***LPS*** of gram-negative bacteria is composed of three parts: central uniform ***polysaccharide core*** connected with ***lipid A*** on bottom side and with external *variable polysaccharide chains* generally known as ***O-antigen***.

Complex lipid that is called ***lipid A*** is attached to inner leaflet of outer membrane by hydrophobic interactions. It is formed within cytoplasmic membrane with next transportation towards the outer membrane. Next it becomes linked to bacterial polysaccharide core.

Lipid A is responsible for high toxicity of LPS for mammals. LPS as ***endotoxin*** of gram-negative bacteria retains its toxic activity after degradation of bacterial cell.

The ***polysaccharide core*** has very similar composition in all gram-negative bacteria that possess LPS.

Terminal polysaccharide chains, which are composed of variable oligosaccharide residues, play a role of ***major surface antigen*** of gram-negative bacteria. Taken together, they are termed as ***O-antigen***. Bacterial antigenic specificity largely depends on oligosaccharide repeatitive units that form the external layer of hydrophilic polysaccharides covering bacterial cells.

Total number of polysaccharide antigenic variations is extremely high (eg, more than 2500 for *Salmonella enterica* species).

Also LPS supports proper activity of many proteins located within the outer membrane.

Bacterial cells carry special hydrolytic enzymes, or ***autolysins***, that destroy their own peptidoglycan. They comprise peptidases, amidases, and glycosidases. Autolysins actively impact on cell growth and division. They perform degradation and lysis of bacterial cell after its death (***autolysis***).

Bacteria can lose the cell wall under various external influences. Gram-positive bacteria become lack of the cell wall by treatment of antibiotics, inhibiting cell wall synthesis (eg, beta-lactams) or by lysozyme action. The resulting cells deprived of the cell wall are named as ***protoplasts***. Usually they are spherical in shape, and without capability to next cellular division.

In gram-negative bacteria the degradation of the cell wall ordinarily leads to formation of ***spheroplasts***. Spheroplasts only partially lose their cell walls.

Another particular morphological variations of microorganisms were disignated as ***L-forms***. They look like protoplasts, making spherical or thread-like structures without cell wall. L-forms were discovered in 1935 at the Lister Institute in Great Britain. They are actively generated under influence of penicillins or in some cases of division of bacterial cells.

Despite L-forms of bacteria closely resemble protoplasts, they are *capable of reproducing* and may reverse to initial vegetative state. As deprived of the cell wall, L-forms display increased resistance to several

groups of antibiotics. Thus, they are able to extend the infectious process resulting in chronic forms of infection.

Bacterial Capsule

In natural conditions many bacteria produce abundant quantity of extracellular polymers. With some exceptions (e.g., *B. anthracis* expresses poly-D-glutamic acid capsule), the extracellular sheet is made of *polysaccharides*.

In case of synthesis of huge polymeric external layer that enwraps the cell, it is named as bacterial ***capsule***. A capsule is an optional structure of the bacteria that is usually formed in worsened environmental conditions.

But in part of bacteria the external polysaccharide sheet doesn't show firm attachment to the cell wall, being relatively thin and loose. In these situations it is characterized as a "***slime layer***."

External polymeric substances are synthesized by superficial enzymes located within bacterial envelope.

The capsule is a *potent virulence factor* of bacteria as capsulated microbes demonstrate high *resistance against phagocytosis*. Similarly, they are poorly available for antibodies.

The "slime layer" takes part in *bacterial adhesion*, including microbial binding to living cells in course of infection.

Owing to its polysaccharide nature, bacterial capsule is poorly stained with aniline dyes. In standard cases it is negatively stained by Gin's method that creates Indian ink background for capsulated microbial cells.

Certain microbial species produce capsules only inside living hosts (e.g., *Streptococcus pneumoniae*, *Clostridium perfringens*, or *Bacillus anthracis*). Some other bacteria express capsule on constant ground regardless of place of habitation (e.g., *Klebsiella pneumonia*).

Bacterial Flagellum

The ***flagella*** provide the motility for microbes. They are optional bacterial organellas responsible for cell locomotion.

According to the mode of flagellum attachment, the motile bacteria are divided into four basic groups:

– ***monotrichious*** bacteria that carry single flagellum attached to one pole of bacterial cell (*P. aeruginosa*, cholera vibrios);

– ***amphitrichious*** bacteria that possess two polar flagella or carrying the tufts of flagella at both poles (*Spirillum volutans*);

– ***lophotrichious*** bacteria with a tuft of flagella located at one pole (*Helicobacter pylori*);

– ***peritrichious*** bacteria having flagella spread over the total surface of bacterial cells (*E. coli*, salmonellas, etc.)

The flagellum is composed of ***flagellin*** – a contractile protein resembling cellular actin.

Antigenic activity of motile bacteria related with flagella is generally termed as ***H-antigen***.

The flagellum has several parts in its structure – *basal bodies*, attached to cytoplasmic membrane and cell wall, flagellar *hook* and terminal *filament* that endows the cell with spinning movement.

Motility of bacteria can be readily observed by light, dark field or phase contrast microscopy making "hanging drop" slide with native (live) bacterial cells. Also the motility of bacterial culture can be determined after stab inoculation of bacteria into tube with agar medium – the spread of bacterial cells from the line of primary inoculation is easily detected.

Direct flagella presence in bacteria is revealed by electron microscopy or by means of special method of bright field microscopy that uses Loeffller's stain. The latter technique causes the enlargement of thin flagellar filaments; and they become visible under light microscopy stained in pink color.

Bacterial Injectisome

Also many species of gram-negative bacteria, like *Enterobacteriaceae* members (shigellae or salmonellas), were found to have particular flagellar-like structure termed as ***injectisome***, or ***needle complex***.

Injectisome is a constituent part of special bacterial ***type III secretion system***, which governs microbial invasiveness and intracellular parasitism.

Once attached to the mammalian cells via needle complex, bacteria inject the number of invasive ***effector proteins*** into host cell. These proteins re-build cytoskeleton of affected cell that leads to membrane pocket formation and subsequent bacterial capture. It results in pathogen penetration across cytoplasmic membrane. Effector proteins also promote intracellular microbial movement and lateral intercellular invasion.

Pili and Fimbriae

Bacterial surface is covered by short hairlike appendages termed as ***pili*** or ***fimbriae***. Usually they are about 0.5-1.0 μm in length and less than 10 nm in width. Every microbial cell may carry from 100 to 400 units of pili.

Pili are composed of closely related family of proteins known as ***pilins***. Two basic kinds of pili are present in bacterial cells. Among them are ***ordinary pili*** that provide microbial *adhesion* to the cells and tissues, and ***conjugative*** (or ***sex-pili***), participating in the process of bacterial conjugation.

Ordinary pili also foster bacterial *nutrition* as they highly increase the efficient area of absorbtion of nutrients for microbial cells.

Conjugative pili produce the hollow channel (or *conjugative tube*) that ensures the direct contact between donor and recipient cells. This allows the transfer of genes to the recipient cell as the result of conjugation.

In addition, bacterial pili can play a role of *cellular receptors* (e.g., for bacteriophages) and demonstrate marked *antigenic properties*.

Spores and Sporulation

Endospores are oval or round-shaped bodies of small sizes formed inside microbial cells. The spores are produced in the course of microbial propagation at certain environmental conditions.

Unlike in fungi, the spores in bacteria are not used for their reproduction, but *for survival* in case of worsened living state.

Rod-shaped bacterial forms of ***bacilli*** and ***clostridia*** demonstrate genetic ability to produce spores. These bacterial groups comprise highly virulent human pathogens (anthrax bacilli, agents of tetanus, botulism or gas gangrene) as well as saprophytic bacteria dwelling in water or soil.

The spores in bacteria can be positioned in the center of the cell (***centrally***) as for *B. anthracis*; directly at the ends of their cells or ***terminally*** (*C. tetani*); and ***subterminally*** nearby the ends of bacteria (e.g., *C. botulinum*).

Spore carriage leads to characteristic appearance of many bacterial species. For instance, *C. botulinum* with subterminal spores looks like tennis racket, and *C. tetani* resembles drumstick with its terminal spores.

Sporulation occurs in natural or artificial surroundings (e.g., on nutrient media), but in most cases it doesn't occur within living tissues during infection.

The *sporulation process* evolves in several consecutive stages: ***preparatory stage***; ***forespore stage***; ***stage of cell wall formation***; and ***maturation stage***.

The beginning of sporulation (***preparatory stage***) is determined by concentration of the cytoplasm and condensation of the nucleoid in a certain region of bacterial cell (*sporogenic zone*).

At ***forespore stage*** the nascent spore is gradually separated from vegetative part of bacterial cell.

Afterwards the spore becomes covered by thick and rigid multilayered external coat (the stage of ***cell wall formation***). The newly synthesized external cell wall includes *cortical layer* or cortex; it consists of modified peptidoglycan in complex with spore-specific *dipicolinic acid* and *calcium* salts

At the end of sporulation the vegetative part of the cell dissolves under the action of *autolysins* (***maturation stage***).

Spore formation usually takes from 10 to 20 hours to develop.

Germination of spore occurs in favorable conditions (increased humidity, warming of the local environment, the rise of nutrients concentration, etc.) It is characterized by spore enlargement and swelling after water accumulation that is followed by activation of bacterial metabolism.

Usually the spore is capable of propagating only after several days of rest. The process of germination comprises three consecutive stages.

Activation stage starts from various external stimuli. Amelioration of the environment or, conversely, damage of the spore shell by heat, acidity or abrasion provoke spore activation.

Stressing of spore leads to beginning of spore germination (***initiation stage***). It is followed by autolysins activation that dissolve cortical murein with the release of calcium dipicolinate. Hydrolytic enzymes rapidly eliminate the components of spore wall.

Outgrowth stage is characterized by formation of protrusion out from the spore wall. This leads to spore transformation into rod-like vegetative bacterial cell. The nascent bacterial cell starts to grow and then propagates.

Usually germination cycle of spore completes in 4-5 hours.

The spores are highly stable in the environment. They maintain viability in soil for many years (e.g., more than 40-50 years for spores of *B. anthracis* or *C. tetani*).

The spores of some bacilli can withstand boiling and demonstrate high resistance to conventional disinfectants. They are killed by autoclaving or by exposure to dry heat in Pasteur oven at 160-180°C for 2 hours.

A rigid coat of spore is poorly permeable for aniline dyes. Therefore, the spores are almost not stained by conventional staining methods and look like transparent glistening bodies within colored microbial cells.

To make spore walls more permeable, various mordants can be applied, e.g. hydrochloric acid and carbol fuchsin in *Ozheshko stain* or steamed carbol fuchsin in *Moeller spore stain*. In both cases the spore becomes red on the blue background of vegetative part of the cell.

Basic Methods for Examination of Microbial Morphology

Morphological traits of bacterial cells (their sizes, shapes, details of inner structure, etc.) are studied by versatile methods of ***microscopy***.

The efficacy of any microscopical technique is actually determined by its *resolving power* (***resolution*** of microscope)

Resolving power *is the minimal distance that yet allows to distinguish 2 objects of microscopy as 2 separate images in the field of view.*

Resolving power of ***optical microscopes***, where the visible light is collected, is described by classical formula of E. Abbe:

$$\mathbf{d = \lambda / 2n * \sin\alpha},$$

where **d** is the resolving power of microscope, **λ** designates the wavelength of light, collected by microscope objective lens, **sinα** is the angular aperture of objective lens, **n** is the refractory index of immersion medium for objective lens.

According to that ratio, the maximum resolving power of conventional optical (light) microscope should be around 200 nm. It is approximately equal to the half of light wavelength that is used for microscopy. In case of violet component of visible light with the shortest wavelength of 400 nm it brings resolution to its theoretical value of about 200 nm.

Below this distance, the diffraction of visible light on the examined objects limits the further increase of resolving power.

Standard ***bright field*** microscopes used in bacteriology are usually equipped with 100-power objective lenses and 10 power eyepieces. Therefore, their total magnification is equal to 1000 times.

Current advanced optical systems with immersion objective lenses of power 150 give efficient resolution near 150 nm.

The sizes of microorganisms can be determined by various measuring devices placed inside the eyepieces, e.g. *ocular micrometer* or reticle, containing scale or measuring grids.

The diagnostic value of bright field microscopy in bacteriology is improved with a great number of ***staining methods***.

Various organic dyes (acid, basic, or neutral) can be employed here. They bind to certain microbial structures by physical and chemical interactions (ionic, hydrophobic, covalent and others).

Simple stain technique involves a single dye (methyl violet, methylene blue, fuchsin, etc.)

Differential stain presumes the use of two and more dyes. They stain distinct parts of bacterial cells (***tinctorial properties*** of bacteria) thus fostering microbial discrimination.

Among these methods are Gram stain, Neisser's volutin granules stain, Gin's capsule stain, Ziehl-Neelsen acid-fast bacilli stain and many others.

Gram stain discriminates the differences in structure of bacterial cell wall. The method includes several steps: staining of flame-fixed slide with *crystal violet* (methyl violet, or gentian violet); treatment with Lugol's iodine solution; ethanol decolorization; *fuchsin* counterstain.

Due to large amounts of negatively charged cell wall peptidoglycans and nucleoproteins *gram-positive bacteria* stain ***violet***. They retain the first basic dye (crystal violet) in complex with iodine within their thick cell wall. *Gram-negative bacteria* stain ***pink*** as crystal violet is washed out by ethanol from the thin cell wall that is few of peptidoglycan, and bacterial bodies are counterstained with fuchsin.

Ziehl-Neelsen stain is used for special staining of ***acid-fast bacteria*** that carry exuberant amounts of lipids. Neutral lipids poorly absorb aniline dyes. Great lipid contents (mycolic acids, waxes) is typical for acid-resistant mycobacteria (*M. tuberculosis*, *M. leprae*), and actinomycetes.

The slide is exposed to *Ziehl carbol fuchsin* and steamed upon burner until vapor appearance. Then it is treated with sulfuric acid and counterstained with methylene blue solution.

Due to acid resistance and poor permeability for dyes, only *acid-fast bacteria* retain the primary red color whereas all other bacteria stain blue.

Romanowsky-Giemsa stain is the universal differential technique that is widely applied for discrimination of many bacteria (e.g., spiral-shaped borreliae, treponemas, or leptospira) as well as for protozoans, and

mammalian cells (e.g., for blood cell count). It uses *Romanowsky-Giemsa's complex stain* (mixture of azure, eosin, and methylene blue dyes).

Following this method, the examined *bacteria* stain ***violet-purple***, *protozoan* ***nuclei*** *–* ***red-violet***, their *cytoplasm –* ***blue***; *mammalian cell nuclei –* ***red***, their *cytoplasm –* ***blue***.

The results of other differential stains (e.g., Ozheshko spore stain, Neisser's stain for volutin granules, or Gin's stain for capsule) provide the identification of these optional microbial structures among various groups of bacteria (see above for details).

Standard staining methods usually operate with fixed inactivated specimens that don't allow to observe the functional activity of bacteria. This is possible only by studying of live bacterial cells within native (not fixed) specimens.

For examination of semi-transparent bacterial cells without staining, ***dark-field microscopy*** is broadly used. It is performed on the base of conventional bright field microscope supplied with special condenser. Dark-field condenser blocks the rays that directly come to the aperture of objective lens along optical axis of microscope. Other light rays, mirrored by condenser, pass through focal plane of the slide at large angles, thus missing objective aperture as well. As the result, the field of view becomes dark. When semi-transparent small objects (e.g., native bacterial cells) are placed in focal plane, the oblique rays will be reflected into objective lens by microbial bodies making bacteria visible. For instance, dark-field microscopy can easily determine motile thin and long bacteria, such us numerous species of spirochetes (treponemas, leptospirae, and borreliae) that are in width less than 0.2 μm.

Another powerful and reliable method for visualization of living bacterial cells is ***phase contrast microscopy***. This technique exploits bright field optical microscopes equipped with special phase contrast devices. This device is based on principle of light phase shift when the light passes through media with unequal refractive indices – bacterial cells and aqueous microbial surroundings, cellular cytoplasm and more dense nucleoid or nucleus. Phase contrast device transforms the shift of phase of light into differencies in light intensity.

Phase contrast microscopy permits direct observations of complex bacterial processes, such as growth and reproduction. Also it makes possible the study of internal structures of bacterial cells.

Another highly efficient method for observation of living unstained microbial objects was devised on the base of polarization contrast

principle. This method of ***differential interference contrast*** (or DIC) detects the alterations of polarized light beams when they pass through non-homogenous transparent structures. Microscopy with DIC creates optical images with 3D-like reliefs. Also it allows optical sectioning of thick non-stained specimens.

High perspectives are related with ***luminescent microscopy***. This technique uses the vast number of luminescent dyes for micobial stain (e.g., *fluorescein*, *acridine orange*, *auramine*, rhodamine, ethydium bromide, SYBR Green, the dyes of Alexa Fluor family and many others). Such dyes easily stain either fixed or vital (native) specimens of bacterial or mammal cells allowing the study of bacterial physiology and pathology.

The method exploits luminescent microscope with UV-source of light for excitation of dye fluorescence. After excitation, the dye begins to emit fluorescence of longer wavelength. The method provides differential staining of various microbial structures (nucleoid, volutin granules, spores and others).

Current striking advances of luminescent microscopy primarily ensue from the employment of laser as the source of illuminating light. Laser beams are easily controlled and can be focussed within minimal volume. The advantages of this technology were realized in the method of ***laser scanning confocal microscopy*** (***LSCM***).

The microscope for LSCM uses lasers of various wavelengthes for excitation of object fluorescence. Every moment of time the laser stimulates emission of fluorescence in certain point of stained specimen. The emitted light is collected by objective lens and then passes to the fluorescence detector. In the center of optical path before the detector, a small *pinhole* (or confocal diaphragm) is situated. It allows to pass further only the light generated directly in point of laser excitation within focal plane. This greatly reduce the size of analyzed specimen point and brings LSCM resolution closer to theoretical limit.

Laser beam of LSC microscope rapidly scans one horyzontal plane of specimen and creates its computer optical image. Then it moves along the vertical axis and repeats the operation. Computer analysis of accumulated images (image stack) generates real 3D reconstruction of microscopical objects. Unlike any other method, LSCM provides real-time 3D-scanning of large living microbial communities like biofilms. For instance, it enables to trace bacterial behaviour within biofilms including their complex interactions with antibiotics and other biocides.

Current developments of laser flourescent microscopical technologies open new horizons in all fields of modern cytology and microbiology. The

most advanced novel methods created opportunities to overcome resolving power limitations that are essential for standard optical microscopes.

As an example, *stimulated emission-depletion fluorescent microscopy* (*STED*-microscopy) has seriously higher resolution equal to ~60 nm. Similar high efficacy is characteristic for *multiphoton fluorescent microscopy*. All these methods pertain to *superresolution light microscopy*.

Laser confocal microscopy with technology of *fluorescence resonance energy transfer* (or *FRET*) makes possible to analyse direct interactions of molecules within living cells (e.g., toxins and their receptors), calculating distances between active reactants ("nanometric ruler" function).

Nevertheless, the profound study of intimate details of microbial structure on molecular and atomic levels requires devices and facilities, operating on priciples other than optical microscopy.

Classical technology that demonstrates superior resolving power in comparison with all other methods is ***electron microscopy***.

The extremely high resolution of electron microscope ensues from the situation that the wavelength of electrons is substantially shorter than the wavelength of photons of visible light.

There are two basic kinds of electron microscopes: the ***transmission electron microscope*** (***TEM***), and the ***scanning electron microscope*** (***SEM***).

TEM electron microscopy uses the beam of electrons expelled from an electron gun under the high voltage. The electron beams are oriented and focussed by the electromagnetic condenser lenses onto a specially prepared thin specimen.

After differential scattering of emitted electrons on atomic and molecular groups of the specimen, some quota of electrons passes across the specimen, depending on its local densities. Such electrons are collected and focused by electromagnetic lens of an objective. This creates the image of the specimen that is further processed with the projector electromagnetic lenses for additional magnification. The visualization of an image can be performed with fluorescent screen that emits the light under electron strikes. TEM efficient resolution operates in the range of 0,1-1 nm (at nanometer or angstrem scales). As the result, even small viruses with their diameters of 30-50 nm can be easily detected and characterized.

SEM electron microscopy gains *three-dimensional images* of microscopical objects. However, this technique demonstrates generally lower resolution than transmission electron microscopy.

Possibility of 3D-imaging arises from highly precise focusing of electrons within a smallest point on specimen surface at time of scanning.

The electrons, passing through the surface of investigated objects, generate various forms of secondary radiation (e.g., secondary electrons) that can be registered, amplified and analyzed with subsequent computer reconstruction of 3D object image.

Despite superior resolution of electron microscopy, this technology has essential limitations, as it operates only with artificially modified fixed objects.

Atomic force microscopy (***AFM***) allows to perform *real-time study* of live microorganisms *at high resolution* that might be comparable with scanning electron microscopy.

Unlike all previosly mentioned techniques, AFM doesn't use any kind of radiation (e.g., light or electron beam) to create object image.

AFM estimates the minimal physical forces that arise from AFM-scanning of micoscopical objects on ultra-low distances. These forces act between the surface of studied object and the tip of extremely sensitive sensor of atomic force microscope (termed as *cantilever*).

Laser beams control the position of the sensor (cantilever), when it scans the surface of the specimen. At the end of scanning the detailed 3D-image of the shapes of microscopical objects is generated with resolution near to molecular level.

AFM can study live bacterial cells and viruses in conditions, closer to their natural surroundings. It makes possible to visualize the essential long-term events of microbial life cycle – bacterial division, spore formation, viral entry and reproduction and others.

In addition, AFM provides exclusive data about the state of bacterial external structures – cell wall architecture, cytoplasmic membrane viscosity and fluidity, organization of bacterial flagella and pili, binding activities of adhesins, clustering of surface proteins, etc.

Future perspectives are related with the combination of advantages of atomic force microscopy and advanced methods of fluorescent light imaging.

Chapter 3

MORPHOLOGY AND ULTRASTRUCTURE OF SPIROCHETES, RICKETTSIAE, CHLAMYDIAE, AND MYCOPLASMAS. MORPHOLOGY OF ACTINOMYCETES AND FUNGI

Morphology and Ultrastructure of Spirochetes

Spirochetes (Lat. *spira* – curve, Gk. *chaite* – mane) demonstrate the structure, distinct from other bacteria. They make ***spiral*** cellular forms with a *corkscrew shape*. Their sizes are in the ranges from 0.2 to 1.5 μm in width and from 5-10 to more than 100 μm in length. Spirochetes have an axial filament with the cytoplasm wound around this filament in spiral manner. The bacteria possess triple-layer outer membrane. Electron microscopy revealed a thin cytoplasmic membrane enwrapping the cytoplasm.

Spirochetes are lack of capsules or spores, but they may produce cysts at worse environmental conditions. Also they are devoid of external flagella, but carry *axial filaments* or *endoflagella* located within periplasmic space. Endoflagella are attached to the end of microbial body providing nimble bacterial motility.

The active motility of spirochetes is also maintained by easy flexibility of their thin spiral cells. Spirochetes demonstrate a rotating motion around the axis of microbial cell; an undulating motion that involves the whole body of the bacteria, and a bending motion with the microbial body bendings at various angles.

The methods studying the morphology of spirochetes comprise the number of *staining techniques* and the methods for *detection of live bacteria* primarily based on their active motility (e.g., dark-field or phase-contrast microscopy).

Romanowsky-Giemsa stain is broadly used for differentiation of certain spirochetal groups. In general, many species of spirochetes poorly absorb aniline dyes because of their high lipid contents. Nevertheless, some bacteria easily stain blue or blue-violet, some – pink or pale-pink, several may remain literally unstained.

All these bacteria are *gram-negative* due to their thin cell wall with minimal peptidoglycan. An ultra-sensitive method of stain for spirochetes is performed by *silver* impregnation.

Basic taxnomical division of spirochetes includes the order *Spirochaetales* with its two main families *Spirochaetaceae* and *Leptospiracea*. These families compise three genera with human pathogenic reprsentatives: *Borrelia* and *Treponema* that both belong to the family *Spirochaetaceae*, and *Leptospira* genus from the family *Leptospiraceae*.

The pathogenic members of spirochetes can be discriminated according to their distinct morfological and tinctorial properties. Also they are easily detected by *dark-field* or *phase-contrast microscopy*.

The bacteria of genus ***Borrelia*** look like large rough spirals with the obtuse-angled irregular coils. The number of coils usually varies from 3 to 8-10. The bacteria possess many (from 10 to 30-40) periplasmic fibrillas (*endoflagella*).

Borrelia stain blue-violet with Romanowsky-Giemsa stain due to the relatively high contents of nucleoproteins within microbial bodies.

Pathogenic borrelia cause anthroponotic human disease known as *epidemic relapsing fever* that is transmitted by lice. The causative agent of this infection is *Borrelia recurrentis*. Also they cause zoonotic *endemic relapsing fevers* that may affect humans. These infections are spread by ticks being caused by multiple borrelial species (*Borrelia persica*, *B. hispanica*, *B. duttonii*, etc.).

The repesentatives of genus ***Treponema*** (Gk. *trepein* – turn, *nema* – thread) are slender flexible highly motile cells. They produce thin spirals with 6-20 regular small coils. These microorganisms carry about 2-16 (usually 3-4) endoflagella.

The bacterial ends can be tapered or rounded. Treponemas possess tender triple-layered outer membrane that is poorly resistant to external influences. It is composed of lipids and proteins. Treponemal cell walls have few amounts of peptidoglycan.

Within unfavorable surroundings the bacteria can produce cysts.

Treponemas render *pale-pink* with *Romanowsky-Giemsa* stain. They can be detected by silver stain. Active motility of treponemas fosters their determination by dark-field and phase-contrast microscopy.

Treponema pallidum – the causative agent of *syphilis* – is a typical representative of treponemas.

The bacteria from the genus ***Leptospira*** (Gk. *leptos* – thin, *speira* – coil) demonstrate very thin cellular structure. They are composed of the large number of *primary coils* (12-18 or more) that are very tightly wound to each other. Being supercoiled, leptospira form secondary twists,

resembling letters C or S under microscopy. The cells display hook-like or spiral-shaped ends.

The bacteria carry two axial filaments, which are attached to the basal bodies at opposite ends of the cells. They protrude toward each other from the cellular ends. The middle of the bacteria is lack of axial filaments.

Endoflagella provide complex and active motility of leptospiras. They render rotational movements where the ends of microbial cells rapidly rotate at a right angles to the axis of microbial body.

Due to the tight packing and excessive lipid contents the leptospiras are poorly stained. They may become slightly pinkish when stained with Romanowsky-Giemsa's method. The methods of choice for their determination are dark-field and phase-contrast microscopy.

Typical representative of leptospiras *Leptospira interrogans* is the agent of zoonotic disease *leptospirosis* that also seriously affects humans.

Morphology and Ultrastructure of Rickettsiae and Chlamydiae

Pathogenic Rickettsiae

Rickettsiae are placed into the order *Rickettsiales* that contains the family *Rickettsiaceae*.

Rickettsiae pertain to ***obligate intracellular parasites***. They live and multiply within the cytoplasm or nuclei of cells from different tissue types.

Rickettsiae are *pleomorphic* organisms. Coccoid forms look like tiny single-grain ovoids with the diameter about 0.5 μm; quite often they produce diploforms.

Rod-like rickettsiae make short rods of 1 to 1.5 μm in length with granules on the ends, or they render longer thin curved rods of 3-4 μm.

Filamentous forms are present as the long threads in the range of 10-50 μm in length; in some cases they might be curved.

Rickettsiae are lack of spores, capsules or flagella.

Electron microscopy revealed that rickettsiae possess inner and an outer membranes with elements of peptidoglycan. Their cytoplasm contains bacterial ribosomes.

Rickettsiae propagate by division of rod-shaped and coccoid forms, and by fragmentation of the filamentous forms; the latter creates daughter rod-shaped and coccoid cells.

For growth and reproduction rickettsiae are *cultured within living cells* – laboratory cell lines, or embrionated chicken eggs within yolk sacs. Zoonotic rickettsia can be cultured in laboratory animals, e.g. guinea pigs.

The bacteria are stained with aniline dyes; for instance, by Romanowsky-Giemsa stain.

There are several specially devised staining methods for examination of rickettsiae. *Zdrodovsky method* with carbol fuchsin (as modified Ziehl-Neelsen stain) makes rickettsiae *red* on the blue background of cellular cytoplasm and nuclei. Similar to this technique is *Gimenez stain* that renders rickettsiae *red* by fuchsin inside blue-green cellular background made by malachite green.

The vast number of rickettsia can cause the ailments in humans. Severe anthroponotic disease *epidemic typhoid fever* is caused by *Rickettsia prowazekii*, zoonotic endemic typhoid fever – by *Rickettsia typhi*. They are transmitted by lice or by ticks and fleas, respectively.

The large group of zoonotic infections known as *spotted fever rickettsioses* includes more than 20 pathogenic agents. Among them are *R. rickettsii* – the agent of Rocky Mountain spotted fever, *R. conorii* – the agent of Mediterranian spotted fever, and many others. The representative of one more rickettsial genus *Orientia tsutsugamushi* causes scrab typhus.

Pathogenic Chlamydiae

Chlamydiae as well as rickettsiae pertain to ***obligate intracellular parasites***.

The order *Chlamydiales* includes the family *Chlamydiaceae*; pathogenic represenatives are present within the genera *Chlamydia* and *Chlamydophila*.

These bacteria are of rather small sizes and they have two stages in their life cycle – ***elementary bodies*** and ***reticulate bodies***.

According to the compositon of the cell wall, chlamydiae are gram-negative bacteria. Their cell walls contain LPS and elevated amounts of lipids. However, they have no typical peptidoglycan because of lack of acetylmuramic acid. On the other hand, they carry multiple cysteine-enriched peptide cross-bridges that make bacterial envelope rigid.

Elementary bodies are the round-shaped structures of minimal sizes (0.2-0.4 μm). They possess infectious properties being capable of invading eukaryotic cells. Before the entry into the cell, chlamydiae are metabolicaly inert.

Within the cells, elementary bodies transform into feeding vegetative forms, known as *reticulate bodies*. They actively reproduce nearby the nucleus of the cells, making characteristic inclusions covered by common membrane (Gk. *chlamyda* – mantle or cloak). The sizes of reticulate bodies are about 0.8-1.5 μm.

After several reproductions they convert again into elementary invasive forms that leave the cell across cytoplasmic membrane. Total chlamydial life cycle takes near 3 days. The reproduction of chlamydiae is *asynchronous*, as all of microbial forms (elementary, reticulate, and intermediate) can be found in the cell at the same time.

Chlamydiae grow in cultures of eukaryotic cell lines. *McCoy cells* are commonly used for their culture. Likewise, they propagate within the yolk sac of embryonated chicken eggs and in laboratory animals (e.g., mice).

Chlamydiae are detected by *Romanowsky-Giemsa* stain (reticulate bodies produce blue inclusions, attached to cell nuclear membrane, whereas elementary bodies stain purple).

The monolayers of cell cultures are examined for chlamydial infection by *direct immunofluorescence.*

Chlamydiae cause numerous diseases in humans.

Chlamydia trachomatis is the causative agent of *trachoma* that afflicts eyes. It also cause sexually transmitted *urogenital infections* (in men – urethritis, epididymitis; in women – cervicitis, cystitis, salpingitis, pelvic inflammatory disease and others); venereal disease lymphogranuloma venereum; *inclusion conjunctivitis* of the newborns; arthritis and other diseases.

Chlamydophila pneumoniae causes chlamydial *pneumonia*; *Chlamydophila psittaci* is the agent of zoonotic avian infection *ornithosis* (or psittacosis) that may provoke severe lung or generalized infection in humans.

Morphology and Ultrastructure of Mycoplasmas

The ***mycoplasmas*** pertain to the separate phylum *Tenericutes*, class *Mollicutes* and the order *Mycoplasmatales.*

Family *Mycoplasmataceae* comprises two genera, containing human pathogens: *Mycoplasma* and *Ureaplasma*.

Mycoplasmas are the smallest bacterial representatives, usually 100-200 nm in size; larger microbial forms up to 500-700 nm can be found.

They are lack of spores and capsules; certain members of *Ureaplasma* genus may have flagella.

The outstanding feature of mycoplasmal cells is *the absence of the cell wall* in these bacteria. Phylogenetic analysis revealed that mycoplasmas initially originated from gram-positive ancestor bacteria after gradual loss of essential genes responsible for the cell wall synthesis.

Because of the cell wall absence, mycoplasmas are highly polymorphic microbials. They produce granular, coccoid, rod-like, clustered, pseudomycelial, and filamentous forms. As the substitute of the cell wall, the bacteria possess thick triple-layered cytoplasmic membrane.

At first mycoplasmal infections were detected by E. Nocard and E. Roux, who studied the infectious agents of pneumonia in cattle. The putative causative agent was invisible in light microscope and not cultured on conventional nutrient media. Thus, it long was ascertained as virus, especially as it easily penetrated through standard bacterial filters. Only in 1944 M. Eaton isolated mycoplasma from sputum of patients with pneumonia.

There are many free-living as well as pathogenic species of mycoplasmas. They inhabit the soil, sewage waters, various organic matters, and the living bodies of plants, animals and humans.

Mycoplasmas grow slowly (up to 1-2 weeks) on media containing serum, cholesterol, fatty acids, arginine and other growth factors. Their microcolonies on solid media resemble "fried eggs" with round consolidated central part .

The most common pathogenic representatives are *Mycoplasma pneumoniae* – the causative agents of pneumonia and other respiratory infections, and *M. hominis, M. genitalium, Ureaplasma urealytica* and *Ureaplasma parvum* that may induce infections of urogenital tract. The latter group of bacteria can occasionally cause meningitis in newborns.

Morphology and Ultrastructure of Fungi

Fungi (Lat. *fungus* – a mushroom) are *heterotrophic* microbial representatives of ***eukaryotic*** organisms. They contain a differentiated nucleus separated from cytoplasm by nuclear membrane.

Life cycle of fungi usually consists of 2 stages: ***vegetative*** and ***reproductive***. Reproductive stage may be asexual (***anamorphic state***) and sexual (***theleomorphic state***).

All fungal taxa are categorized within the ***kingdom*** *Fungi*. This kingdom comprises 7 ***phyla***. The phyla *Ascomycota* and *Basidiomycota* include fungal agents that affect humans. The majority of pathogenic fungal species pertain to *Ascomycota* phylum.

The members of phyla *Ascomycota* and *Basidiomycota* are separated into ***sub-kingdom*** *Dikarya* because of their sexual reproduction that is followed by *dicaryon* formation.

Overall, the current number of registered fungal species is extremely high – about 100 000 species are well-defined to date, being described in details. And the estimated total number of fungi inhabiting the Earth is conceived now in the range from 1 to 5 mln species.

Many fungal representatives are not perfectly classified yet on the ground of monophyletic principle. For instance, the numerous order *Mucorales* that harbors more than 300 species and certain human pathogens stays beyond 7 delineated fungal phyla. Previously this order belonged to former phylum *Zygomycota* that is newly re-classified as phylum *Glomeromycota*.

Fungi are hall-marked by their variable structure. In vegetative stage a complex body of fungus, which embraces many cellular elements, is termed as ***thallus***.

There are two basic types of fungal thallus – ***mycelial*** and ***yeast***.

The ***mycelium*** is composed of multiple long cellular filaments or ***hyphae***.

In general, two kinds of mycelium known – ***substrate*** and ***air*** mycelium, or according to its function – ***vegetative*** and ***reproductive*** mycelium.

In several groups of fungi the mycelium is *unicellular* or ***non-septate*** (i.e., without cross-walls).

The *highest multicellular* fungi have the cross-walls that separate the cells within mycelium (***septate*** mycelium).

Mycelium production is characteristic for ***mould fungi***.

Yeast thallus is composed of many groups of single fungal cells that arise from their progenitor cells by ***budding***.

Thus, the ***yeasts*** are oval or rounded unicellular fungi devoid of mycelium.

The fungi, capable of changing their morphology from yeast to mould mycelial forms and vice versa, are termed as ***dimorphic***.

Fungal cells are covered by rigid multi-layered cell wall. Their external layers consist of various hydrophilic substances, such us glycoproteins and α-glucans. Inner sheets of the cell wall are insoluble in water and contain ***chitin*** – non-branching polymer of β-1,4-linked N-acetylglucosamine, and β-glucans (e.g., cellulose).

Cytoplasmic membrane of fungi is enriched with extra amounts of sterols, predominantly of ergosterol, which is the primary target for many anti-fungal drugs.

Fungal cytoplasm contains the whole number of organelles, including ekaryotic 80S ribosomes, mitochondria and differentiated nucleus or

nuclei. Hence, fungal cells might be mononucleate or polynucleate. Unlike in prokaryotes (e.g., in bacteria), fungal genome has exon-intron organization that is realized via RNA splicing.

Numerous inclusions can be found in the cytoplasm of fungi: volutin, drops of fat, starch granules, etc.

Mycelial fungi have sexual and asexual reproduction with sporulation or framentation of hyphae. Yeasts can reproduce by budding or binary fission.

Spores in asexual reproduction are formed by mitosis. Sexual reproduction is followed by meiosis with formation of haploid hyphae; haploid hyphae later fuse resulting in diploid mycelium.

Various ecological groups of fungi comprise the large mass of environmental saprophytes, as well as parasites or facultative parasites that affect plants, animals and humans.

The members from different fungal taxa demonstrate clearly discernible morphological and physiological features.

The fungi from the order ***Mucorales*** have unicellular (non-septate) vegetative and reproductive mycelium. These mould fungi are broadly spread in nature. Many species are saprophytic, living in the soil and water, or upon plants.

The typical genus ***Mucor*** or bread mould have long branching mycelial cells. Among them are fruiting hyphae or ***sporangiophores*** finished with head-like dilatations or ***sporangia***. Sporangia contain numerous ***endospores*** that account for vegetative reproduction. These fungi are also capable of sexual reproduction.

In rare cases the fungi of the certain genera of the order *Mucorales* (e.g., *Apophysomyces*, *Rhizopus* or *Absidia*) can cause human diseases known as *mucormycoses*. These disorders demonstrate severe manifestations with predominant damage of brain and lungs.

Ascomycetes or sac fungi include more than 60 000 fungal species. They are composed of a multicellular mycelium.

These fungi have *sexual reproduction* with *ascospores* (the spores, carried within the special case, or *asc*).

Also ascomycetes have asexual reproduction by means of *exospores*. Exospores make long chains or ***conidia*** (Gk. *konidion* – particle of dust) on the ends of sporulating hyphae.

The members of the genus ***Aspergillus*** are the typical representatives of ascomycetes. Aspergilli have septate vegetative mycelium, and a unicellular reproductive hyphae or ***conidiophores***. On the ends of

conidiophores a fan-like rows of short pedestals or ***sterigmata*** are finished with the terminal chains of spores – ***conidia***.

Microscopy of these fungi demonstrates that the arrangement of conidia someway resembles the jets spurting out from a watering can; that's why aspergillus is often called as the *sprinkler mould* by its morphology.

A vast number of aspergillus species is widespread in nature. Among them are *A. flavus*, *A. niger*, *A. fumigatus* and many others.

In some cases, especially in predisposed patients with chronic bronchopulmonary diseases, these fungi (such as *A. fumigatus*) cause specific infection or *aspergillosis*. It is severe disorder that affects lungs with tendency to chronic course and invasive spread.

The genus ***Penicillium*** also belongs to ascomycetes. The fungi of this genus have both multicellular and septate vegetative and reproductive mycelium. Their sporulating hyphae with conidia resemble a brush; hence, these fungi are termed as *brush moulds*. Like other ascomycetes, penicillium representatives are common habitants of external environment. They contaminate the surfaces of moist objects, plant and animal-derived matters, various foodstuffs, e.g. dairy products, jam, or bread.

Certain species *(Penicillium notatum*, *Penicillium crustosum* and some others) are the active producers of β-lactam antibiotics (penicillin group).

Some penicillium species may cause *penicilliosis* – the disease, affecting predominantly immunocompromised hosts, e.g. AIDS patients.

The ***yeasts*** fungi pertain to the phylum *Ascomycota* and the order *Saccharomycetales*. They produce large round-shaped or oval cells. Yeasts have well-defined nucleus. The cytoplasm of yeasts contains numerous inclusions (volutin, starch, lipids). They multiply by sporulation, budding, or binary fission. Some yeast species are capable of sexual reproduction.

In the process of sporulation the true yeasts produce from 2 to 16 endospores located inside the cells. The yeast cell harboring the ascospores is termed as *ascus.*

Some yeast species are reproduced by continious budding, where the buds are not easily separated and make elongated chains. The progressive budding without bud detachment results in formation of elongated yeast chains called as ***pseudohyphae*** (or ***pseudomycelium***). Such species are often termed as ***yeast-like fungi***.

One genus of yeast-like fungi, *Candida*, includes the causative agents of high-rate *opportunistic* mycosis – ***candidiasis***. The main causative species of candidiasis is *C. albicans*; more seldom this infection is caused by *C. parapsilosis*, *C. glabrata* or other candidae. In most of clinical

situations candidiasis ensues from the suppression of the normal human microflora by massive antibiotic treatment. At the same time it may develop from severe secondary immunosuppression, like in AIDS patients.

A mass of yeasts species demonstrate the excellent capacity of fermentation of various carbohydrates. Thus, for a long time they are broadly used in food industry in bread baking, beer brewing, or wine making. The most common representative of such yeasts is *Saccharomyces cerevisiae* species

Basidiomycetes are the mushroom-producing fungi with multicellular mycelium and fruiting bodies. They are reproduced sexually by means of *basidiospores* (*basidiae* – special reproductive organs in these fungi, where a certain number of spores is developed). In natural conditions they occupy the decaying humus, being in symbiosis with trees and other plants. The ample number of species are tree parasites. The fruiting bodies (or mushrooms) of many basidiomycetes can be used as food. About two hundred species of mushrooms are edible but dozens of them are poisonous and may cause severe *food poisoning*.

Deuteromycetes or *Fungi imperfecti* is an artificial grouping of a large set of fungi that consist of a multicellular mycelium without the asco- or sporangiophores, but carrying only the conidia. Their *reproduction is asexual*, sexual reproduction (teleomorph) is not discovered.

Deuteromycetes comprise the vast number of human pathogens – the agents of dermatomycoses: ***trichophytosis*** (*Trichophyton violaceum*), ***favus*** (*Trichophyton schoenleinii*), ***microsporosis*** (*Microsporum canis*) and many others.

Most of medically relevant fungi are easily discriminated according to their morphological properties by standard methods of *optical micoscopy*.

Similarly, the majority of fungal species are successfully cultivated on liquid ***Sabouraud broth*** or solid medium ***Sabouraud agar*** that contain elevated concentrations of glucose (up to 3%) and peptone.

Morphology and Ultrastructure of Actinomycetes

Actinomycetes (Gk. *mykes* – fungus; *actis* – ray) pertain to the bacterial order *Actinomycetales*, family *Actinomycetaceae*, and genus *Actinomyces*. More than 35 microbial species are known in this genus, among them are *A. viscosus*, *A. bovis*, *A. israelii*, and many others.

Actinomycetes are organized as the large complex of thin rod-like cells and long branching filaments that make *air* and *substrate* ***mycelium***.

Mycelium of actinomycetes is composed of non-septated filamentous cells or ***hyphae***. Mycelial cells germinate by ***spores***.

Unlike fungi, actinomycetes are *prokaryotic* microorganisms *without nucleus*. As for all of the bacteria, they contain *nucleoid* as the main genomic structure.

Actinomycetes have firm and thick cell wall that leads to their *gram-positive* stain. Many of these bacteria synthesize pigments of various colors.

They efficiently grow on blood or serum agar and *Sabouraud medium* (*broth* or *agar*). Within affected tissues actinomycetes produce so-called ***druze*** – rim-like mycelial structure with interwoven filamentous microbial cells.

The reproduction of actinomycetes is performed by germinating spores located within the sporulating hyphae or *sporophore*, and by direct fragmentation of hyphae.

When the culture of actinomycetes becomes senescent, the cells of bacteria accumulate cytoplasmic vacuoles, granules, and various inclusions (fats, starch, etc.) Their mycelium becomes brittle and easily breaks down, followed by partial lysis of microbial cells.

In humans actynomycetes cause specific inflammatory disease, generally known as ***actinomycosis***.

Other families of the order *Actinomycetales* also harbor pathogenic representatives that cause serious human disorders. For instance, certain pathogenic mycobacteria from *Mycobacteriaceae* family are the agents of tuberculosis, leprosy and mycobacterioses; the basic member of *Corynebacteriaceae* family *C. diphtheriae* causes diphtheria; the members of the family *Nocardiaceae* are the agents of nocardioses.

Numerous representatives of the family *Streptomycetaceae* (more than 500 bacterial species) are the valuable sources for antibiotic substances.

Chapter 4
NUTRITION OF BACTERIA. BIOLOGICAL OXIDATION IN BACTERIA. GROWTH AND REPRODUCTION OF BACTERIA

Metabolism of Bacteria

The ***physiology of bacteria*** *studies the vital activity of microbial cells – processes of their nutrition, respiration, growth, and reproduction*.

Metabolism *is a complex of biochemical pathways providing energy accumulation and cell structure synthesis*. It is composed of two closely related sets of reactions: catabolism and anabolism.

The catabolism (energy metabolism) is a process of degradation of large molecules into more simple ones, resulting in the energy accumulation in the form of proton-motive force, ATP, or GTP.

Anabolism (the synthetic metabolism) ensures the synthesis of macromolecules the cell is created from. It uses the energy, accumulated from catabolism. Metabolism of bacteria is of high speed and provides fast microbial adaptation to varying environmental conditions.

Nutrients present in the environment or growth media must contain all the elements necessary for the microbial biosynthesis.

Autotrophs (Gk. *autos* – self, *trophe* – nutrition) are photosynthetic and chemosynthetic microorganisms capable of producing organic molecular substances from inorganic precursors. They don't need carbon of organic origin, and they build the structures of their cells by utilization of *carbon dioxide*, water, and primary nitrogen-containing inorganic compounds (ammonia and its salts, nitrates, etc.) For instance, environmental nitrifying bacteria and sulphur-containing bacteria pertain to the autotrophic microorganisms.

Heterotrophs uptake the carbon for their growth and development from any external organic source (from carbohydrates, proteins, lipids and fatty acids, etc.). It is worthy to note that organic carbon of these substances should be easily available for next assimilation.

Heterotrophic microorganisms fall into 2 basic groups depending on their source of organic matter – *saprophytes and parasites*.

1. ***Saprophytes*** (Gk. *sapros* – decaying, *phyton* – plant) assimilate organic compounds acquired from non-living matter of the environment. The majority of bacteria belongs to saprophytes. Their activity is

absolutely essential for global turnover of basic chemical elements as well as any complex substances on the Earth.

2. ***Parasites*** inhabit another living body (host organism) and exploit the host for their nutrition and/or energy donation. This group comprises relatively small amount of species of microbes that in the process of evolution have adapted themselves to parasitic mode of life.

Parasites can be divided into ***obligate*** and ***facultative***.

The ***obligate parasites*** are able to survive only intracellularly without possibility to change their parasitic mode of behavior (e.g. rickettsiae and chlamydiae).

The ***facultative parasites*** in proper situations can change their source of nutrition being capable of propagating not only within the live host, but also on artificial nutrient media.

According to possible *energy source*, ***chemotrophic bacteria*** gain energy from transformations of various chemical substances. ***Phototrophic*** bacteria obtain energy from light.

Lithotrophs (Gk. *lithos* – stone, *trophe* –nutrition) utilize some inorganic substrates as electron donors (e.g., hydrogen, sulfur, or ammonia serve as reductants), whereas ***organotrophs*** use the wide number of organic substances.

The majority of pathogenic microorganisms pertain to ***chemoorganoheterotrophs***.

Many bacteria need special ***growth factors*** for their optimal growth and development. They use vitamins; essential amino acids and fatty acids; peptides, purine and pirimidine bases, etc.

Bacteria that require one or several growth factors for their propagation are termed ***auxotrophs***.

The Mechanisms of Bacterial Nutrition and Transport of Nutrients into Bacterial Cells

Bacterial cells are generally characterized by ***holophytic type*** of nutrition. This mode of nutrition has some common essential traits:

a) there are no specialized cellular organelles for nutrition in bacteria;

b) the nutrients are absorbed by the whole surface of bacterial cell; this requires special mechanisms for their transport across the layers of bacterial envelope;

c) only relatively small molecules (usually about 600 Da or even less) can be easily delivered into bacterial cell.

In latter case many saprophytic bacteria and fungi produce the number of exo-enzymes that make *extracellular digestion* of various polymeric substrates (proteins, carbohydrates, lipids and others). These substrates undergo transformation into low molecular weight substances (amino acids, mono- and oligosaccharides, etc.) that become available for microbial cells. This is known as ***saprotrophic nutrition***.

A hydrophobic phospholipid nature of bacterial cytoplasmic membrane poses the impermeable barrier for hydrophilic nutrients delivered from outside. This resulted in creation of versatile transport systems harnessed for the delivery of nutrients into the cells and backward transportation of wastes out of the cells.

Usually such transport systems act against a concentration gradient resulting in accumulation of nutrients inside the cell. This process ultimately requires the energy in some available form.

There are four basic mechanisms providing the transport of substances across the bacterial membranes: ***facilitated diffusion, chemiosmotic-driven transport, binding protein-dependent*** (***active***) ***transport, group translocation.***

Facilitated diffusion does not need energy for transportation. It is driven by established *concentration gradient* of substances, where the external concentration of the substance is higher than internal one. This stimulates the passive diffusion of a nutrient through the cell membrane. It is evident, that substrate internal concentration never overcomes the levels of its external concentration. As an example, glycerol is one of the few substrates that enter into bacterial cell by the mechanism of facilitated diffusion.

Chemiosmotic-driven transport provides the translocation of molecules across the cytoplasmic membrane using the energy of primarily established membrane gradient of protons, known as ***proton-motive force***. It also involves other ions, such as Na+.

Three basic kinds of chemiosmotic-driven transport are determined: ***uniport, antiport*** and ***symport***.

Uniporters carry the substrate across the membrane regardless of any other accompanied substance. ***Antiporters*** stimulate the parallel delivery of two similarly charged substances in opposite directions using the same carrier (e.g., H+ and Na+). And ***symporters*** provide the simultaneous movement of two substances towards the same direction by a common carrier. For example, an established H+ gradient activates the symport of certain oppositely charged compounds (like amino acid glycine) or the neutral nutrients (such as galactose).

Chemiosmotic-driven mechanism plays a substantial role in trans-membrane transport in bacteria. For instance, more than 40% of nutrients, acqusited by *E. coli*, exploits chemiosmotic-driven transport.

Binding protein-dependent (or active) transport is *energy-dependent* nutrient delivery across the cytoplasmic membrane against the existing concentration gradient. It is governed by specific substrate-binding proteins. They transfer the substrate to specialized membrane-located protein transport complex. In gram-negative bacteria these complexes are present within the periplasmic space. The process of transportation requires ***ATP energy*** or in some situations other high-energy substances (e.g., acetylphosphate).

Another 40% of nutrients, delivered for *E. coli*, uses this universal mechanism.

Group translocation as the mechanism of trans-membrane transport is characterized by temporary change of structure of translocated substances. It is used, for example, for successful uptake of nutrient sugars by bacterial cells (e.g., glucose or mannose). This process is performed by bacterial phosphoproteins. They posphorylate the sugars outside the membrane and move them into the cell in posphorylated form.

Secretion Systems for Transport of Proteins and Other Substances Outside the Bacterial Cells

Intensive metabolism of bacteria requires continious controlled transport of bacterial high molecular weight substances (primarily, proteins) out of the microbial cells to the extracellular environment. A tremendous number of biologically active molecules is secreted by bacteria (enzymes, toxins, signalling messengers, genetic elements and plenty of others). They play the decisive role in bacterial physiology and pathology.

Well-studied are various ***types of protein secretion systems***, organized in ***gram-negative*** bacterial cells.

To date, ***7 types*** of secretion systems for proteins are found; six of them are the attributes of gram-negative bacteria, whereas type VII is determined in mycobacteria.

Bacterial secretion systems include *translocator* and *effector* proteins. ***Translocator proteins*** build the structural units of secretion systems and serve for their proper function, thus providing the transport of effector proteins. ***Effector proteins*** are biologically active molecules (enzymes ot

toxins) that are secreted by bacteria and develop their specific activity outside the bacterial cell.

Types I-VI of protein secretion in gram-negative bacteria are different in their structure and function. Overall, gram-negative cell wall with its hydrophobic outer membrane and LPS creates a serious barrier on the way of translocated proteins.

In this vein, the types I, III and VI perform *one-step secretion* of proteins across the envelop of gram-negative bacteria, whereas the types II, IV and V elaborate *two-step secretion*; in latter case the proteins are first delivered into periplasmic space and next transported out of the microbial cell across the outer membrane.

The most simple is ***type I*** secretion system (or T1SS). It includes 3 distinct proteins – cytoplasmic membrane *ATPase* with ATP-binding cassette (*ABC-transporter protein*) that initiates the process and provides energy for molecular transport; *membrane fusion protein* that makes the channel, penetrating the periplasmic space; and *outer membrane protein*, located within the outer membrane. The last protein plays a role of a "gatekeeper" of channel outlet, switching its activity into proper state.

T1SS provides the excretion of certain groups of toxins (predominantly, hemolysins) by gram-negative bacteria.

The ***types III*** and ***VI*** of ***protein secretion*** are the basic systems for ***delivery of bacterial virulence factors*** into affected cells. They make ***injectisome*** or "*needle complex*" protruding outside from bacterial cell. After primary contact with the membrane of the host cell, the needle complex activates and injects the effector virulence proteins into target host cells.

Most of gram-negative bacteria (e.g., shigellae, salmonellae, or *Pseudomonas aeruginosa*) use these pathways for secretion of multiple virulence factors.

The systems of ***II***, ***V*** and partially of ***IV types*** (with two-step secretion of molecules) use special *secretory or Sec proteins* for initial transport of proteins from the cytoplasm into periplasmic space. The transported molecules are primarily synthesized on the ribosomes as *pre-proteins*. These pre-proteins bear additional *signal sequence* that prevent them from degradation during transport. At first step Sec-proteins deliver them into periplasmic space, where signal sequence is removed by proteolysis. At second step the molecule is transported across the outer membrane outside the cell.

T2SS facilitates the secretion of extremely high variety of molecules by gram-negative bacteria. Among them are enzymes

(e.g., phospholipase C of *P. aeruginosa*) and toxins (e.g., *Vibrio cholerae* toxin). Thus, T2SS is denoted as "***general secretory pathway***" in gram-negative bacteria.

T5SS is responsible for secretion of several enzymes and toxins, such as vacuolating or Vac toxin of *Helicobacter pylori*. Unlike other pathways, the secreted proteins of T5SS, if appeared in periplasmic space, play a further role of *autotransporters* – the tail part of transported molecule makes a channel within the outer membrane and provides its final excretion.

The activity of ***T4SS*** resembles to some extent the bacterial conjugation. Therefore, T4SS delivers not only the vast number of virulence proteins (e.g., Cag toxin of *H. pylori*, the toxins of pertussis bacteria or legionellas) but also the mobile genetic elements to the recipient bacteria. The exchange of genetic material accelerates the adaptive capacity of bacterial strains including the spread of the resistance to antibiotics and antiseptics.

The secretion of proteins in ***gram-positive bacteria*** is not completely elucidated. They may perform direct protein secretion via the channels within peptidoglycan cell wall. Futhermore, according to the currently known data, gram-positive microbes use the similar principles of protein secretion as gram-negative bacteria. For instance, they use Sec-proteins for protein translocation across the cytoplasmic membrane. After the removal of signal sequences, the transported proteins are introduced into external layers of the cell wall.

Recently a new ***type VII*** secretion system (***T7SS***) was described in *M. tuberculosis*. It is capable of secreting mycobacterial toxic proteins that provide the survival of mycobacteria within phagocytes. A homologous systems were also found in pathogenic cocci, e.g. *S. aureus*.

Bacterial Cultures – Optimal Conditions

Basic methods of laboratory diagnosis in microbiology presume the ***isolation of the microbial culture*** for its further *identification* (i.e., the determination of microbial genus and species).

To aim this, in laboratory conditions the bacteria are cultured in various nutrient media at a constant optimal temperature.

The ***optimal temperature*** *range* is of great value for the successful propagation of bacterial cells.

Depending on temperature, suitable for their growth and reproduction, all microbial agents are divided into several groups:

– ***psychrophilic*** microorganisms (Gk. *psychros* – cold, *philein* – love) that have the permissible temperature range from -10-0^{o}C up to 25-30^{o}C with optimum about 10-20^{o}C;

– ***mesophilic*** microorganisms (Gk. *mesos* – intermediate) that live in the range from 10-25^{o}C up to 40-45^{o}C with optimum about 20-40^{o}C;

– ***thermophilic*** microorganisms (Gk. *thermos* – warm) that prefer the temperature range from 25-45^{o}C up to 70-80^{o}C with optimum with 50-60^{o}C.

These grades indicate that the bacteria cover a broad temperature scale for their growth – at least, from +10 to +80°C.

Most of *pathogenic representatives* pertain to *mesophilic bacteria*. The are cultured within the moderate temperature range of 20 to 45°C with optimal temperature near 37^{o}C.

Likewise, the optimal *concentration of hydrogen ions* or ***pH*** *of the medium* is of great significance for microbial propagation as well. Overall, the saprophytic microbes can live within the long range of pH values – from extremely acidic (pH~0.6) up to highly alkaline (as pH about 11). Pathogenic bacteria are characterized in most cases by relatively narrow range of optimal pH – within 6.0-8.0.

Similarly, the *total concentration of ions* or ***ionic strength*** (e.g., concentration of Na^{+} or Cl^{-}) as well as *osmotic pressure* play a substantial role in normal growth and function of bacterial cells. In general, the bacteria demonstrate the high limits of salt tolerance. Nonetheless, for optimal growth of the most of bacteria, the medium sholud be closer to isotonic (~0.15 M NaCl). By contrast, some groups of bacteria termed as ***halophiles*** prefer the increased concentration of salts – in the range from 0.3 M of sodium chloride to more than 5 M that is equivalent to 30% of NaCl. As an example, the causative agent of cholera, *Vibrio cholerae,* is the halophylic bacterium.

The microbial agents that can live and propagate far beyond the natural limits for habitation of conventional microorganisms are called ***extremophiles*** – for their life within the extremal conditions. For instance, hyperthermophils can thrive at temperature 100-120^{o}C, deep psychrophiles – at -15^{o}C, alkaliphiles – at pH>9.0, already mentioned halophiles – at 20-30% NaCl concentration, xerophiles – under extremely dry desert conditions. The existence of such bacteria clearly expands the borders for life; on the other hand, they are used as valuable sources of biological

products with outstanding characteristics (e.g., microbial strains for wastes degradation, thermostable enzymes, etc.)

Main Principles of Microbial Culture. Nutrient media

Nutrient media for culture must be easily assimilable by microorganisms.

Therefore, they have to contain a certain amount of ***organogens*** (*vitally required elements – oxygen, carbon, hydrogen, nitrogen, phosphorus*), ***growth factors*** (vitamins, some carbohydrates, microelements, amino acids etc.), and necessary concentration of salts. In addition, they should be sterile, isotonic, maintain optimal buffer properties, viscosity, and proper reduction-oxidation (or ***redox***) potential.

Notably, the bacteria should be cultured at optimal temperature. The closed chamber, mantaining the required temperature for culture, is known as *thermostat*.

L. Pasteur and his collaborators introduced non-protein media for the cultivation of microbes. Then R. Koch with co-workers proposed meat broth, peptone, and sodium chloride as essential components for satisfactory microbial growth.

As the result, a ***meat-peptone broth*** or ***MPB*** (otherwise, beef-peptone broth) and ***meat-peptone agar*** (***MPA***) were successfully introduced into microbiological practice as *basic nutrient media*. Meat-peptone agar is prepared by adding to the medium 2-4% of agar.

Agar (from Malayan – *jelly*) is a fibrous matter produced from certain seaweeds that creates a solid gel, when solidifies in water solutions. Agar is predominantly composed of polysaccharides (70-75%); also it includes proteins (about 2-3%) together with other nitrogen-containing substances, and 3-4% of ashes. The major constituents of agar are the highly polymeric carbohydrates – *agarose* and *agaropectin*. Agar easily dissolves in hot water and hardens at room temperature. It is distributed as colorless powder ready to use.

In order to design an appropriate liquid medium M. Hottinger proposed to use the tryptic breakdown of proteins that contain free amino acids and short polypeptides. Similarly, L. Martin used plant derived proteolytic enzyme papain for the breakdown of proteins.

All the scope of nutrient media is classified into certain groups depending on their laboratory use.

According to ***composition*** the nutrient media are divided into *ordinary* (*simple*) and *complex* media.

Ordinary or ***simple media*** usually have single basic component. There are only few examples of such products, e.g. gelatin or coagulated serum.

Complex media contain many components, being the combination of various compounds.

All complex media are composed on the ground of ***basic nutrient media*** supplemented with other nutrients and growth factors. Essential basic nutrient media included into the most of other complex media are ***meat-peptone broth*** and ***meat-peptone agar***.

In ***consistency*** nutrient media may be ***solid*** (meat-peptone agar, coagulated serum, gelatin, etc.), ***semisolid*** (the medium contains about 0.5-1% of agar), and ***liquid*** (meat-peptone broth, sugar broth, Mueller Hinton broth, etc.)

According to their ***destination*** the nutrient media are also divided into several groups.

Differential media are employed for determination of differencies in *biochemical activity* of bacteria.

There are the media for the determination of fermentation of carbohydrates, for instance, liquid ***Hiss' media*** for identification of enterobacteria.

In tube version of these media every tube includes MPB, one kind of mono- or disaccharide, the tube (float) for gas accumulation and acid-base ***indicator dye***, for example – fuchsin, decolorized with 1 M NaOH. If grown bacteria ferment carbohydrate, the color of indicator is changed due to appearance of aldehydes and acid products of carbohydrate degradation.

The multi-well plate version of similar media includes the broad panel of sugars for bacterial identification. This enables parallel mass screening of biochemical activities of many isolated microbial strains, followed by automated colorimetric registration of the results and their computer analysis.

Also there are numerous solid media for the differentiation of bacteria by *lactose* fermentation (***McConkey*** agar, ***Endo*** agar, ***eosin-methylene blue*** or ***EMB*** medium, etc.); for determination of the proteolytic action of microbes (gelatin, MPB with indicator dyes), media for the determination of hemolytic activity (blood agar); media for evaluation of oxidative and reductive activity of microorganisms and many others.

Selective media create the growth conditions permissive to only definite species of bacteria; and all other microbial species should be suppressed or don't grow at all. These media include *biocide* components

that inhibit the majority of outside species except the investigated one (for instance, bismuth sulphite agar for *Salmonella enterica var. typhi* culture, or egg yolk salt agar for *S. aureus* with 7,5% NaCl).

Nutrient media that contain antibiotics are selective for antibiotic-resistant strains, inhibiting the propagation of strains, susceptible to these antibiotics.

Special media are used for the growth of fastidious bacteria that are hard for culture. Usually they include a plethora of growth factors ensuring the beneficial conditions for microbial reproduction (***ascite agar*** for gonococci, complex hemin-containing media for *Hemophilus influenza* culture, Mueller Hinton agar with blood for testing antibiotic susceptibility of streptococci, etc.)

Enrichment media are also implicated for culture of the questioned species, which grows more actively and more rapidly than concomitant bacteria. They can be used for primary inoculation of clinical specimen. In number of cases they include biocide to suppress accompanying bacteria. In this vein, for instance, ***alkaline peptone broth*** serves as enrichment and selective medium for cholera vibrio.

Transport media provide the delivery of microbial-containing clinical specimen to the laboratory without damage of microbial cells. They may support the growth of pathogenic bacteria and damp the activity of saprophytes. As an example of such a medium, it can be glycerol-containing phosphate buffered saline.

Synthetic media are composed of chemically-defined synthesized components of known standard structure.

Now all nutrient media as well as their components are manufactured and commercially delivered as dry powders. They are stable, convenient for routine laboratory work and demonstrate high efficacy of microbial isolation.

Evaluation of growth properties (or ***cultural characteristics***) of isolated microorganisms enhances the accuracy of their final identification.

When cultured on solid nutrient media, the microbes produce the ***colonies*** of different sizes and shapes.

Microbial ***colony*** is the *isolated group of microorganisms*, grown on *solid* nutrient media, which are the *offsprings of one microbial cell*.

Hence, the microbial cells of one colony form the single genetic clone. Clonality of colonies provides successful discrimination and identification of representatives of all microbial taxa present in clinical specimen.

The colonies possess the number of intrinsic descriptive characteristics.

According to their ***sizes***, the colonies can be separated into *large* (more than 4-5 mm in diameter), *intermediate* (2-4 mm), *small* (1-2 mm), and *dwarf* (less than 1 mm).

The ***shapes*** of the colonies are also variable – the basic types are regular or irregular *round-shaped*, also they can be *rosette-shaped*, *tree-like*, *star-shaped*, etc.

The ***edges*** of colonies are characterized as *straight* or *irregular*, *fibrous*, *serrated*, *tasseled* and others

According to their ***plane***, the colonies may be *flat*, *convex* or *pitted*, *dome-shaped*, etc.; depending on colony ***surface*** – *smooth* (***S-forms***) or *rough* (***R-forms***), *bumpy* or *ridged*.

The colonies differ in their density, ***consistency***, and ***color***. They may appear as *slimy*, *moist*, or *dry*; *transparent* or *opaque*, *pigmented colored* or *colorless*.

Inner structure of colony is studied by optical microscopy with low-level magnification.

The *growth* of bacteria *on liquid media* displays diffuse turbid suspension, or visible bottom precipitate, or the biofilm, attached to the inner surface of the well or test tube

The Production of Pigments by Microorganisms

Many species of bacteria and fungi that live in various environmental conditions are able to produce ***pigments***.

Colonies of these bacteria can be colored gold (as for *Staphylococcus aureus*), yellow (*Sarcina flava*), white (*Staphylococcus saprophyticus*), blue (*Pseudomonas aeruginosa*), red (as for *Serratia marcescens*, or actinomycetes and yeasts), pink (*Micrococcus roseus*), violet (*Chromobacterium violaceum*), black and brown (*Prevotella melaninogenica*, yeast and mould fungi). Some microorganisms can synthesize two or more pigments.

The production of pigments by bacteria and fungi is stimulated by sunlight, culture aeration and temperature shift to 20-25^{o}C or less.

The pigments can be further analyzed according to their solubility – soluble in water (produced by *Pseudomonas aeruginosa*), soluble in alcohol and insoluble in water (*Serratia marcescens* and *Staphylococcus aureus* pigments), insoluble in water and alcohol (*Prevotella melaninogenica*, black and brown pigments of yeasts and moulds).

According to their chemical structure, pigments are divided into several distinct groups.

Carotenoid pigments of orange or yellow color are produced by *Mycobacterium tuberculosis*, *Actinomyces spp.* and some other bacteria.

Quinone pigments are usually yellow. They are synthesized by actinomycetes, yeasts, etc.

Melanin pigments are produced by some kinds of fungi (*Aspergillus niger*), bacteroids and related bacteria (*Prevotella melaninogenica*).

Pyrrole pigments are red or orange. The example of bacteria producing this pigment is *Serratia marcescens* (*prodigiosin* pigment).

Phenazine pigments are green-blue. Such a pigment (***pyocyanine***) is produced by *Pseudomonas aeruginosa*.

Pigment production in bacteria has the evident physiological value. In the process of bacterial respiration the pigments are supposed to work as electron and hydrogen acceptors. Also they are able to confer protection against natural ultraviolet radiation and were shown to possess certain antibiotic activity.

Microbial Enzymes and Their Role in Metabolism

Enzymes as ***biological catalysts*** are the key proteins of cellular metabolism. Usually they have a *complex protein nature*, and demonstrate relatively strict catalytic *specificity* .

In large part the enzymes predetermine the total behaviour and properties of bacterial cell. They catalyze (speed up) all the scope of cellular chemical reactions. In the absence of an enzyme, the ***substrate*** is transformed into a reaction product so slowly that it is even less likely to measure the product's formation. By contrast, the enzyme converts the substrate into a product in a short time.

Enzyme action leads to chemical changing of substrate molecules. It results from stabilization of substrate transition state by weak but specific binding forces within enzyme-substrate complex that accelerates substrate transformation. Enzyme-substrate binding stresses chemical bonds of the substrate enough to break them down with subsequent formation of new bonds.

Enzymes act in two steps. Initially the substrate binds to a specific location of the enzyme, known as ***active*** or ***catalytic site***, to create an *enzyme-substrate complex*, according to *"lock-and-key"* or *“induced fit”* mechanisms. After chemical transformation the products of the reaction are

released, making the enzyme active site free to bind to new substrate molecules.

International classification divides enzymes into 6 major classes:

1. ***Hydrolases***, which catalyse the cleavage of the links between the carbon, oxygen, nitrogen or sulphur atoms in watery solutions with addition of one molecule of water (esterases, proteases, glycosidases, nucleases, etc.).

2. ***Transferases*** perform the transfer of certain groups and residues from one molecule to another – *intermolecular transfer* (aminotransferases, transacylases, transglycosidases, etc.)

3. **O*xidoreductases*** catalyse the reactions of oxidation and reduction, resulting in electron transfer from the reductant (electron donor) to the oxidant or electron acceptor (oxidases, oxygenases dehydrogenases, catalases and others).

4. ***Isomerases*** create substrate isomers resulting from *intramolecular* substrate *rearrangement*. They play an important role in bacterial carbohydrate metabolism (phosphohexoisomerase, phosphoglucomutase, racemases, etc.) Also they may cause conformational substrate changing.

5. ***Lyases*** cleave the bonds in molecules via non-hydrolytic manner with intramolecular double bond formation (e.g., bacterial hyaluronate lyase).

6. ***Ligases*** or *synthetases*, which catalyze synthetic reactions, thereby coupling molecules together (DNA ligase, acetyl-CoA synthetase, chelatases, etc.)

Some enzymes are excreted by bacteria into external environment (***exoenzymes***) to participate in extracellular digestion of nutrients or toxicants, whereas other enzymes work inside the microbial cell (***endoenzymes***).

The ***constitutive*** enzymes of bacteria provide their basic metabolic reactions, being constantly expressed regardless of presence or absence of substrate. This group comprises the essential enzymes of cellular metabolism (ATP synthase, nucleases, proteases, oxidases, lipases, etc.) The synthesis of ***adaptive*** enzymes commences only after the appearance of corresponding substrate (beta-galactosidase, alkaline phosphatase, penicillinase, and many other adaptive enzymes).

An important role is played in bacteria by ***aggression and invasion enzymes***, which facilitate microbial spread in the affected host, destroying body tissues (***hyaluronidase***, ***collagenase***, etc.) or blocking the action of antimicrobial drugs (***beta-lactamases*** for beta-lactam antibiotics), or

rendering ***toxic effects*** (phospholipase C of *C. perfringens* or zinc endopeptidase of tetanospasmin toxin of *C. tetani*).

Overall, there is a tremendous variety of enzymes produced by microorganisms that demonstrate extremely high efficacy. The enzymes of microbial origin are broadly used in industry and agriculture, biotechnology and medicine.

For instance, microbial enzymes (e.g., restriction endonucleases, polymerases, proteases, and many others) are actively used in genetic engineering, antibiotic synthesis, production of polysaccharides and alkaloids, synthesis of steroid hormones and other valuable substances. Enzymes as biological products for medicine have an expanding field of clinical applications including the treatment of certain diseases (e.g., administration of streptococcal streptokinase in myocardial infarction).

Practical Use of Enzymatic Properties of Microbial Cultures

As was mentioned above, closely related species of bacteria can often be distinguished by their biochemical activity.

In laboratory practice the specific biochemical properties of bacterial cultures are used for their precise identification. In most of cases carbohydrate fermentation, protease and oxidoreductase activity of microbes are evaluated.

Carbohydrate fermentation is estimated on panels and plates containing dozens of various chemical substrates for fermentation like in Hiss' media rows. Accumulation of acid products of fermentation is indicated by dye color change, and gas appearance can be revealed within the special tube (float) or by automated gas analyzer.

Proteolytic activity can be visually assessed in special protein-containing media (e.g., gelatin hydrolysis) or according to monitoring of the end-products of protein chemical decay. In lattter case the indicator stripes with necessary reagent (lachmus for ammonia, oxalate for indole detection, and lead acetate for hydrogen sulphide determination) are placed into the wells or near the outlet of the test tubes with substrate meat peptone broth (MPB). Being cultivated, the bacteria hydrolyze the peptones of broth releasing the end products of protein depolymerization (ammonia, indole or hydrogen sulphide).

Also various *synthetic labelled substrates* are used either for assessment of carbohydrate hydrolysis or proteolytic activity. After the

incubation of substrates with microbial cultures the results are registered by colorimetric or fluorimetric detection.

Catalase activity is usually assessed by a simple test, where the loop of microbial culture is added into the drop of hydrogen peroxide. Bubbles of gas indicate the hydrogen peroxide conversion into O_2 and H_2O.

Urease microbial activity is observed in urea test hydrolysis. The accumulation of ammonia that released after urea decay eventually rises medium pH, and the change of color of indicator dye (e.g., phenol red) is observed.

Biological Oxidation in Bacteria

The nutrients, acquisited by microbial cells, contain low amounts of energy in each of chemical bonds within their molecules. The total energy is distributed throughout the molecule and it is not readily available for direct assimilation.

The pathways of energy metabolism comprise the sets of versatile enzymatic reactions resulting in slow stepwise concentration of diffuse energy of many low-energy chemical bonds of various nature into a few high-energy equivalents (such as macroergic phosphate-containing substances or proton-motive force). The latter forms can be easuly utilized by the cell.

The energy is eventually transferred to *adenosine diphosphate*, (***ADP***) to form ***adenosine triphosphate,*** abbreviated ***ATP,*** which is used like universal energy storage in the cells. The same function is maintained by ***guanosine triphosphate***, or ***GTP***.

Within ATP molecule the energy is concentrated by two high-energy bonds (tagged by the symbol ~). After ATP or GTP enzymatic cleavage these bonds break down, and the large amounts of energy are liberated.

ATP-derived release of energy can be outlined according to the following general scheme of the reaction:

ATP = ADP + (inorganic phosphate P) + *Energy*

Accumulation of energy in bacteria is predominantly realized via the process of ***biological oxidation***.

Chemoheterotrophic bacteria, because of their dependency on some organic source for energy, exploit ***two basic pathways of biological oxidation*** that collect the diffuse energy of “fuel” nutrient molecules within the high-energy of ATP bonds.

One pathway is termed ***fermentation***, the second one is ***respiration*** (aerobic or anaerobic).

Together with energy gain, all the pathways create a number of valuable *precursor metabolites* – intermediate organic substances that should be further transformed into the structural subunits of biopolymers (e.g., amino acids) after a set of specific biochemical reactions.

Fermentation

Fermentation pathway is characterized by its essential trait, where an ***organic compound*** (e.g., pyruvic acid) is the *final electron acceptor* for the electrons removed from substrates of this pathway. Furthermore, this final acceptor is directly *produced in the pathway*; thus, no external acceptor required.

As the result, by contrast to aerobic respiration, fermentation doesn't need molecular oxygen as common electron acceptor. When accomplished, it produces a variety of low-energy compounds, termed ***end products***; the nature of these products strongly dependent on the species of bacteria.

Different species of bacteria can utilize extremely broad number of organic substances to obtain energy and reducing power by means of various metabolic pathways.

Glycolysis (Gr. *glycos* – "sugar" and Gr. *lysis* – "dissolution") is the most common pathway for degrading sugars. It is often termed as ***Embden-Meyerhoff pathway***, named after two scientists, who identified its major steps in the 1930s.

Many bacteria and yeasts use this pathway to degrade glucose and other sugars. Here *one glucose molecule* is converted into *two molecules of pyruvic acid*, as well as into reducing power equivalents such as hydrogen atoms.

Pyruvic acid, generated from a primary set of reactions, is used further for both fermentation or respiration.

The differences in molecular energy between glucose and pyruvic acid are accumulated in high-energy bonds in ATP. ATP synthesis results from ***substrate phosphorylation***, where energy-rich phosphate anhydride bond is directly transferred from organic donor molecules to ADP. The final ATP yield in fermentation is equal to 2 ATP molecules.

In the absence of respiration or photosynthesis, mirobial cells are completely dependent on substrate phosphorylation to gain energy. Therefore, in this case ATP synthesis ensues from chemical

transformations of primary organic substances. A great variety of substrates are metabolized within the diverse fermentation pathways.

For instance, a lot of bacteria may perform the lactic acid fermentation. Many of them produce only a single product (lactic acid) being called as ***homofermenters.*** Nonetheless, some other lactic acid-producing bacteria are ***heterofermenters***, releasing CO_2 and ethanol, as well as lactic acid end products.

Closely related species of bacteria can be reliably discriminated by their products of fermentation.

Due to the fermentation activity of acetic acid bacteria *(Acetobacter spp.)* acetic acid is formed (***acetic acid fermentation***).

Ethanol or alcoholic fermentation takes place under the influence of the enzymes of yeasts *Saccharomyces cerevisiae* (yeasts), mucor moulds, *Zymomonas mobilis* bacteria, etc.

Lactic acid fermentation is caused by the fermentative activity of *Lactobacillus casei*, *Lactococcus lactis*, etc. The enzymes of lactic acid bacteria break down glucose with the production of lactic acid. The representatives from family *Enterobacteriaceae* are heterofermenters and produce lactic, succinic, and acetic acids, ethanol, carbon dioxide and hydrogen.

Butyric acid fermentation is performed with the anaerobes *Clostridium perfringens* or *Clostridium butyricum* resulting in the production of butyric acid

Propionic acid fermentation is demonstrated by anaerobes from genus *Propionibacterium spp.*

Other Pathways of Glucose Degradation

The major scheme for the degradation of glucose harnesses the glycolytic pathway. However, not all bacteria are able to use this process. They derive energy through another pathways.

The alternate route is termed the ***Entner-Doudoroff pathway*** or 2-keto-3-deoxyphosphogluconate pathway. It gives pyruvic acid from glucose oxidation via 2-keto-3-deoxyphosphogluconic acid intermediate. This pathway generates only 1 molecule of ATP after transformation of 1 molecule of glucose as substrate.

Another sugar metabolizing pathway that is present in most bacteria is termed the ***pentose phosphate pathway***. The pathway is not of great significance for the production of energy, but it is valuable as the products

of the pathway are 5-carbon and 4-carbon molecules that serve as precursor metabolites for nucleic acid and amino acid synthesis. Further, it provides reducing power required in the biosynthesis of cell components. This pathway is important in the organisms that carry out fermentations where reducing power (NADH) is not available for biosynthetic reactions.

Both of these pathways as well as glycolysis, can operate in the presence or absence of oxygen, Nevertheless, Entner-Doudoroff pathway is most likely found in aerobic bacteria, e.g. *Pseudomonas aeruginosa*.

Bacterial respiration

The second metabolic pathway of biological oxidation in bacteria is termed ***respiration*.**

In this pathway, an inorganic substance serves as the final acceptor of electrons or hydrogen atoms.

When oxygen plays a role of the final electron acceptor, it is known as ***aerobic respiration***. This case the end products of respiration pathway are H_2O and CO_2.

In anaerobic bacteria *inorganic substances* or ions *other than* O_2 serve as terminal oxidants in respiration process. This ability, essential for many microbial groups, is termed as ***anaerobic respiration***. Suitable electron acceptors for anaerobic respiration include *nitrate*, *sulfate*, *carbon dioxide*, and some others.

Respiratory metabolism dependent on carbon dioxide as final electron acceptor is an intrinsic property of members of distinct prokaryotic domain of archaebacteria. In particular, archaebacteria are capable of reducing carbon dioxide to acetate, thus generating energy for their cells.

Basic chemical mechanism for generation of ATP associated with respiration includes the addition of inorganic phosphate to ADP molecule. In ground state this reaction is energetically unfavorable; therefore, it needs energy support. Energy for ATP synthesis is acquired from proton gradient, established on opposite sides of bacterial cytoplasmic membranes and their specialized structures (mesosomes).

Therefore, respiration is a *membrane-located pathway*. In process of biological oxidation electrons pass from a chemical reductant to a chemical oxidant by specific set of electron carriers associated with cytoplasmic membrane. This leads to the creation of electrochemical proton gradient across the membrane, resulting in generation of *proton motive force*.

Backward flow of protons through the membrane is coupled with the synthesis of ATP by ATP synthase enzyme.

In aerobic respiration the biologic reductant is commonly NADH, and the oxidant is oxygen.

In the process of fermentation bacterium extracts only a very small fraction of the total energy available in glucose molecule. The energetic yield of fermentation is 2 molecules of reduced NADH and final 2 molecules of ATP – 4 are newly synthesized, whereas 2 are consumed within the pathway.

In respiration the large amounts of energy are gained as the result of biological oxidation, following the transfer of electrons from a high-energy to a low-energy levels through the set of membrane-associated enzymes of ***electron transport chain***, also termed as the ***respiratory chain***. Proton-motive force, generated by this mechanism, causes phosphorylation of ADP into high-energy ATP molecules. This reaction is named as ***oxidative phosphorylation***.

The high yield of energy of oxidative phosphorylation ensues from the further oxidation of pyruvic acid into compounds with less sum of bond energy. This process is accomplished by the enzymes of ***tricarboxylic acid*** (***TCA***) ***cycle***, or the ***Krebs cycle***.

The TCA cycle generates a lot of energy as the acetic acid (in form of acetyl CoA) is oxidized to final low-energy products CO_2 and water. Total ATP yield from oxidative phosphorylation here is in the range of 30-36 ATP molecules.

In addition, several compounds formed in the TCA serve as the important precursors for the next cellular metabolic reactions.

In case of anaerobic respiration, the total energetic yield is expectedly lower than in aerobic one.

Overall, the processes of respiration in bacteria are very complex and include a long chain of oxidation-reduction reactions with the participation of many enzymatic systems transporting the electrons. The detailed biochemical mechanisms of respiration are described elsewhere within biochemistry course.

Classification of Bacteria According to Their Respiration Type

Bacteria can be classified into five major groups based on their requirements for oxygen. Usually the bacteria are supplied with air that contains about 20% of oxygen .

1. ***Obligate*** (or ***strict***) ***aerobes.*** These microorganisms have an absolute requirement for oxygen because they metabolize sugars through aerobic respiration. Since oxygen is not enough soluble in watery solutions, aerobes grow best in cultural vessels with continuous stirring or shake that enhances oxygen circulation. Members of this group pertain to the genera *Bacillus, Pseudomonas* and to many other microbial taxa (brucellae, meningococci, mycobacteria, etc.)

2. ***Obligate*** (or ***strict***) ***anaerobes.*** The representatives of this group can't propagate even at small amounts of oxygen. Some of them are literally killed by the contact with traces of oxygen as they are unable to detoxify the active forms of oxygen generated in metabolism. Moreover, some of their enzymes are inactivated by oxygen. The anaerobic bacteria encompass many microbial genera, such as the sporeforming genus *Clostridium* (causative agents of tetanus, botulism, etc.), or the numerous group of non-sporeforming gram-negative anaerobic bacteria that comprises bacteroids, prevotellae, porphyromonads, fusobacteria, and others.

3. ***Facultatively anaerobic bacteria***. These microbial agents can utilize oxygen as terminal oxidant if available, but can also propagate in its absence. It is obvious, that their metabolism is more rapid under oxygen consumption – more ATP molecules are synthesized by aerobic respiration. Typical examples of facultative anaerobes are *Escherichia coli* and *Saccharomyces* yeasts.

4. ***Microaerophilic bacteria***. They need small or moderate amounts of oxygen, (2% to 10%), as the higher concentrations are deleterious. Not so many pathogenic microorganisms pertain to this group, e.g., *Helicobacter pylori,* the agent of gastritis and gastroduodenal ulcer. Inside its natural habitation site, mucous gastric epithelium, only the low levels of oxygen are available. In addition, microaerophiles usually grow more actively in presence of elevated concentrations of carbon dioxide (***capnophiles***). To support this, microaerophiles can be cultured in anaerobic jars with controlled supply of CO_2 to final concentrations of 3-5%. At the same time the concentration of remaining oxygen is enough to prevent the growth of strict anaerobes.

5. ***Aerotolerant bacteria***. These microbials are indifferent to the presence or absence of oxygen, capable of growing in both conditions. However, they dont't use oxygen for biological oxidation. Many aerotolerant bacteria also grow more actively under the increased concentrations of carbon dioxide (*capnophilic bacteria*). Therefore, they

are better cultured in conditions of high humidity within the special chambers – CO_2-incubators with microprocessor gase control.

As an example, *Streptococcus pyogenes*, the causative agent of streptococcal sore throat, is an aerotolerant microorganism.

Enzymatic Differences Between Aerobes and Anaerobes

Aerobes require oxygen because they have metabolic pathways that utilize oxygen to convert the energy of primary substrates into the readily available form of ATP. Anaerobes have another pathways that may result in similar end products, but they don't require molecular oxygen for their realization.

On the routes of cellular metabolism oxygen can be transformed into a number of forms that are highly biochemically active and therefore toxic, mainly *hydrogen peroxide* and *superoxide*. Cells that survive in the presence of oxygen contain enzymes converting oxygen metabolites into non-toxic forms. For instance, aerobic bacteria contain the enzyme ***catalase***, which breaks down hydrogen peroxide, and ***superoxide dismutase***, which neutralizes superoxide anion. Strict anaerobes usually don't possess these enzymes. As an exception, some of strict anaerobes may carry certain amounts of superoxide dismutase or catalase.

Aerobes have got the adaptation to live at higher redox potential of the medium, anaerobes – at a lower one. It is worthy to note that anaerobic bacteria are not the passive bystanders on the growth – they are capable of active decreasing of the medium redox potential after intensive metabolic reactions. Thus, anaerobes demonstrate the marked capacity to re-organize the microsurroundings for their own needs.

Anaerobic culturing

Anaerobes demand the special methods for their culture. Since the growth of anaerobes is inhibited by oxygen, they should be protected against its deleterious activity.

For successful culturing of anaerobes it is mandatory to inoculate the large amounts of clinical specimen into the nutrient medium. The specimen is commonly taken by tissue puncture into closed syringe and immediately transferred into appropriate anaerobic medium. The nutrient medium should maintain a proper viscosity by balancing of certain agar

concentrations. The dissolved air is eliminated by boiling prior to inoculation, and subsequent contact with air is prevented by covering of the medium with mineral oil (liquid vaseline) with a layer of 0.5-1 cm thick. Anaerobiosis can be created by the adsorption of oxygen with reducing chemicals or by adding of porous absorbing substances (like the charcoal).

For anaerobic culture transportation the ***thioglycolate broth*** is widely used. It contains sodium thioglycolate (the salt of mercaptoacetic acid) that is a strong reducing agent for elimination of dissolved oxygen. After primary inoculation the medium-containing test tubes with specimens should be tightly closed.

Another medium for anaerobic cultivation is ***iron sulphite agar***, that contains MPA, glucose, iron citrate or chloride, and $NaHSO_3$. It is prepared as high butt of agar covered with liquid vaseline. Microbial inoculation is made like deep agar culture. The growth of anaerobes (e.g., *Clostridium perfringens*) is followed by the blackening of the medium from the production of hydrogen sulfide.

The growth of the anaerobes can be also produced within a ***Kitt-Tarozzi medium*** composed of broth, 0.5% of glucose and pieces of animal organs (as liver) or minced meat for oxygen absorption. The top of medium is covered with liquid vaseline.

Deep agar cultures are performed by ***stab inoculation*** of the specimen into long tubes filled with solidified anaerobic nutrient medium, covered with mineral oil.

For plate inoculation anaerobe ***Shaedler agar*** is commonly used. It includes protein enzymatic digest, yeast extract, D-glucose (dextrose), hemin, L-cystin, and agar. For anaerobic culture Shaedler agar sholud be put into anaerobic jar.

The most advanced and effective are the methods of *apparatus-based anaerobic culturing*. They use special closed cylinders – ***anaerobic jars*** (or ***anaerostats***), where the air oxygen has been pumped out. A number of Petri dishes with inoculated anaerobic media are placed inside the jars for incubation.

In addition, the air from anaerobic jar might be substituted with certain inert gas (nitrogen, argone, etc.)

Another common mode of anaerobic jar culture employs the ***chemical reagent*** placed into the jar as a disposable sachet (gas-pack or anaeropack). This reagent *removes oxygen* from closed jar volume. For instance, in some techniques the chemical reagent produces hydrogen that (under the action of catalyst) binds to any free oxygen inside the jar to form water.

Sometimes ***biological method*** of culture can be used, where Petri dish with appropriate media like blood agar is inoculated. At first, *aerobic* culture is plated upon the one half of Petri dish and the material, containing *anaerobes*, is inoculated onto opposite side of the medium. Primarily aerobes grow and consume the oxygen inside tightly sealed Petri dish; subsequently, anaerobic bacteria begin to propagate.

Now this method is mostly of historical interest, as the maintenance of proper anaerobic conditions poses serious difficulties in such "hand-made" system.

Growth and Reproduction of Microorganisms

The ***growth*** of microorganisms means the increase of all the components and total mass of microbial cell resulted from the enhanced synthesis of a new cellular material.

Cellular growth in bacteria and fungi is stimulated by active microbial metabolism with prevalence of anabolic reactions over the cellular catabolism. This is supported by favorable conditions for microbial propagation. The continuous growth of the cells ultimately leads to their division and microbial reproduction.

Reproduction of microorganisms means their ability of self-multiplication that results in the rise of the number of microbial cells in the same volume of the medium.

Bacterial cells reproduce by simple non-mitotic *transverse division* known as ***binary fission***. It happens in different planes and generates the great variety of morphological forms of bacteria.

The process of reproduction starts from cellular DNA replication and formation of at least two nucleoids migrating to the opposite poles of bacterial cell. Then they are separated by deep cytoplasmic insertions of the cell wall and cytoplasmic membrane resulting in cell separation. The new *daughter cells* appeared after binary fission *are genetically identical* if the mutations are absent in the process of replication.

The rate of DNA replication and bacterial fission is an intrinsic characteristic of each bacterial genus or species.

The rate of the cell multiplication differs strongly among the bacteria. It depends not only on the species origin, but also at the nutrient medium contents, the age of the culture, and the state of the environmental conditions (temperature, humidity, concentration of oxygen and carbon dioxide, etc.)

The growth and reproduction of bacteria in artificial nutrient media can be efficiently managed and controlled. When cultured in standard conditions, the bacterial populations demonstrate the uniform or similar behavior that can be predicted and regulated depending on various external or internal stimuli.

Bacterial reproduction in liquid media demonstrates characteristic ***growth curves*** that include a number of basic ***reproduction phases***.

Primary ***lag phase*** is the initial cultivation stage that proceeds from the moment of bacterial inoculation into nutrient medium. The adaptation of the bacteria to growth conditions occurs. Reproduction is absent or low intensive, albeit the growth rate is accelerated. This phase may last about 3-4 hours.

Acceleration phase demonstrates the initial increase of growth rate.

Exponential (or ***logarithmic***) ***phase*** is characterized by a maximal and constant division rate. Usually the duration of this period is about 5-6 hours. Due to the intensive mictrobial reproduction, two growth-limiting factors begin to prevail – the exhausting of nutrient medium and accumulation of toxic metabolites.

The next ***retardation phase*** renders the gradual inhibition of microbial propagation, where the reproduction rate becomes slower. It lasts about 2 hours.

In the *maximum* ***stationary phase*** the quantity of newly produced cells is equivalent to the number of dead bacteria. A total concentration of bacterial cells remains the highest through this stage. This phase usually covers about 2-3 hours. As the result, the insufficiency of nutrients or the increased concentration of toxic products causes the termination of culture growth. However, a certain cell turnover is observed in the stationary phase: a slow loss of microbial cells is still balanced by generation of new bacteria.

And finally, the ***decline*** or ***death phase*** comes, where the equilibrium between the microbial accumulation and their death rate is interrupted, and the cells progressively die. In many cases, after the death of the majority of cells, the death rate falls down, and some number of survived microbial cells may stay alive for a long time.

Actually, the duration of these phases is greatly arbitrary, depending on microbial species and the conditions for growth.

As above indicated, the ***generation time*** (or the ***doubling time***) is an intrinsic characteristics of certain bacterial species. It differs strongly between various bacteria.

Thus, for instance, *E. coli* duplicates every 15-17 minutes, salmonellas – about 23 minutes, streptococci – near to 30 minutes, pathogenic *C. diphtheriae* – 34 minutes, and the most slowly growing *M. tuberculosis* – as long as 18 hours.

Microbial Growth in Biofilms

Typical growth curves of microbial cultures are readily observed, when the bacteria propagate in artificial liquid nutrient media. However, in real conditions the most of bacteria form the complex microbial communities that exist in closest contact with surrounding non-living or living matter. The bacteria demonstrate outstanding capacity of adherence – they easily attach one to another, or settle and bind to any underlying surface (inorganic grounds, natural or artificial polymers, or body tissues). Futhermore, the nascent microbial clusters and microcolonies produce a vast number of substances that become an integral part of growing culture. This multicomponent united microbial complex, demonstrating the common behavior and the evident tendency to spatial expansion, is termed as ***biofilm***.

The International Union of Pure and Applied Chemistry (IUPAC) gives the next definitions and essential characteristics for microbial biofilms:

biofilm is: "***Aggregate of microorganisms*** in which cells that are frequently embedded ***within a self-produced matrix*** *of extracellular polymeric substance* (EPS) adhere to each other and/or to a surface";

and gives further clarifications:

"Note 1: A biofilm is a system that can be *adapted internally* to environmental conditions *by its inhabitants*.

Note 2: The self-produced matrix of extracellular polymeric substance, which is also referred to as ***slime***, is a polymeric conglomeration generally composed of *extracellular biopolymers in various structural forms*."

An alternative to biofilm is the existance of bacteria in their *free*, or ***planktonic forms***, where the cells are not directly connected to each other.

Now it is generally ascertained that the largest majority of infections (not less than 60-80%) are caused by bacteria, growing as biofilm. Among them are the most deleterious and resistant microbial pathogens, such as *S. aureus*, streptococci, *P. aeruginosa*, numerous enterobacteria. Likewise, motile microorganisms are more active producers of the biofilm.

Every biofilm community evolves through certain common steps: a) initial and irreversible ***attachment***; b) biofilm ***maturation***; c) biofilm ***dispersion***.

Biofilm formation can be triggered not only by microbial contact with some external surface, but by any stress affecting microbial population (e.g., heat exposure, antibiotic treatment, etc.)

Biofilm maturation is followed by intensive synthesis of various kinds of extracellular biopolymers as the essential components of biofilm ***matrix***. The main components of matrix are usually *DNA* and *polysaccharides*.

After primary biofilm establishment it begins to demonstrate quasi-multicellular behavior reacting as "a single whole" against external or internal challenges. This ensues from the activity of a *system of intercellular microbial signalling and coordination* known as ***quorum sensing***. Various environmental challenges, affecting microbial population within biofilm, stimulate active synthesis of *signal quorum sensing molecules* by most of bacteria (e.g., N-acyl-homoserine lactone or regulatory oligopeptides). These inducer molecules spread throughout the biofilm and trigger coordinated gene expression by microbial cells. This, in turn, leads to the concordant changes of individual bacterial reactions within the biofilm.

As the result, microbial biofilm is characterized by active release of depolymerizing enzymes that provide bacterial feeding and invasion; it also develops intensive synthesis of extracellular polymeric matrix and elevated production of toxic substances. Enhanced lateral gene transfer between cooperating microbial cells ensures their rapid adaptation to worsened environmental conditions.

After maturation, microbial biofilm becomes poorly permeable for the most of biocides. By fact, it usually demonstrates the increase of antibiotic resistance more than 10-100 times over the resistance of respective planktonic forms of bacteria. The dispersal of biofilm accelerate microbial contamination of various prosthetic devices and artificial appliances in clinics (e.g., catheters, drains or implants) that is responsible for about 60-70% of hospital-acquired infections.

In sum, the problem of bacterial biofilm formation still remains unsolved and poses serious difficulties for existing health care services.

Chapter 5
MICROBIAL GENETICS. METHODS OF MOLECULAR GENETIC ANALYSIS.

Microbial genetics. Genotype and Phenotype

Microbial genetics studies the mechanisms of ***heredity*** and ***variability*** of different kinds of microorganisms (bacteria, viruses, fungi, or protozoans).

The basic unit of heredity is ***gene***, *the segment of DNA, controlling one specific microbial property and usually encoding one protein molecule or certain polypeptide chain.*

Genotype *is the total combination of genes in particular organism*.

Phenotype *is the realization of genotype via its interaction with the environment; it comprises all structural and functional traits of microbial cell*.

Molecular Organization of Bacterial Genome. Regulation of Gene Expression

Genetic information is encoded in a sequence of ***deoxyribonucleic acid*** (***DNA***) nucleotides. RNA viruses contain genetic information in ***ribonucleic acid*** (***RNA***) sequence.

DNA molecule is folded as the double-stranded helix, where ***complementary bases*** (***A-T***, ***G-C***) are linked by hydrogen bonds. The bases are coupled to deoxyribose-phosphate resulting in ***nucleotide formation***. ***Supercoiling*** of nucleic acid chains provides compact DNA structure in vivo conditions. The size of a DNA molecule is usually expressed in thousands of base pairs (***kilobase pairs***, ***kbp***). The size of genome of smallest viruses doesn't exceed 5 kbp, whereas bacterial DNA molecule of nucleoid comes to 5000 kbp (*Escherichia coli* genome is about 4600 kbp).

Double-stranded DNA is reproduced by ***semiconservative replication***. Each maternal strand serves as the template for newly synthesized strand of DNA, and the ***replication fork*** is formed. After replication each daughter DNA molecule contains one maternal strand and one newly formed strand.

Genome of bacteria contains circular DNA of bacterial chromosome (nucleoid).

Genomic ***ribonucleic acid*** (***RNA***) of viruses can exists both in single-stranded or double-stranded form. The base uracil (U) in RNA plays the same role as thymine (T) in DNA for hydrogen bonds formation, and complementary base pairs look like A-U and C-G. RNA is the source of genetic information in RNA-genomic viruses.

Genetic code is realized into final protein sequence via ***messenger RNA*** **(*mRNA*)**. Usually RNA polymerase forms a single polyribonucleotide strand of messenger RNA (mRNA), using DNA as a template. This process is called ***transcription*****.** The mRNA attains a nucleotide sequence complementary to the template DNA strand. In bacteria the newly formed mRNA is ***polycistronic***, containing the information about the group of related proteins encoded by several genes.

The ribosomes composed of ***ribosomal RNA*** (rRNA) and proteins transfer the genetic information from mRNA into the primary structure of proteins by the action of aminoacyl-***transfer RNAs*** (***tRNAs***). The latter process is known as ***translation***.

Regulation of expression of bacterial genes is quite complex and intricate.

Genes in bacterial genome can be organized into ***operon*** clusters. ***Operon*** *is a structural and functional unit of bacterial genomic organization*. It encodes and therefore controls a set of related structural proteins and chemical reactions.

Operon usually comprises ***promoter***, ***operator***, ***structural genes*** and in many cases ***regulatory gene***.

Specific regulatory proteins (e.g., ***repressors***) encoded by ***regulatory genes*** influence the expression of *structural genes*.

Structural genes predominantly encode enzymes and structural proteins. DNA transcription resulting in polycistronic mRNA synthesis is initiated from the ***promoter*** site – a specific sequence of DNA capable of binding RNA polymerase for transcription initiation.

Regulatory proteins that attach to the regions of DNA nearby promoters also actively participate in expression of structural genes. Short DNA sequence between promoter site and structural genes known as ***operator*** binds to regulatory proteins (e.g., ***repressors***) to control transcription.

Inhibition of transcription by repressor proteins is termed as ***negative control*****.** The opposite case – initiation of transcription by binding of so-called ***activator proteins*** to bacterial DNA is termed as ***positive control***.

A single regulatory gene governs the transcription of several structural genes. For instance, five genes affecting tryptophan biosynthesis are clustered within the *trp operon* in *E. coli*.

Trp operon of *E. coli* is regarded as ***negative respressible operon*** in bacteria. In this case the genetic expression is controlled by ***repression mechanism***.

For *trp operon* of E. coli it functions as follows:

if tryptophan amino acid is sufficiently present in the medium and thereafter within bacterial cell, it leads to tryptophan binding to the ***repressor protein***. This results in changes of repressor conformation ensuring repressor protein binding to DNA sequence of ***trp operator***. This binding of the repressor blocks the transcription of ***structural trp*** genes responsible for tryptophan synthesis.

In opposite case of tryptophan lack in the medium, the repressor protein doesn't block operator sequence, and the expression of structural genes starts, resulting in the synthesis of tryptophan that needs for bacterial cell growth.

The variations of bacterial ***negative inducible operons*** are also well-presented in bacterial genomes.

For instance, E. coli harbors the ***lac operon***, responsible for lactose metabolism in bacteria.

This operon carries three structural genes. Among them, the *lacY* gene governs the transport of lactose across the membrane into the bacterial cell. The *lacZ* gene codes for beta-galactosidase, the enzyme that hydrolyzes lactose resulting in production of galactose and glucose. Glucose is further used by bacterial cell in the pathways of energy metabolism. In addition, some limited amounts of lactose can be converted by beta-galactosidase into its isomer ***allolactose***.

In case of lactose absence the ***lac operon*** is almost silent and only the trace amounts of its proteins (e.g., galactosidase) can be expressed.

This is achieved by binding of the ***repressor protein*** (encoded by regulatory gene) to ***operator*** DNA sequence thereby blocking transcription of structural genes (***negative*** *transcriptional control*).

When the lactose concentration arises, the initially present beta-galactosidase produces some amounts of allolactose from available lactose. ***Allolactose*** is the direct ***inducer*** of the *lac* operon. It binds to the repressor, causing the dissociation of repressor-operator complex. This in turn makes available the promoter site for RNA polymerase that begins the transcription of structural genes. Structural genes products (e.g., galactosidase enzyme) utilize lactose for cellullar needs.

In addition, the expression of many operons is directly stimulated by the transcriptional ***activator proteins***. For the regulation of transcription they interact with a specific DNA fragment called *enhancer sequence*.

The ***enhancer sequence*** is located near the promoter site within the regulated operon. Activator proteins ensure ***positive transcriptional control*** stimulating RNA polymerase activity.

As an example, *E. coli* expresses regulatory molecules of ***cyclic AMP-binding protein*** (***CAP***). It is stimulated by specific cellular metabolite *3',5'-cyclic* ***AMP*** (or ***cAMP***). This substance (cAMP), shown to arise in energy-exhausted cells, activates the CAP thus enhancing the expression of catabolic enzymes that elevate the yield of metabolic energy.

Structural Organization of Bacterial Genome

Prokaryotic genome is composed of circular DNA molecules. Single bacterial chromosome is called ***nucleoid*** (the details of nucleoid structure and function are given in corresponding part of General Microbiology section). It size varies from 500 kbp up to 5000 kbp of DNA.

The possible number of chromosomes per growing cell is from one to four. Unlike eukaryotes, bacteria don't possess mitotic spindle apparatus that provides the segregation of eukaryotic chromosomes to the different progeny cells.

Bacterial genome is ***haploid***.

Many bacteria harbor additional genetic elements (***plasmids*** and ***episomes***) that are capable of autonomous replicating. All the bacterial genomic structures that contain genetic information sufficient for their own replication are termed ***replicons***.

The replication of bacterial DNA begins at starting point (***ori*** locus) and spreads in both directions from the initiation site. Chromosome duplication is stopped in the locus named as ***ter***. The newly formed nucleoids are separated before cell division, and each offspring cell gains one DNA molecule.

There are two basic modes of replication for the nucleoid or plasmids in bacterial cells. One is determined as ***theta-type*** of replication of bacterial circular chromosome through the replicative intermediate resembling Greek letter **θ** (*theta*). This bidirectional replication results in formation of two identical circular replicons (e.g., nucleoids). Theta-type of replication is characteristic for standard bacterial division by binary fission providing the daughter microbial cells with two identical copies of genomes.

Another type of genomic replication in bacteria is known as ***rolling circle*** or ***sigma-type*** of replication (from Greek letter **σ** – *sigma*). This unidirectional DNA replication primarily results in creation of one copy of circular DNA and one copy of linear DNA molecule. Rolling circle mode of DNA replication is essential for bacterial conjugation since the linear DNA copy can move into the conjugation tube. It ensures the transfer of genetic information from donor to the recipient cell via their direct contact (e.g., by structures of type 4 secretion system).

The genes required for essential bacterial metabolism are usually found within the nucleoid, whereas plasmids predominantly harbor genes with certain specialized functions.

Plasmids and Episomes

Genetic elements apart of nucleoid, which possess capacity of independent replication with high incidence of transmission, are termed ***plasmids*** and ***episomes***.

It is considered that episomes are able to integrate with nucleoid, whereas plasmids not.

Plasmids were discovered in 1958 by F. Jacob and E. Wollman.

These elements play a definite role in the evolution of bacteria. In large part due to the broad use of antibacterial agents, the natural environment of bacterial habitation harshly changes. To withstand these unfavorable conditions, many representatives of pathogenic and non-pathogenic microflora acquire drug resistance. Despite the high variability of resistance mechanisms, they rapidly spread among the bacteria owing to the permanent exchange of genetic transmissible elements, such as plasmids

Most of bacteria contain one or more of different plasmids. Plasmids vary in size from a few genes to several hundred. The properties, encoded by the plasmids, endow the bacterial cell with many useful adaptive properties. Plasmids can possess infectivity, being transmissible from one cell to another.

The group of plasmids and episomes includes the ***fertility factor***, the ***resistance transfer factor*** that controls the multiple bacterial resistance to antibiotics and other drugs, the ***factor of bacteriocinogenesis***, the ***hemolytic*** and ***enterotoxigenic*** factors, and many others.

The number of copies of a plasmid in the single bacterial cell can be various, depending on the plasmid nature. For instance, tetracycline

resistance plasmid is a ***low copy number plasmid*** being present in only 1-2 copies per microbial cell. Other plasmids can exist in the cell in quantity of more than 100 copies (***high-copy number plasmids*****)**.

Narrow host range plasmids can propagate only in a certain microbial species (e.g., F factor). But some other plasmids, termed as ***wide host range plasmids***, can propagate in a great variety of different species of bacteria.

Some plasmids and episomes (e.g. R or F factors) contain the information necessary for their transfer from one bacterium to another by conjugation. They are named ***conjugative plasmids***. Conjugative plasmids carry so-called ***tra-operon***, which encodes structural elements (sex-pili, different proteins), responsible for conjugation. These plasmids are ***self-transmissible***. Others are ***non-conjugative plasmids*** that are non-transmissible.

Fertility factor (F factor)

This factor governs bacterial conjugation, and its action is described in details in the corresponding paragraph, devoted to conjugation (see below).

R plasmids

Resistance plasmids, or ***R factors*** confer resistance to the great variety of antibiotics and metals (e.g., copper, arsenic or mercury).

They are usually composed of two elements: a ***resistance transfer factor*** (***RTF***) that encodes the transfer of the plasmid by conjugation, and ***resistance genes*** (***R genes***), which control the resistance properties. The RTF part is based on *tra*-operon that governs the transfer of the plasmid into the recipient bacteria. Therefore, these plasmids are conjugative. R genes are responsible for the resistance to commonly used antibacterial drugs (sulfonamides, beta-lactams, aminoglycosides, tetracyclines), as well as to a number of different metals.

They can encode antibiotic-degrading enzymes, capable of destroying the antibiotics: ***beta-lactamases*** for beta-lactam antibiotic hydrolysis, ***acetyltransferase*** for chloramphenicol inactivation, etc.

R factors can be rapidly transferred to neighbouring sensitive bacteria of various species or genera (***lateral*** or ***horizontal gene transfer***), thus spreading the resistance among microbial cells.

Many R factors can be transferred and reproduced in closely related bacteria (e.g. *Enterobacteriacea* family representatives – *Escherichia*, *Shigella*, *Salmonella*, *Yersinia*, *Klebsiella*, *Serratia*, or *Proteus*). Some R

factors can also be transmitted to less related genera such as *Pseudomonas* or *Vibrio*. These R factors pertain to ***wide host range*** plasmids. Hence, R factor can develop interspecies transmissibility. It is obvious that easy transfer of R factors in some ecological niche spread the drug resistance to all of the susceptible inhabitants of the certain biotope. Thus many different microorganisms in a hospital environment become resistant to wide number of antimicrobial agents.

The factors of bacteriocinogenesis

These genetic elements encode the proteins with specific inhibitory activity towards various species of bacteria.

The ***factors of bacteriocinogenesis*** are responsible for synthesis of different inhibitory proteins: ***colicins*** in *E. coli* (this genetic element is called ***Col-factor***); ***staphylocines*** in staphylococci; ***vibriocines*** in *Vibrio cholerae*; ***pesticins*** in *Yersinia pestis*, the causative agent of plague; ***corynecins*** in *Corynebacterium diphlheriae*, etc.

They are transferred by conjugation from bacteriocinogenic to non-bacteriocinogenic strain. Bacteriocin synthesis ensures selective ecological advantages for the bacterial cells.

Bacteriocins produce severe disorders in affected bacterial cells, destroying target cell DNA or impairing their cell wall. For instance, bacteriocinogenic microflora of gut can suppress susceptible enteropathogenic bacteria. Therefore, the bacteriocinogenic property of normal gut microflora is the important factor that supports intestinal ***colonization resistance*** and blocks the ability of pathogenic bacteria to attach and colonize the intestinal wall.

Other types of plasmids

Many other types of plasmids can be produced by bacteria. They may encode certain virulence factors (like toxins, capsule, etc.)

Ent-plasmids as well as some ***temperate bacteriophages*** contain ***tox-genes***, responsible for gram-negative bacteria enterotoxin production. ***K88 plasmid*** controls bacterial capsule synthesis. ***Hly plasmid*** is shown to encode hemolysins, produced by enterobacteria strains and streptococci.

Mobile Genetic Elements of Bacterial Genome

There are several specialized genetic structures in bacteria able to move within the genome.

Insertion elements (also known as ***insertion sequence elements*** or ***IS elements***) code only their ability for intra-genomic migration, i.e. carry the genes that account for transposition. The products of these genes include the ***transposase*** enzyme with endonuclease activity and regulatory proteins, controlling transposition.

IS element movement triggers a number of important genetic events, affecting bacterial genomic function. They induce mutations in the sites of their integration, being responsible for most of insertion mutations in bacteria. Also they can alter gene expression regulating the activity of promoter sequences.

Another type of mobile genetic sequences, known as ***transposable elements*** or ***transposons***, is more complex. They also can migrate from one genetic site to another. By action of transposases, transposons are incised and further inserted into another part of the same replicon or move to integrate with another replicon (*cut-and-paste mechanism*). This process of transposon movement is called ***transposition***. In some cases the sequences of transposons can be duplicated.

A special class of ***retrotransposons*** is hallmarked by the synthesis of intermediate RNA copy from primary transposon DNA sequence. Next this RNA serves as the template for synthesis of a new DNA copy of the transposon by the enzyme *reverse transcriptase*. Retroviruses behave as retrotransposons when infecting eukaryotic host cells.

Transposons are composed of 2500-20000 bp of DNA. Besides genetic sequences controlling their transposition, large transposons can include additional genes conferring antibiotic resistance and some other special properties. Transposons are flanked by insertion sequences, and various insertion mutations occur in places of transposon integration. Thus, these mobile elements are the potent biological mutagens.

The specificity of transposon integration is rather low. Usually transposons block the activity of genes in sites of their integration.

Unlike plasmids, transposons are lack of genetic information that needs for their own self-replication. Hence, their propagation depends on the activity of bacterial replicon, which contains the transposon. Many conjugative plasmids are transferred among bacterial cells, and insertion of a transposon into such a plasmid can lead to its dissemination throughout bacterial population.

Transposable elements play an extremely important role in gene movement within the same cell and also between different cells. They accelerate strongly the evolutionary process of microbial populations.

Genetic Organization of Adaptive Immunity in Bacteria – CRISPR/Cas System

The genome of near to 50% of bacterial species and about 90% of archaebacteria harbors the unique genetic region responsible for so-called "***adaptive immune system***" of bacterial cells. For instance, this ability is essential for bacteria, which are the threatening pathogens of humans, e.g. *M. tuberculosis*, *Y. pestis*, or *S. pyogenes*.

The genes and genetic elements of this system govern the acquired defensive reactions of bacterial cell against the invaded foreign nucleic acid of bacteriophage or plasmid. Furthermore, the genetic information of invaded DNA is memorized in bacterial genome and becomes heritable. As the result, next entry of the same foregn nucleic acid leads to the activation of specific RNA-mediated defensive reactions that destroy invaded exogenous nucleic acid of bacteriophage or plasmid. This way the bacterial cell becomes "immune" against its specific pathogen.

It was determined long ago that after the infection of certain bacterial population with specific bacteriophage the minor amount of cells survives and confers protection against this phage thus generating new resistant bacterial population.

Genome of these bacteria contains individual genetic elements known as ***CRISPR cassettes***. In most of cases they are located within bacterial nucleoid.

Acronym "CRISPR" is deciphered as "*clustered regularly interspaced short palindromic repeat*". This indicates the genetic structure of CRISPR cassette – it comprises genetic ***spacers*** of similar length but ***different DNA sequence*** interspersed with almost ***identical direct repeats*** of DNA. A single CRISPR cassette may contain more tham 100 spacers.

In addition, DNA sequence of the large part of spacers appeared to be quite similar to fragments of DNA of bacterial pathogens, e.g., bacteriophages.

Finally, CRISPR genetic region containing one or more of CRISPR cassettes borders with ***cas*** genetic locus (*cas* means *CRISPR-associated*) that encodes *Cas proteins*.

Cas proteins are multimeric enzymes with metal-dependent ***nuclease*** and ***integrase*** activities.

When the bacteriophage delivers its DNA into the cytoplasm of bacterial cell, the process of a new ***spacer acquisition*** begins.

It consists of ***hydrolysis of viral DNA*** by Cas1/Cas2 protein complex followed by the ***release*** of so-called ***protospacer*** sequence from phage's

DNA and its further insertion into CRISPR region of bacterial genome. All these events lead to ***acquisition of the new spacer*** by bacterial cell, which harbors specific foreign DNA from bacteriophage.

Similarly, new spacers from the same phage might be inserted nearby the first one. This way the bacterial cell becomes immune to the next infection caused by this phage.

When another episode of phage infection occurs, it stimulates the transcription of the large part of CRISPR locus. It results in the synthesis of primary long RNA transcript that is further cleaved by Cas proteins with formation of ***CRISPR-RNA*** (***crRNA***). This crRNA is complementary to the phage sequence.

At next step crRNA accociates with ***Cas protein complex with nuclease activity*** and delivers it to phage nucleic acid ("***guide RNA***"). Here crRNA recognizes specific sequence of phage DNA and binds to it allowing ***Cas nuclease*** to ***destroy*** *the invaded nucleic acid*.

Depending on their types, specific Cas proteins besides foreign DNA may destroy also RNA phages and phage messenger RNAs.

These mechanisms of genetic adaptive immunity protect bacteria from phage infections as well as from insertion of undesirable genetic material delivered by natural transformation or conjugation (see below).

Specially designed CRISPR-Cas systems (e.g., CRISPR/Cas9) were realized as the extremely powerful tools for genetic engineering allowing precise and non-expensive editing of both prokaryotic and eukaryotic genomes.

Genotypic and Phenotypic Bacterial Variations

There are two main types of bacterial variation. One is ***phenotypic***, which is not inherited by the microorganism. Second is ***genotypic***, it comprises different genomic changes that can be transferred to next generations of bacterial cells.

Phenotypic Variations of Bacterial Properties

Phenotypic variations or ***modifications*** can appear due to the influences of environment on microbial cells. They change the rate of various metabolic reactions in response against external or internal challenges. Phenotypic variations are controlled by genome but *don't affect*

primary sequence of genome structures (genomic DNA). Sometimes modifications depend on alternative expression of different microbial genes.

Usually modifications are based on concerted variation of bacterial enzymatic activity, their induction or repression.

Basic factors, stimulating phenotypic diversity in bacterial populations, are related with the cultivation conditions for the microbial strain (temperature, humidity, salt concentrations, medium chemical composition, etc.) Also antibiotic and disinfectant action potentially increases the incidence of modifications. For instance, bacterial cells can transform into L-forms, devoid of cell wall.

Modifications are considered to be the *temporary changes in microbial reactivity*. However the stable forms of modifications exist, which can be preserved in several bacterial generations.

The discrimination between genotypic or phenotypic alterations rests on several assumptions. Genotypic changes are rare and result from the new order of the nucleotide sequence in DNA (e.g., after mutations); therefore, only a few cells in a large population will be altered towards the acquisition of a new trait. In contrast, phenotypic changes will involve almost all cells in the population (e.g., by the action of *quorum sensing* mechanism). Further, *phenotypic variations are readily reversible*, and most of the bacteria in the population can reverse back to their initial property as the environmental conditions resolves to the original state. On the contrary, genotypic changes are generally stable.

Genotypic Variations

Three main types of genomic alterations are demonstrated in bacteria. There are ***mutations***, ***recombinations***, and bacterial ***dissociation***.

As the result of mutations and recombinations, heterogeneity of microbial populations is permanently maintained thereby accelerating evolutionary process.

Mutations

Mutations *are the changes in primary sequence of genomic DNA.*

Mutations that emerge in the absence of the certain mutagen are called ***spontaneous***, whereas the mutations occuring after the exposure to definite mutagenic factor (radiation, temperature, chemical and other agents) are termed ***induced***.

Mutagens

Mutagens *are the substances, agents and factors, causing mutations*.

The frequency of mutations accelerates greatly by exposure of bacterial cells to mutagens.

Mutagens can be divided into three broad categories: ***chemical agents***, ***physical*** factors (most important is ***radiation***) and ***biological*** mutagens (e.g., ***genetic transposable elements***).

Ultraviolet (UV) *light* is a potent ***physical mutagen*** that alters DNA sequence by conjugation of thymine bases resulting in thymine dimer formation. Two nucleotides produce dimers, which impair normal replication of DNA. Photoreactivation of DNA structure by visible light is performed by special enzyme, photolyase, which breaks down thymine dimers. Sequence recovery is not completely precise; therefore, various mutations can arise.

Chemical mutagens exert mutations by changing either the chemical structure or folding of DNA molecules. Various chemical substances directly modify the bases within DNA. For instance, nitrous acid (HNO_2) reacts with hydroxyl groups resulting in formation of amino groups. That leads to incorrect DNA replication during cell life cycle.

Mutations, affecting reparation enzymes, change their specifity and activity. In that case these enzymes play a role of ***biological mutagens***. Other biological mutagens are transposons, IS elements and temperate bacteriophages that alter DNA sequence after their incorporation into a new site of bacterial genome.

A great number of versatile microbial traits may be affected by the mutations – auxotrophy to various growth factors (vitamins, nucleotides, or amino acids), antimicrobial resistance, sensitivity to bacteriophages, enzyme expression, etc.

Types of Mutations

Spontaneous mutations affecting the certain gene can occur with a frequency of 10^{-6}-10^{-8} in microbial population generated from a single bacterial cell.

Mutations comprise ***base insertions***, ***deletions***, ***duplications***, ***substitutions***, ***inversions***, ***translocations*** and some others.

Deletions eliminate the genetic sequences from the genome. They can affect the large genetic regions and usually not revert to the initial state.

Insertions occur after the addition of a new genetic material into primary DNA sequence; ***duplication*** presumes the addition of the same or closely related DNA fragment. The latter mutations are largely unstable resulting in spontaneous reversions. Some other mutations invert the sequences of DNA (***inversions***) or deliver DNA fragment to another location (***translocations***).

Substitutions result from the mispairing of complementary bases in the process of replication. The frequency of substitutions is about one base for 10^{10} nucleotides incorporated into DNA molecule during replication.

The deletion or insertion of one nucleotide into DNA molecule leads to the shift of the triplet sequence of DNA. This creates new codons resulting in translation of altered protein sequence because of incorporation of incorrect amino acids. This type of genetic alterations is known as ***frame shift mutations*.**

According to their size there are ***large*** (e.g., ***gene***), and ***small*** (***point***) mutations. The large rearrangements rest on the deletion or insertion of a considerable portion of the gene. ***Point*** mutation are located within the gene itself, usually resulting in ***deletion***, ***inclusion***, or ***replacement*** of one nucleotide pair within DNA molecule. Large mutations in bacteria are commonly lethal, albeit point mutations more easily repaired.

Many point mutations are not detected readily at the phenotypic level, as they don't alter significantly the biological function of the translated enzyme or protein. It concerns the ***missense mutations***, followed by the substitution of one amino acid for another in final protein sequence. Nevertheless, phenotypic results of such a mutation might be negligible. ***Nonsense mutations*** terminate the process of protein translation in mutational site. This usually leads to substantial misfunction of final protein molecule.

The recovery of normal biological activity of the protein after mutation is denoted as ***phenotypic reversion***. It may happen due to the restoration of primary DNA sequence in the mutated site (***genotypic reversion***). Sometimes phenotypic reversion arises from the mutation within another genetic site that shuts down the effects of primary mutation (so-called ***suppressor mutation***). ***Intragenic suppression*** is the secondary mutation within the same gene that was initially affected by primary mutation. In some cases it may normalize the function of defective protein. ***Extragenic suppression*** occurs after next mutations in genes beyond the affected one.

For detection of mutant phenotypes, various permissive media supplemented with antimicrobial agents are used. The mutant bacterial

strain can be resistant to the antibiotic and hence propagates, but the growth of wild type bacterial populations is inhibited.

Mutations affecting the biochemical properties of bacteria could be detected by their cultivation on minimal nutrient media containing a very limited number of carbohydrates or other essential substances. For instance, as the result of mutations the switching from prototrophic to auxotrophic type of nutrition may arise.

Recombinations

Recombination *is the transfer of genetic material from donor to the recipient cell or from one to another replicon*. Recombinations provide the regular exchange of genetic information between bacterial cells.

Recombinant bacteria acquire the genetic properties of both parental cells: the basic number of the recipient's genes and a small amount of genes from donor.

Recombination is controlled by special genes for recombination (or ***rec genes***).

There are different types of recombination based on *DNA homology*.

General recombination occurs with the participation of rather large ***homologous*** (complementary) ***sequences*** of DNA.

Site-specific recombination is induced via the short homologous defined sequences of DNA.

Illegitimate recombination results from cross-change of ***non-homologous*** strands of DNA.

The basic kinds of recombination events found in bacteria comprise ***transformation***, ***transduction*** and ***conjugation***.

The Molecular Mechanisms of Recombination

Donor DNA interacts with the recipient DNA to become integrated into genome of the recipient cell. This requires genes and proteins with specific functions that govern recombination

RecA, *recB*, *recC*, *recD* genes encode the synthesis of specific enzymes (***recombinases RecA***, ***RecBCD***) that promote recombination processes.

RecA is the multifunctional protein that is activated after DNA binding. It acts as DNA-helicase (unwinds DNA double helix) and

destroys several repressors, which block recombination. It catalyzes DNA cross-structure rearrangement.

RecA mutations lower recombination incidence for more than 1000 times.

RecBCD nuclease, encoded by *recB, recC, recD* genes, splits one DNA strand, allowing *RecA* binding. Also it accomplishes recombination by final cut of heteroduplex DNA cross-structure.

Recombination is based on the exchange of two complementary fragments between parental DNA molecules of donor and recipient cells. It includes incorporation or a donor DNA sequence into recipient one with the parallel transfer of the homologous recipient sequence backward into the donor DNA molecule. The fragment of DNA containing the complementary strands both from donor and recipient is termed as DNA ***heteroduplex***.

At first, both of parental duplex DNA chains become unwound. Then they interact by their complementary DNA fragments, forming transition cross-like structure (so-called *Holliday junction*). The hydrogen bonds, maintaining DNA conformation, break in both parental molecules but lock again between primary and newly coming complementary DNA. The ***heteroduplex*** DNA fragment is formed, which carries the genetic sequences from donor and recipient DNA molecules.

Recombinases maintain the proper orientation and then split the complex of cross-reacting donor-recipient DNA strands.

Finally, DNA lygase links the free ends of phosphate backbone of recombinant DNAs thus restoring strands integrity.

Genetic recombinations in bacteria occur as the result of ***transformation***, ***transduction***, and ***conjugation***.

Transformation

Transformation *is the direct uptake of donor's DNA by the recipient cell.*

F. Griffith discovered the process of transformation in 1928. He studied the experimental infection of mice triggered by the injection of the bacterial mixture, composed of a live decapsulated non-pathogenic type II *Streptococcus pneumoniae* and pathogenic capsulated type III *S. pneumoniae*, previously inactivated by heat. As the result, infected mice died due to septicemia caused by the virulent infection.

F. Griffith has found that type II *S. pneumoniae* acquired virulent properties being able to produce the capsule essential for *S. pneumoniae* type III. F. Griffith supposed that bacterial capsular polysaccharides were responsible for transformation.

In 1944 O. Avery, C. McLeod, and M. McCarthy revised the experiment of F. Griffith. They isolated transformational substance of high viscosity, resistant to proteases but sensitive to DNAse. It induced the transition of any type of pneumococci to type III *S. pneumoniae*. The substance was confirmed to be desoxyribonucleic acid. The scientists were the first, who proved DNA transforming activity and demonstrated the role of DNA as the possible substance of heredity.

In nature only under special living conditions the bacteria become able to capture the relatively large molecules of DNA. These bacterial cells were designated as ***competent.*** Natural occurrence of this state is seldom among the bacteria. Some of them can undergo transformation only under the influence of ***competence factors,*** produced at a certain point of bacterial growth. This is followed by the marked changes of bacterial phenotype including the increased permeability of bacterial cell wall for nucleic acids and the expression of protein receptors on the membrane for DNA uptake.

The production of competence factors is not common among the bacteria; therefore, many bacterial species are poorly transformed.

Essentially competent bacteria can be found in different bacterial genera or species. Among them are *Streptococcus pneumoniae, Neisseria gonorrhoeae, Hemophilus influenzae, Bacillus subtilis* and others.

Many bacteria can be stimulated for transformation by external stimuli (temperature stress or calcium chloride exposure).

Electroporation is an artificial method to induce transformation of bacteria. Free DNA is added to bacterial cells and the electric current is applied. The electric current increases the permeability of the bacterial envelope (cytoplasmic membrane and cell wall) thus facilitating DNA uptake. Once appeared in the cytoplasm, DNA becomes incorporated into the recipient chromosome as the result of the homologous recombination.

Transduction

Transduction *is bacteriophage-stimulated genetic recombination in bacteria.*

Transduction phenomenon was first described by N. Cinder and J. Lederberg in 1952. Bacteriophages as the specific viruses of bacteria

were demonstrated to deliver genes from donor to the recipient bacterial cells. Phage genome may harbor genes encoding the resistance to antimicrobial agents, virulence factor synthesis (e.g., exotoxin and adhesin expression), flagella and pili formation, production of enzymes, etc.

The *donor bacteria*, the ***temperate*** *phage*, and *recipient bacteria* are the participants of the transduction process.

Three types of transduction have been revealed: ***general*** transduction, ***specific*** transduction and ***abortive***.

As the result of ***general*** transduction the transfer of any bacterial gene may happen. The frequency of this rare genetic event is about of 10^{-4}-10^{-7} per single phage particle. The incidence of general transduction can be arisen by pre-treatment of the phage with UV-light or other activators.

Specific transduction is performed by the temperate phage particles. They are generated after the excision of DNA sequence of the temperate phage from the nucleoid of bacterial lysogenic cells. It should be noted that lyzogenic bacteria have the genome with integrated DNA of temperate bacteriophage. When liberated from the nucleoid, phage DNA is further incorporated into capsids of nascent phage particles.

In case of specific transduction only the definite gene clusters can be transduced (e.g., galactosidase locus, controlling the utilization of lactose in *E. coli*). After occasional non-proper excision, temperate bacteriophages can capture the bacterial genes flanking phage nucleic acid sequence. In that case the phage becomes ***defective*** but enables to transfer different host bacterial genes to other susceptible bacteria.

Abortive transduction occurs, when the genetic material delivered by the phage is not included into the genome of the recipient. It retains in the cytoplasm of the recipient cell. After next cell division DNA of the phage remains non-replicated and stays only in one of the progeny cell, the second cell is free of phage DNA. Thus the phage genes become lost for next bacterial generations. Abortive transduction is considered to be about 10 times more frequent event, than transduction types with integration of phage nucleic acid.

Transduction occurs between the bacteria of the same or different microbial species. Interspecies transduction has the evident biological value. Here bacteriophages enhance the diversity of living systems, thereby accelerating microbial evolution.

Conjugation

Conjugation *is a one-sided transport of genetic material from one microbial cell to another by direct cell-to-cell contact.*

Plasmid of certain type (or, more correctly, episome) termed as ***F factor***, or ***fertility factor***, ensure the conjugation.

F factor replicates independently of nucleoid within bacterial cytoplasm.

Harboring F factor bacteria are the genetic donors, designated as F^+ cells, whereas F^- cells are the recipients. They are lack of F factor.

F plasmid of donor cell contains the genetic information for the synthesis of ***sex pili*** – special extracellular protrusions that promote binding of donor cell to the recipient bacteria. F plasmid also carries some additional genetic elements that need for the successful transfer of DNA.

The transfer of F factor into the recipient cell takes place only in case of direct contact of the bacteria.

F factor can exist in two forms: ***autonomous*** in bacterial cytoplasm and ***integrated*** into the bacterial nucleoid. Therefore, besides F^+ donor cells, containing free F factor in cytoplasm, bacterial donors with integrated F factor sequence are found. They were designated as ***Hfr*** (***high frequency of recombination***) cells. These cells are characterized by essential high frequency of recombination (10^{-1}-10^{-4}), whereas the frequency of recombination between the F^- and F^+ strains is in the range between 10^{-4} and 10^{-6}.

Thus, there are major ***two variants*** of the conjugation.

In first case ***autonomous F factor*** initiates the formation of the *conjugation tube* and reduplicates itself by the *rolling circle mechanism*. One linear strand of newly synthesized donor's DNA is transferred into the conjugation tube. The recipient cell completes the structure of F factor's DNA by synthesis of the novel DNA strand on the transferred donor's DNA template. The remaining strand of F factor within the donor cell retains its circular form after duplication. As F factor copy has been delivered, the recipient cell becomes converted into the donor F^+ cell.

Another variant of conjugation proceeds within ***Hfr cells***. DNA sequence of Hfr cell is incised nearby the integrated F factor. But after the formation of conjugation tube the transfer of single-stranded linear DNA begins from the side of bacterial DNA localization. Thus F factor can be transported into recipient cell only after complete transference of nucleoid DNA. The latter is almost unlikely, so the recipient cell cannot obtain the properties of genetic donor.

Nevertheless, the nucleoid DNA fragment of the Hfr cell can be included in the genome of the recipient cell (F^-) by recombination. As the result, an *incomplete zygote* (or ***merozygote***) is formed that is composed of the whole genome of the recipient and some part of donor's genome.

After conjugation both cells remain viable.

Similar to other recombinations, conjugation may occur not only between the cells of the same species, but among the cells from various species, thus leading to the production of interspecies recombinants.

Bacterial Dissociation

Bacterial dissociation is the complex set of alternative changes affecting microbial structure and metabolism. This type of variation is specific only for the bacteria. It is based upon some modifications as well as on different genotypic alterations. During dissociation bacterial population is splitted into ***S*** (***smooth***) or ***R*** (***rough***) microbial forms. They show almost opposite morphologic and cultural properties.

S forms are characterized by *smooth* and convex colonies. Motile S form species possess flagella. Capsulated strains have well-defined capsules. S forms usually render intensive biochemical activity. They are considered to be more virulent because of their enhanced resistance against phagocytosis. S forms are often isolated in the acute stage of the disease.

R forms produce *rough*, irregular and in most cases flattened colonies. They are less biochemically active. In course of infection the host phagocytes efficiently engulf and digest them as the bacteria are lack of capsules.

Most R forms are regarded as less or non-virulent. Nevertheless, the certain pathogens of outstanding virulence are registered. *Plague*, *anthrax*, *diphtheria* and *tuberculosis* causative agents are extremely virulent in R forms.

Dissociation is provoked mainly by the number of ***related mutations***, which appear after the integration of temperate phages, episomes, IS-elements and transposons into nucleoid sequence. These mutations can impair the process of LPS synthesis within the cell wall, thus leading to the creation of microbial R forms. Nonetheless, this forms are regarded as more resistant to the environmental harmful effects.

For instance, *Corynebacterium diphtheriae* comes into toxic R form after lysogenization with temperate bacteriophage, carrying ***tox-genes***. Therefore, lysogenic conversion of corynebacteria is responsible for the virulence of corynebacterial population.

Methods of Molecular Genetic Analysis

Investigation of genomic structure and function requires highly sensitive and specific methods of molecular genetic analysis. Furthermore, these methods play a pivotal role in laboratory identification of any microbial representative, if to take into account that modern classification schemes are based on the analysis of genetic relatedness of tested microorganisms.

Overall, the discovery of specific microbial DNA is the most sensitive and reliable test for detection of past or current presence of pathogenic agent in clinical specimen.

Molecular Hybridization of Nucleic Acids

One of the most powerful methods of genetic analysis is ***hybridization*** technique. It develops high sensitivity, allowing the discovery of about 10^{-10} g of investigated nucleic acid. Hybridization method is based on complementary interaction of single-stranded DNA or RNA molecules resulting in specific formation of double-stranded complex.

At the initial step of hybridization test (e.g., ***dot*** or ***spot hybridization***) the sample, containing unknown nucleic acid sequence is heated or treated by alkali to produce single-stranded DNA molecules (*DNA melting*).

Then single-stranded DNA is adsorbed on some solid phase (e.g., nitrocellulose paper sheet).

Afterwards the sample is treated by specific ***hybridization probe***.

The probe *is the known short sequence of one-stranded DNA molecule, complementary to investigated nucleic acid sequence and labeled with highly sensitive tag – fluorescent or chromogenic dye, or radiochemical label.*

The labeling with fluorescent dye is the most commonly used now. Also radioactive ^{32}P phosphate isotope can be applied as radiochemical tag.

If the investigated specimen contains the nucleic acid of interest, the probe will bind to its complementary sequence. After thorough wash the specimen fluorescence or radioactivity is analyzed. Positive samples demonstrate the increased levels of activity.

Hybridization probes are in routine use for investigation of complex mixture of nucleic acids. Specific DNA sequences separated in agarose gel can be detected by ***Southern blots***, a method that uses ***hybridization of DNA to DNA***.

Likewise, ***hybridization of probe to RNA*** by ***Northern blots*** can evaluate RNA synthesis.

Hybridization in situ is employed to discover microbial DNA and RNA in cells and tissues of different origin. In these cases frozen microscopic sections of tissue samples treated by fluorescent probes are tested.

The most advanced methods of nucleic acid hybridization exploit ***DNA microarray*** technologies. They are based on detection of mass parallel hybridization of thousands of nucleic acid probes and clinical samples. In this case an array of DNA probes taken in micro-quantities is absorbed on glass or plastic solid phase thereby producing ***DNA biochip***. It is next treated with the mass of clinical samples containing microbial nucleic acid with subsequent fluorescent registering of dot hybridization. This technology allows parallel testing of thousands of clinical specimens for specific microbial DNA or RNA as well as simultaneous detection of all of microbial pathogens present in clinical sample.

Polymerase Chain Reaction (PCR)

In early 1980th K. Mullis created a revolutionary molecular technology, which made possible to raise the sensitivity of genetic methods literally up to several molecules of nucleic acid in tested sample. This method was named "***polymerase chain reaction*** (***PCR***)".

Polymerase chain reaction allows amplification of exactly specific DNA fragments. About one billion copies (***amplicons***) of tested DNA fragment can be produced in one hour starting from initial single DNA.

PCR is elaborated in several stages.

First, DNA is isolated from a cell and heated to approximately 95-97°C, causing the separation of two DNA strands breaking down the hydrogen bonds between A-T and G-C (***DNA melting***).

In the second step, the temperature is decreased to about 65-70°C. It allows the attachment of two short specific fragments of DNA, termed ***primers***. The ***primers*** (forward and reverse) are complementary to the amplified DNA sequence. They bind to the ends of complementary DNA (***primer annealing***) and play a role of initiators of DNA polymerization.

The third step (***amplification***) is the synthesis of complementary strands of new DNA molecules on the templates of both parental DNA strands. The process begins from the place of primer attachment. This reaction requires all number of nucleotide substrates and thermostable

DNA-polymerase (***Taq-polymerase***). After the first cycle of enzyme action the single original DNA is converted into two identical DNA molecules. Thus the duplication of original genetic material is achieved.

Next amplification cycle is stimulated by heating of the reaction mixture again up to 95-97°C to dissociate all of existing strands of DNA. And the amplification cycle is repeated again.

Each cycle of heating, cooling and doubling of tested DNA segment lasts about several minutes. The method demonstrates an extreme sensitivity – DNA quantity in single bacterial cells is enough to be amplified.

The registration of PCR results was primarily made by agarose electrophoresis of DNA amplification products (amplicons) followed by their fluorescent stain with DNA-specific fluorescent dyes (e.g., ethydium bromide, propidium iodide, Sytox Green and many others).

The more advanced and convenient version of PCR known as ***real-time PCR*** test is broadly used now especially in clinical settings. This quantitative fluorescent technique allows to determine the quantity of microbial DNA directly in reaction tube during the process of its amplification. Computer analyzer of this test registers the growing accumulation of fluorescent signal and builds the curves of DNA amplification resulting from the incorporation of fluorescent dye into the nascent DNA strands.

In order to determine RNA-containing viruses (like HIV) the first reaction step of PCR involves complementary DNA synthesis on viral RNA template (***reverse transcription*** step). This reaction is catalyzed by ***RNA-dependent DNA polymerase*** (or ***reverse transcriptase*** enzyme). Subsequent PCR steps are similar with the above described.

Actually, PCR opened the new horizons in microbiological investigations. All microbials, including the agents that are unable for culturing can be identified. The particular microorganism, causing a disease, can be detected even if it is present in extremely small amounts. For instance, the technique makes possible to reveal less than 100 viral genome copies in 1 ml of serum in diagnostics of viral hepatitis, HIV-infection, etc.

DNA and RNA Sequencing

As mentioned above, the current radical changes covering literally all the fields of modern microbiology are stimulated greatly by the practical

implementation of new technologies of molecular genetic analysis with special emphasis on ***nucleic acid sequencing***.

Nucleic acid sequencing produces an absolute identification of microbial nucleic acids, and therefore, discovers the causative agents that reside in clinical samples.

Sequencing methods determine the direct order of nucleotides in nucleic acid chains. This clarifies the organization of genes within microbial genome and allows to deduce the structure of corresponding gene products.

Currently known technologies of nucleic acid sequencing demonstrate a tremendous progress in concern of their efficacy.

The group of so-called "*first generation methods*" comprise two classical techniques.

Maxam-Gilbert DNA sequencing is based on the treatment of studied DNA with several chemicals that cleave DNA molecule by position of certain nucleotide (C, T+C, G, and G+A). This action leads to the production of four numbers of DNA fragments of various lengths each finished by specific nucleotide.

Sanger (or ***dideoxy termination***) ***method*** uses four types of fluorescently labeled dideoxy-nucleotides (ddNTP) that terminate DNA synthesis by DNA polymerase in position of definite nucleotide (A, T, G, or C).

When complementary DNA strand is synthesized, incorporation of ddNTP stops DNA elongation by position of corresponding nucleotide. As the result, four numbers of various lengths DNA fragments are created with specific ddNTP on their ends (similar to that of Maxam-Gilbert method).

After that, in both methods of sequencing 4 numbers of newly produced DNA fragments labeled with fluorescent or radioactive tags undergo *gel electrophoresis*. Four various mixtures of DNA fragments each bearing specific terminal nucleotide (A, T, G, or C) are separated by electrophoresis according to their fragment lengths running along 4 parallel lanes.

Finally, the comparison of positions of fluorescent DNA fragments within four parallel gel lanes allows to assemble primary sequence of investigated DNA.

More convenient Sanger sequencing method was actively used for a long time in practical genetics. Its capillary version was applied for first sequencing of full human genome in 2001.

However, the expanding efforts in full-genomic sequencing of the vast number of prokaryotic and eukaryotic genomes have required the development of new high-throughput methods of ***massive parallel DNA sequencing***. They were eventually termed as ***next-generation sequencing*** (***NGS***) or *second generation sequencing* methods.

There is an impressive variety of NGS methods, highly different by their chemistry and miniaturized technical platforms. They are organized as ***automated DNA sequencers***. Most of them apply fluorescent labels and register fluorescent signals in sequencing process.

NGS methods comprise the number of following steps.

At first a large genetic library containing multiple copies of fragments of investigated DNA is created by PCR on solid or lipid phase reaction template.

After the dissociation of generated DNA copies into the single-stranded molecules the reaction of synthesis of a new double-stranded DNA molecules is performed (e.g., by PCR or ligase chain reaction).

The process of synthesis of complementary DNA strand is followed by consecutive attachment and incorporation of complementary nucleotide or probe into the sequence of growing DNA strand. Here every act of the attachment generates various fluorescent signals specific for the labels of all types of nucleotides (A, T, G, or C). These signals are registered by sensitive fluorescent detectors, and their order corresponds to the primary sequence of investigated nucleic acid.

NGS methods can analyse up to several billion of *overlapping fragments of sequenced DNA* (known as ***reads***) per 1 run of the test.

The reads can be of various lengths – from 50 base pairs (bp) to 400-700 bp and even more. Every read is repeatedly analyzed from 8-10 times (known as ***deep sequencing***) up to more than 100 times per run (***ultra-deep sequencing*** mode that is used by the supreme sequencing methods).

All deep sequencing methods generate enormously large amounts of primary data. They are further analyzed by the methods of computer ***bioinformatics*** using highly sophisticated computer algorithms. Computer analysis performs the alignment of read sequences and constructs the most probable sequence of investigated nucleic acid.

As the result, full-genome analysis of certain microbial DNA isolated from clinical sample covers about several hours or days.

A great number of second generation sequencing methods are actively used now making genomic analysis fast and low-cost (pyrosequencing, Illumina and SOLiD platforms, and many others).

Currently emerging NGS technologies imply ***single DNA molecule sequencing***. Sometimes they are termed as "*third generation*" methods.

For instance, *single molecule real time sequencing* (SMRT) uses microchip with thousands of nanoholes (waveguides) each of the volume about 20 zeptoliters (or $2*10^{-20}$ liters). Every such cell contains one molecule of single-stranded analyzed DNA, one molecule of DNA polymerase and all 4 types of nucleotides with fluorescent tag. Here every act of DNA strand elongation results in new fluorescent signal that is registered by detector, thus accumulating the information for reconstruction of DNA primary sequence.

SMRT technology made possible to analyze seriously longer DNA reads (10,000-15,000 bp).

Likewise, if it needs to determine the sequence of long-length genomes, the procedure known as ***shotgun technique*** can be applied. In this method the DNA of interest is broken down into random smaller overlapping fragments thus making random fragment library. The fragments are next processed by automated DNA sequencers deciphering their nucleotide sequences. These overlapping fragments are further placed into the correct order by the computational methods of bioinformatics resulting in determination of the whole primary DNA sequence.

The "next-gen" sequencing technologies revolutionized the practice of modern microbiological laboratories. They created the opportunities for microbial transcriptome analysis, for investigation of individual variations of the large microbial communities (microbiomes), epigenetic regulations of microbial genomes, and evolutionary interplays between various microbial taxa (see below for the details).

Genetic Engineering

Genetic engineering is one of the most advanced branches of biotechnology. It uses modern microbiological and biochemical techniques based on genetic manipulations to solve practical problems in medicine, biology, agriculture and industry. Key procedure of genetic engineering is the construction of the recombinant DNA – ***recombinant DNA technology***. It results in creation of DNA molecules with new primary sequencies and therefore, with new properties.

Recombinant DNA is defined as *DNA molecule that contains new DNA fragments artificially incorporated into original DNA sequence*.

The recombinant DNA technology resulting in production of recombinant biologically active proteins comprises several basic procedures. Among them are:

1) isolation of the DNA of interest from the host cells;

2) incorporation of the required DNA fragment into the *vector*;

3) the delivery of DNA into the *producer cells*;

4) selection of the cells that contain recombinant DNA and synthesize the required *recombinant protein*;

5) accumulation of the producer cells (their *cloning*),

6) assessment of the rate of recombinant protein synthesis, its isolation and final purification.

The process of great importance is ***gene cloning***. At the first step of this procedure the genes, encoding necessary protein sequence, are taken from their initial DNA molecule. Also it is possible to use messenger RNA, encoding the protein of interest, which should be extracted from specific cells or tissues. In this case the primary step includes complementary DNA synthesis on RNA template by reverse transcriptase.

In order to incorporate a new gene into the recipient ***producer cell***, it must be delivered into it by ***vector,*** or ***cloning vehicle***. Vector contains DNA molecule that is capable of reproducing within the host cell.

A great number of natural and artificially engineered vectors are used for gene cloning. They comprise DNA molecules, plasmids, temperate bacteriophages, viruses (e.g. baculovirus or vaccinia virus), and combined vectors. The latter include ***cosmids***, ***phagmids***, ***phasmids***, and others.

Cosmids contain small plasmid vector; *cos*-sequences of lambda-phage, responsible for DNA incorporation into phage's head; and the large fragment (up to 30-45 kbp) for DNA of interest. ***Phagmids*** and ***phasmids*** are constructed in similar manner, but the source of vector replication for phagmids is encoded by bacteriophage, whereas in phasmids – by plasmid sequence.

Now in eukaryotes genetic engineering the method of direct microinjection of DNA into recipient nucleus is used.

Two groups of specific enzymes perform the insertion of DNA into the cloning vehicle. The first group contains *site-specific endonucleases* or ***restriction enzymes*** (***restrictases***). A great variety of such enzymes was derived from the different bacteria. Restrictases recognize and bind to specific base sequences in double-stranded DNA and break the phosphodiester bonds at the place of attachment.

When the DNA of the vector and the fragment of the DNA for cloning are cut with the specific restriction endonuclease and next mixed together the *recombination initiates*. The next enzyme, ***DNA-lygase***, links both molecules into the continious chain; and vector ***recombinant hybrid molecule*** appears.

The vector transfers the cloning DNA into a host cell, where it should be reproduced. Frequently used prokaryotic recipient cells are *E. coli*, eukaryotic – yeasts fungi *Saccharomyces cerevisiae*, different plant cells, embryonic mammalian cells, etc.

The vehicle used for DNA delivery must carry a gene providing successful selection of the producer recipient cells that acquired the hybrid DNA. It is related with the low frequency of host cell genetic transformation. A proper marker is a gene, which code for antibiotic resistance, thus the recipient cells maintaining functional vector DNA can be selected directly on nutrient medium with the appropriate antibiotic.

By this technique a single gene of the request from the total genome of a thousand genes can be expressed in the clone of the recipient cells.

In case of successful manipulations, the gene *product* (*biologically active protein*) will be expressed by bacterial of fungal strains and accumulated in the nutrient medium. The last point will be the indication of protein production with its subsequent purification, concentration and storage.

Genetic engineering achievements stimulated great progress in biology and medicine.

Vaccine against hepatitis B infection, based on recombinant surface viral antigen, HbsAg, was shown to be strong effective, making possible the control of the hepatitis B spread. Another recombinant anti-rabies vaccine is under clinical trial now.

Also many human recombinant proteins (hormones, enzymes, cytokines and others) were obtained by genetic engineering methods. Insulin, human growth hormone, erythropoetin, streptokinase, various kinds of interferons, interleukins, colony-stimulating factors, humanized monoclonal antibodies and many other valuable substances are used now to treat patients suffering from certain diseases. The application field of genetically engineered products is being expanded continually.

Bacterial Genomics

Great successes in full-genomic sequencing opened the ways to decode complete genomic sequence of living organisms. This extremely difficult scientific problem was solving gradually from the middle of 90th of XX century and finally reached its node point with the deciphering of human genome in 2000-2001. This outstanding result has been achieved only on the base of experiments that identified more simple genetic sequences of bacteria and viruses.

Tens of thousands of bacterial and viral genomes were sequenced up to the present date. The characteristics of some investigated microbial genomes are shown in Table 2.

Table 2.

Characteristics of several known genomes of bacterial pathogens

Microorganism	Genome size, millions of base pairs	Gene quantity	Pathogen description
Mycoplasma genitalium	0.580	468	Urogenital tract pathogen
Mycoplasma pneumoniae	0.816	677	Pneumonia causative agent
Ricketsia provazekii	0.112	834	Epidemic typhoid fever causative agent
Treponema pallidum	1.138	1.041	Syphilis causative agent
Helicobacter pylori	1.668	1.590	Provokes gastric and duodenal ulcer and gastric tumors
Hemophilus influenzae	1.830	1.073	Causative agent of meningitis, acute respiratory diseases, etc.
Mycobacterium tuberculosus	4.412	3.924	Tuberculosis causative agent
Escherichia coli K 12	4.639	4.288	Autotrophic enterobacterium
Metanococcus jannaschii	1.660	1.738	Anaerobic thermophilic bacterium. Methanogen

Knowledge of microbial genome clarified a great number of unclear circumstances in infection pathogenesis and treatment, including bacterial capacity to produce toxins, adhesins and other virulence factors, mechanisms of bacterial invasion and persistency, spread of multidrug microbial resistance, etc. Determination of unique genetic clusters in microbial genomes ensures rapid precise diagnostics of the infectious diseases.

Also it was proven that the closest relationships exist between microbial and human genomes. Human genome was found to contain a great number of prokaryotic genes. For instance, many genes, which are very similar in humans and bacteria, control essential metabolic pathways. Nevertheless, vast representation of viral genes within human genome remains unclear. Maybe, it gives human beings some selective advantages in evolutionary process.

And finally, the next task of ultimate importance is to conceive the basic mechanisms of genotype realization towards corresponding phenotype. This is the subject of ***functional genomics***, ***transcriptomics***, ***epigenetics***, ***proteomics***, and ***metabolomics***.

For instance, the new scientific direction of ***proteomics*** investigates the *proteome*. In this vein, ***proteome*** means *the continiously changing array of cellular proteins and a complex of their interactions at the certain stage of cell development*.

Similarly, ***transcriptome*** is the *complete number of messenger RNAs* in microbial cells. And ***metabolome*** is the *total array of microbial chemical metabolites* taken at the certain time point and resulted from all cellular metabolic pathways.

While the genome is rather stable, the transcriptome, proteome and metabolome states are *dynamic*.

Of the extreme importance for the genome proper function is the influence of *epigenetics* and *epigenetic factors*.

Epigenetics investigates the *heritable changes in the genome* that are *not related with the changes of primary sequence of microbial DNA*. Epigenetic alterations ensue from various environmental impacts on the microbial cell that affect the expression of microbial genes. Epigenetic changes become fixed, being demonstrated in the next several generations of microbial offspring cells.

Overall, the elucidation of bacterial cell response to the different types of internal and external signalling, the discovery of the mechanisms of microbial reactivity and adaptation should result in design of new methods of infectious disease control and treatment.

Chapter 6
NORMAL MICROFLORA OF HUMAN BODY.

MICROFLORA OF ORAL CAVITY
(***for students of Dentistry Faculty***)

Normal Microflora of Human Body

Normal human microflora is the result of a long-term adaptation of microorganisms and their human hosts following the common process of their evolution.

A total amount of microbial cells inhabiting the body as well as the total number of their genes (also indicating non-cultivable microbes) is termed as human ***microbiome***.

The human microbiome is enormously large – it has been estimated that the total sum of microbial cells harbored within the human host is over 40 trillions ($4*10^{13}$ cells). This quantity really exceeds the total number of our own cells (nearby 30 trillions) that compose human body. There is no doubt that such a powerful force impacts on all the sides of human existence.

The Human Microbiome Project started at 2008 "...set the goal of identifying and characterizing the microorganisms, which are found in association with both healthy and diseased humans."

Relatively stable ensembles of normal human microflora occupy various body compartments as constant residents (***autochthonous*** or ***indigenous*** microflora). On the other hand, many microbes occasionally appear in various body parts and leave them after some time of dwelling. They pertain to ***allochthonous*** (or ***transient***) microflora, temporary for this site.

Nevertheless, the composition of normal human microflora is not constantly fixed but rather flexible, at least depending on the immune state, nutritional conditions and the age of individuals. It is especially influenced by various diseases encountering the body.

Microflora of Skin

Staphylococci, streptococci, micrococci, pseudomonads, numerous non-pathogenic corynebacteriae, and various fungi (yeasts and moulds)

usually inhabit the skin surface. Most of these agents pertain to aerobic or facultatively anaerobic microorganisms. Deep layers of skin including glandular ducts and hair follicles harbor non-sporeforming anaerobic bacteria, e.g. propionibacteria, bacteroids, prevotellas, and others. All of them gain the nutrients from the desquamated epithelium, secretions of the sweat and sebaceous glands, microbial waste products, etc.

The number of microbes on 1 cm^2 of the skin is about 80,000 of microbial cells.

In most of situations the opened areas of skin are available for exogenous infection being contaminated with staphylococci, streptococci, multiple fungi, spores of aerobic and anaerobic bacteria.

When the human body is exposed to soil and dust, the skin becomes contaminated with spores of bacilli and clostridia including the causative agents of gas gangrene and tetanus.

Suppurative infections of the skin and underlying tissues (e.g., boils, pyoderma, or abscesses) usually occur from the poor hygienic conditions of the skin on the background of secondary immunodeficiency.

Microflora of Eye Conjunctiva

The conjunctival mucosa is always washed by lacrimal fluid (tears) that contain many active antimcirobial substances (mucins, lysozyme, lactoferrin, IgA antibodies). This blocks the active growth of the most of bacteria. Relatively few microbial representatives, such as *Corynebacterium xerosis* or other diphtheroids, *S. epidermidis* and *S. saprophyticus*, nonhemolytic streptococci, non-pathogenic neisseriae or moraxellas may inhabit the surface of the conjunctiva.

Microflora of Respiratory Tract

When breathing, humans inspire a large number of aerosol dust particles contaminated with microorganisms. It has been found that the number of microbial cell within inspired air exceeds 200-500 times the amount of microbes in expired air. The penetrated bacteria are easily trapped or expelled out by ciliated epithelium of the nasal cavity, larynx or large bronchi. Therefore, only less amount of the microbials enters the bronchial tree. As the result of successful clearance, the terminal bronchioles and alveoli are not available for microorganisms.

In general, the nasal cavity confines only a moderate or small amounts of microorganisms. It depends in part from bactericidal activities of mucosal mucins, lysozyme and secretory IgA. Various staphylococci, diphtheroids, hemophiles, viridans streptococci are capable of growing there. In addition, many viruses maintain their viability in these conditions for a long time.

The upper respiratory tract (nasopharynx and larynx) harbors relatively stable composition of a limited number of microbial species. Among them are *S. epidermidis* and *S. saprophyticus,* various streptococci, diphtheroids and some others.

The lowest parts of respiratory tract that include bronchioles and alveoli are normally sterile.

When the body protection dampens from some internal or external challenge (like cooling, starvation, or secondary immune suppression) the facultative pathogens – normal inhabitants of the respiratory tract – can be re-activated and cause certain respiratory infections such as sinusitis (the common agents are haemophilic bacteria and pneumococci), tonsillitis (induced by streptococci), bronchitis, or pneumonia (caused by pneumococci or staphylococci).

Microflora of Urinary Tract

In healthy individuals the renal calyces, pelvis, urethers, bladder and proximal parts of urethra are sterile.

In the distal part of male urethra occasional presence of *Staphylococcus saprophyticus*, viridans streptococci, diphtheroids, neisseriae, and some gram-negative rods is registered. In most of cases they appear in this area from skin and perineum.

The female urethra is normally sterile; rarely it may contain a limited number of coccoid microflora.

Mycobacterium smegmatis and saprophytic mycoplasmal species can be ordinarily found on the mucous membranes of genitalia.

Microflora of Vagina

First 1-2 days after birth the vagina of newborn is sterile. Next several weeks pH of vaginal content becomes slightly acidic that activates the growth of lactobacilli. In some time pH value changes to neutral range and

holds this level until puberty. This stimulates the growth of coccoid flora; the balance between cocci and lactobacilli supports the state of vaginal microflora this time.

At puberty lactobacilli compose a predominant part of vaginal microorganisms (*Lactobacillus crispatus*, *Lactobacillus jensenii* and others). They intensively produce acids from vaginal carbohydrates (mainly, from glycogen), thus shifting pH levels to acidic range of 4-5. Therefore, they demonstrate the evident antagonistic properties against transient vaginal bacteria including pathogenic species.

The vaginal secretion of the healthy woman has an increased concentrations of glycogen and other sugars with relatively low amount of proteins; this state is maintained by normal endocrine function of ovaries. Acidification of vaginal content is an important protective condition that prevents the propagation of pathogenic and facultatively pathogenic bacteria. The established pH level about 4.7 inhibits their growth.

During the menstrual cycle vaginal pH temporarily becomes alkaline that fosters the progression of coccoid bacteria. They, in turn, create the favorable conditions for other groups of bacteria that may be pathogenic. Sexual activity also results in alterations of vaginal microflora with appearance of extraneous microbial representatives from outside.

Together with lactobacilli, other microbial species in various proportions may present as the part of normal vaginal microflora. Among them are group B streptococci (*S. agalactiae*), mycoplasmas, *Gardnerella vaginalis* and *Mobiluncus* species, anaerobic bacteria (bacteroids, prevotellas, peptostreptococci and others). In case of poor hygiene the microbes from perineal and perianal areas may appear.

Intensive antimicrobial treatment with antibiotics of broad spectrum of action can suppress normal vaginal bacteria, primarily lactobacilli, that results in burst growth of concomitant resistant microflora. It may lead to vaginal *dysbiosis*, where the fungal species usually prevail. Among them are yeast-like fungi from *Candida* genus (e.g., *C. albicans*) that cause serious infectious disorder known as *vaginal candidiasis*.

The abrogation of protective function of lactobacilli may also trigger an excessive growth of many other vaginal microorganisms. When they start to dominate, they may develop an extensive genital non-inflammatory syndrome termed as ***bacterial vaginosis***. It is caused by broad microbial association of *Gardnerella vaginalis* and *Mobiluncus mulieris* with non-sporforming gram-negative anaerobes (*Prevotella bivia,* bacteroides and some others). Above 1/3 of women may suffer from bacterial vaginosis. If not controlled, this pathology leads to serious complications, e.g.

endometritis or pelvic inflammatory disease. Their progression cause profound negative effects on normal vaginal microflora..

Microflora of Oral Cavity

In the oral cavity more than 1000 species of microbes are present. Less than half of them are only cultivable. Total quantity of microbes exceeds 1 billion per 1 ml of saliva. The detailed characteristics of oral microbiota are presented in the section "Microflora of oral cavity" (see below).

A tremendous variety of saprophytic and facultatively pathogenic microorganisms (streptococci, staphylococci, diphtheroids, treponemas, fungi, protozoa and many others) is found upon oral mucosa.

The oral cavity is a favorable medium for the most of microbes; it has an optimal temperature, a sufficient amount of nutrients, and a weakly alkaline reaction.

The groups of bacteria, associated with the healthy state of dental tissues, include the vast number of streptococci (e.g., *S. sanguis*, *S. mitis*, *S. gordonii*, or *S. intermedius*) and some other bacterial species (e.g., *Veillonella parvula* and *Actinomyces odontolyticus*).

The majority of bacteria can readily attach to dental tissue forming ***dental plaque*** – a special kind of microbial *biofilm*. The role of oral streptococci should be emphasized here, as they produce large amounts of long-chain polysaccharides from food sugars, thereby promoting microbial adhesion.

When oral hygiene is inadequate, the deep teeth lesion, or ***caries*** develops. In conditions of food carbohydrate excess (so-called "table sugars") ***cariogenic oral streptococci*** *S. mutans* and *S. sobrinus* metabolize sucrose and other carbohydrates with lactic acid production. Decrease of pH leads to teeth enamel decay. Various lactobacilli species promote further caries progression.

The presence of carious teeth is the condition for deep change of normal oral microbiota. It is characterized by gradual expansion of anaerobic bacteria that accelerate decaying processes. Finally, this may lead to various kinds of periodontal pathology (e.g., ginigivitis and acute or chronic periodontitis).

Among the most common pathogens, causing gingival pathology, are *Prevotella intermedia*, *Peptostreptococcus micros*, and several species from *Fusobacterium* genus (*F. nucleatum*, *F. periodonticum*).

The causative agents of periodontites comprise pathogenic microbial species *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, as well as *Eikenella corrodens*, *Aggregatibacter actinomycetemcomitans*, *Capnocytophaga spp*., *Actinomyces naeslundii* and many others. They actively stimulate the progression of periodontitis resulting in tissue destruction.

Microflora of Gastrointestinal Tract

Initially sterile in newborns, gastrointestinal tract is rapidly colonized by microorganisms, uptaken with food. In breast-fed infants the intestinal microflora largely comprise lactobacilli, lactic acid streptococci and bifidobacteria.

In healthy adults the esophagus has only accidental transient microflora passing from oral cavity.

In stomach the normal acidity of gastric juice (in the range of 1.5-3.5) greatly diminishes the total amount of microorganisms. Actually, the gastric juice demonstrates remarkable microbicidal properties, being an efficient barrier on the way of incoming microbial agents.

Nevertheless, the protective function of the gastric juice is flexible, depending on food habits and preferences, the volume of water consumed, and many other factors, including the state of gastric mucosa. In hypoacidic patients with chronic atrophic gastritis the defensive barrier of the gastric juice is seriously weakened.

In healthy individuals the medium concentration of microorganisms in gastric juice doesn't exceed 10^3-10^5 cells per 1 g of gastric contents. Various groups of bacteria and fungi, such us *Sarcina ventriculi*, lactobacilli, sporeforming *Bacillus subtilis*, yeasts may be present there.

In 1980s a causative agent of chronic gastritis and duodenum ulcer was discovered in gastric mucous layer and then isolated. This bacterium was named *Helicobacter pylori* according to its spiral form. It is motile microaerophil persisting in gastric mucosal membrane. Stomach of children is usually free of *Helicobacter* but among adults almost 50% of humans are carriers of *Helicobacter spp*. About 30 species of *Helicobacter* are discovered to date, some of them may persist in humans.

In the duodenum and other parts of small intestine the pH of lumen contents becomes alkaline, thereby rising the opportunities for microbial propagation. However, the small intestine carries moderate amounts of microbes in the range of 10^4-10^8 cells per 1 g of contents with gradual

increase towards the large intestine. In upper parts of the intestine lactobacilli and enterococci are found, in cecum the fecal microflora prevails.

The large intestine is literally overwhelmed with bacteria. About one-third of the dry weight of feces is made up of microbial bodies.

In distal parts of the bowel (sigmoid colon and rectum) about 10^{11} microbial cells per 1 g of feces is determined.

Strictly anaerobic bacteria dominate within the large intestine comprising 96-99% of total microbial mass.

Among them are non-sporeforming gram-negative anaerobic bacteria (genera *Bacteroides*, *Prevotella*, *Bilophila*, *Porphyromonas*, *Fusobacterium*), anaerobic sporeforming clostridia (*Clostridium perfringens*), anaerobic gram-positive peptostreptococci, anaerobic lactobacilli and bifidobacteria.

The minority of facultatively anaerobic bacteria comprises the strains of *E. coli* and other coliform bacteria, *Enterococcus fecalis*, candida fungi and some others.

Normal microflora of the large intestine supports many important physiological functions of the bowel.

For instance, bifidobacteria and lactobacilli are the natural antagonists of pathogenic enteric microflora like salmonellas and shigellae.

Non-sporeforming gram-negative anaerobic bacteria play a significant role in food digestion, transforming carbohydrates and other nutrients into short-chain fatty acids that are used by the host as the substantial source of energy. These bacteria also stimulate local intestinal immune response and support intestinal ***colonization resistance*** that hinders pathogenic bacteria to attach and colonize the intestinal wall.

Similarly, *Clostridium perfringens* produces a number of digestive enzymes (e.g., proteases and lipases); *E. coli* and some other species synthesize the essential vitamins (primarily, of the groups B and K).

However, in case of intestinal damage by trauma or inflammation all these bacteria cause the serious pathology of the human body. For instance, the members of genera *Bacteroides* (mainly, *Bacteroides fragilis*), *Fusobacteria*, *Prevotella*, or *Bilophila* as well as *E. coli* actively participate in many infalmmatory disorders. They are found in acute appendicitis, postoperative infectious complications within the peritoneal cavity (abscesses and peritonitis), inflammatory diseases of the gastrointestinal tract, and in the emergence of sepsis.

Likewise, a long indiscriminate use of antibiotics especially of broad spectrum of action suppresses normal gut microflora, resulting in ***dysbiosis***

of the intestine. In these cases *candida fungi* are most commonly registered. Serious complications after long-term antibiotic treatment followed by dysbiosis are provoked by *Clostridium difficile* that cause ***antibiotic-associated diarrhea*** and severe antibiotic-associated ***pseudomembranous colitis*** with the deep damage of the intestinal wall.

Dysbiosis (**Dysbacteriosis**)

Quantitative and qualitative disturbances of normal microflora of human body that follow infectious and somatic diseases, long-term and indiscriminate use of antibiotics results in ***dysbiosis*** (or ***dysbacteriosis***).

Many factors may lead to dysbiosis. Of main medical importance is long antibiotic and antiseptic treatment especially with drugs of wide spectrum of action administered in improper doses. Among other causes are chronic somatic and infectious diseases, cancer, immune suppression, irradiation, stress etc.

This state is characterized by profound disorder in digestion products assimilation, impairment of enzyme activity, physiological secretion cleavage, etc. The territorial deviations of microflora cause a whole series of complications: intestinal dyspepsia, secondary immune deficiency, toxic infections, suppurative processes, inflammation of the respiratory tract, various forms of candidiasis, etc. In dysbiosis the number of lactobacteria declines, the number of anaerobes arises; fungi, resistant to conventional antibacterial treatment, begin to grow actively.

Current researches try to establish dysbiosis associations with obesity, colitis, various forms of cancer, bacterial vaginosis, inflammatory bowel disease or chronic fatigue syndrome.

The treatment of dysbiosis includes cancellation of antibiotics usage, and administration of special diet, vitamins or immunomodulatory drugs. Most effective is treatment with ***probiotics***. These biological products contain *live bacteria of symbiotic intestinal microflora that possess antagonistic activity against pathogenic microbial agents*.

Colibacterin as biological product contains living *E. coli* from strain M17 that produce bacteriocins (***colicins***) against shigellae, salmonellas, enteropathogenic colibacteria, etc.

Bifidumbacterin is composed of live bifidobacteria of the same features.

Bificol is a complex probiotic product of *E. coli* and bifidobacteria.

Bactisporin contains the spores of *Bacillus subtilis*; it develops antimicrobial and favorable enzyme action for food digestion.

Some other broadly used probiotics may contain the strains of *Lactobacillus rhamnosus* or the yeasts *Saccharomyces boulardii*.

Nevertheless, if to take into account the numerous entangled relationships within microbial biota of human body, it becomes obvious that not every disturbance in normal microbial population must be treated, and the microbial balance may be rehabilitated due to its natural processes.

MICROFLORA OF ORAL CAVITY
(***for students of Dentistry Faculty***)

Normal Oral Microbiota

Oral cavity provides favorable conditions for growth and propagation of multiple microbial inhabitants. They can be found in great amounts on mucous membranes of tongue, cheeks, teeth, gingival crevices and pockets. Species composition of oral microflora is extremely variable (see Table 3).

Table 3.
Typical representatives of oral microbiota

Microbial species	**In saliva**		**In gingival crevices (detection rates and grades)**
	Detection rates, %	***Quantity, cells/ml***	
Group A. Resident autochthonous microflora			
I. Aerobic and facultatively anaerobic:			
S. mutans	100	$1,5*10^5$	100
S. salivarius	100	10^7	100
S. mitis	100	10^6-10^8	100
Saprophytic neisseriae	100	10^5-10^7	++
Lactobacilli	90	10^3-10^4	+
Staphylococci	80	10^3-10^4	++
Diphtheria-like corynebacteria	80	No data	+
Actinomycetes	100	No data	++
Candida and other yeast-like fungi	50	10^2-10^3	+
Mycoplasmas		10^2-10^3	No data
II. Obligate anaerobes			
Veilonellas	100	10^6-10^8	100
Anaerobic streptococci (peptostreptococci)	100	No data	100
Bacteroids	100	No data	100
Fusobacteria	75	10^3-10^4	100
Group B. Transient allochthonous microflora			
Aerobic and facultatively anaerobic:			
Gram-negative rods			
Klebsiella spp.	15	10-10^2	0
Aerobacter spp.	3	10-10^2	0

Oral cavity harbors above 1000 of diverse bacterial species. Their absolute quantity is also enormously high. For instance, total salivary microbial count exceeds 1 billion cells per 1 ml.

These bacteria encompass mixed microflora from various compartments of oral cavity. Most of them participate in dental plaque formation.

There are two main groups of bacteria that make oral microbiota – *autochthonous* and *allochthonous* microflora.

Autochthonous or ***indigenous*** bacteria are the resident inhabitants of oral cavity (*obligate* microflora), whereas *allochthonous* microorganisms are temporary for this site (or *transient*) arising largely from external source. Nevertheless, transient oral microflora comprises more likely pathogenic and opportunistic bacterial species in comparison with resident ones.

Allochthonous microorganisms enter oral cavity from other biotopes of human body (e.g., large intestine) or from external environment.

The group of resident aerobic and facultatively anaerobic gram-positive cocci encompasses mainly ***viridans streptococci***. They produce green zone of hemolysis when grown onto blood agar medium. Most common here are *S. mutans, S. mitis, S. sanguis, S. salivarius*. Their quantitative distribution depends on many variable external and internal factors: person's diet, oral cavity personal hygiene, state of local immune response, genetic factors, etc.

Streptococci can produce hydrogen peroxide and ferment carbohydrates yielding organic acids. This lowers local pH below 5.0 resulting in dental enamel demineralization and teeth decay. Furthermore, streptococci are capable of making polysaccharides from sucrose taken from sucrose-containing foodstuffs. Soluble oligosaccharides are metabolized by other bacteria thereby intensifying acid formation. Non-soluble polysaccharides actively promote adhesion of oral streptococci to dental surface thus fostering dental plaque growth.

Gram-positive anaerobic cocci are represented by peptococci that intensively utilize peptides and amino acids. Unlike streptococci they demonstrate slow carbohydrate fermentation.

Resident oral gram-negative anaerobic cocci, e.g., *Veillonella* genus members, play important role in metabolic balance within oral cavity. They don't ferment mono- and disaccharides but utilize numerous organic acid (lactate, pyruvate, acetate and others) yielding CO_2 and H_2O end products. This leads to acid content neutralization and pH rise that ameliorates local environment. Taking into account virtually similar amount of viridans

streptococci and veillonellas in saliva the latter degrade lactic acid produced after streptococcal fermentation thus protecting against caries.

Gram-negative diplococci from *Neisseria* genus are facultatively anaerobic. They can be found at early stage of dental plaque initiation and growth. Unlike streptococci they demonstrate slow rate of propagation. Their most common species are *N. sicca* that produce various polysaccharides and *N. subflava*.

Oral gram-positive aerobic and facultatively anaerobic rods comprise lactobacilli, corynebacteria and some other representatives.

The members of *Lactobacillus* genus generate ample quantities of lactic acid upon carbohydrate fermentation that actively stimulates caries progression. Corynebacteria lower redox potential in local dental surroundings ensuring beneficial conditions for anaerobic bacteria overgrowth (e.g., bacteroids, prevotellas, porphyromonads, fusobacteria, spirochetes, and many others). Moreover, corynebacteria produce vitamin K that is used as potent growth factor by many oral bacteria.

Two genera from *Actinomycetaceae* family, namely *Actinomyces* and *Bifidobacterium*, can be found in oral microflora as well.

Actinomycetes easily settle upon mucous layer of oral cavity; they are typical microbial constituents of dental plaque and dental stone. Actinomycetes are commonly isolated from ducts of salivary glands, gingival pockets, and carious cavities. These bacteria possess weak proteolytic activity but intensively ferment carbohydrates accumulating broad spectra of organic acids (lactate, acetate, succinate, formate and others).

The species *A. israelii*, *A. naeslundii genospecies 2* (former A. *viscosus*) contribute to caries and periodontal disease progression.

Bifidobacteria ferment numerous carbohydrates with lactic and acetic acid end products predisposing to decay of dental enamel and caries.

Gram-negative rods predominantly comprise obligate anaerobic bacteria from genera *Bacteroides*, *Porhyromonas*, *Prevotella*, *Fusobacteium*, *Leptotrichia*. These agents are autochthonous representatives of oral microbiota. They lack of catalase, ferment carbohydrates with gas and hydrolyze proteins to amino acids.

Multiple *bacteroidal* members of microbial community belong to *B. forsythus*, *B. gracilis*, *B. urealyticus*, and many other species. In association with streptococci and fusobacteria they may exert periodontal disorders.

Pigment bacteria *Porphyromonas gingivalis* and *P. endodontalis* are isolated from periodontal tissues. They are typically indole-producing.

P. gingivalis expresses collagenase that acts detrimentally on dentin layer and destroys fibrinogen. These pathogens are found in gingivitis and periodontal pathologies.

Very common oral pathogens are *Prevotella melaninogenica, P. oralis, P. denticola, P. buccalis*. Their carbohydrate-fermenting capacity is low. *P. melaninogenica* is the constant habitant of dental pockets in adults. By secretion of phospholipase A it breaks cell membrane integrity thereby stimulating periodontal diseases.

Fusobacteria are the spindle-like polymorphic rods that grow in dental pockets in association with other bacteria (e.g., spirochetes). They weakly ferment carbohydrates and peptone, releasing butyric, and lesser amounts of lactic and acetic acids; prodice indole. Typical representative is *F. nucleatum*.

Leptotrychia are granular polymorphic rods, some of them are filamentous. Most frequent agent here is *L. buccalis*, capable of glucose fermenting with large amounts of lactic acid. The amount of these bacteria raises in case of periodontal disease progression..

Conventional members of dental microflora embrace numerous spirochetal species of *Treponema, Borrelia* and *Leptospira* genera. Typical oral treponemas are *T. oralis, T. macrodentium, T. denticola* and others.

Treponema vincentii is ordinarily found in oral folds and dental pockets. It produces modest amounts of acetic and butyric acids. In persons with weakened immunity *Treponema vincentii* together with prevotellas and fusobacteria exerts acute necrotizing ulcerative gingivitis (or ANUG), demonstrating sudden onset.

Gingival pockets often harbor *Borrelia buccalis* – large spirochetes that live in symbiotic associations with fusiform bacteria.

Most of oral mycoplasmas pertain to saprophytic *M. orale* and *M. salivarium* species.

Candida fungi participate in colonization of oral mucosa in closest interrelationships with neighboring bacteria. In most situations they don't evoke pathological changes. However, in cases of indiscriminate use of antibiotics or secondary immune deficiencies *oral candidiasis* can arise thus indicating deep shift in local microbiota composition that results in dysbiosis.

Ontogenesis of Normal Oral Microflora

Bacterial entry to newborn's oral cavity occurs initially at the time of delivery. Primary oral microflora is composed of lactobacilli, enterococci,

micrococci, staphylococci and some others. In first weeks these casual microorganisms will be displaced by certain bacterial species inhabiting maternal oral cavity. Likewise, medical personnel of obstetrics care settings becomes the next source of microbial contamination. Aerobic and facultatively anaerobic microflora dominates in newborn's oral cavity. Among them are streptococci, lactobacilli, neisseriae and candida fungi. Their total count rises up to 4th month of life; then it gradually declines. Initial amount of anaerobic bacteria is very low (veillonellas, fusobacteria, and some others). Usually they stay within the folds of oral mucous membranes.

Dentition creates new opportunities for anaerobic bacteria propagation. Anaerobes begin to spread throughout the all compartments of oral cavity. At puberty the number of anaerobic bacteria arises; bacteroids, prevotellas and spirochetes become typical this time.

In elderly people with multiple comorbidity and lowered immunity the composition of normal oral microflora is profoundly altered. The number of staphylococci as well as candida fungi elevates substantially; *E. coli* and enterococci can be found. The presence of removable dental prosthetic devices facilitates the shift in microbial composition resulting in emergence of prosthetic stomatitis. The plaques made of settled microorganisms and organic matrix under partial dentures accumulate acidic substances that favor candidal propagation. Oral candidiasis in patients with dental prostheses can occur in more than 70% of cases. In these situations candidae may spread from initial colonization site towards any oral compartment. In fact, they cause angular stomatitis when located in angulus oris.

Similarly, the bacteria colonizing oral cavity, can afflict airways and gastrointestinal tract.

Microflora of Saliva, Tongue, Dental Plaque and Gingival Crevice

Saliva and gingival crevicular fluid are the main liquid substances washing oral cavity. The saliva is crucial in balancing oral microbial ecology. All the properties of saliva (secretion rate, viscosity, mineral contents, ionic potential, buffering capacity, multiple organic matter – amino acids, polysaccharides, vitamins, nucleotides, potent antimicrobial factors – mucins, secretory IgA antibodies, lysozyme) contribute to microbial composition of oral cavity.

Besides saliva, the bacteria are located preferentially in three zones of oral cavity: dental plaques upon tooth crown (or inside carious lesions in case of caries); within gingival crevices; and upon lingual body especially covering its back side.

Total amount of bacteria in saliva varies in the range from 40-50 mln to more than 5 billion per 1 ml, for about 750 mln an average. Microbial concentration in dental plaques and gingival crevices is almost 100-fold higher – nearly 200 bln microbial cells per 1 g of medium content. Besides microbial cells, the latter harbors about 80% of water.

As mentioned above, numerous microbial species and genera reside in oral cavity. More than one-half pertain to vast number of streptococcal species, e.g., *S. mutans*, *S. mitis*, *S. sanguis*, *S. sobrinus* and others – except beta-hemolytic streptococci that can be found solely as transient microflora. Various coccal species occupy certain compartments within the mouth. For instance, most of enterococci are located inside gingival crevice and upon the body of tongue; *S. mutans* is typically found in dental plaque upon crown.

Viridans streptococci and veillonellas produce the great mass of salivary microflora. Mostly they shed there from tongue body. The number of gram-negative anaerobic rods (bacteroidal species and fusobacteria) together with diphtheroids increases in gingival crevices.

Total quantity of microbial cells undergoes daily alterations. It depends mainly on amount of saliva secretion that greatly declines at night. Dental loss leads to marked reduction of dental microflora.

Multiple factors can impact the certain members of oral microbiota. For example, any antibiotic treatment inhibits the target group of defined microbial species thus impairing normal microbial balance. Protein-enriched diet doubles the number of facultatively anaerobic gram-positive rods. Large part of bacteria needs vitamins or other supplements for their successful propagation; lack of growth factors results in suppression of activity of selected bacterial groups.

Qualitative and quantitative composition of dental microflora is greatly influenced by various diseases. For instance, *C. albicans* recovery from oral samples is made with highest rate in diabetes patient (up to 80% against 50% in healthy individuals). Lactobacilli grow high in caries patients and fall down after lesions treatment.

It can be indicated also that *S. mutans*, *S. sanguis*, lactobacilli, yeasts and spirochetes seriously disappear after massive dental loss, whereas the amount of *S. salivarius* elevates in the course of time. In first 2 weeks after mounting of removable dentures the levels of streptococci look high,

whereas the quantity of lactobacilli and yeasts rapidly goes down. In 3-5 weeks the count of lactobacilli and yeasts tends to restore but the number of streptococci declines. Overall, the total number of streptococci doesn't alter significantly in all periods of life.

Polymicrobial adherence to dental surface leads to *dental plaque* formation.

Dental plaque is a complex matrix (or ***microbial biofilm***) made of immensity of microbial bodies, their extracellular products and wastes, and salivary compounds.

Dental plaques are divided into ***supragingival*** and ***subgingival***. Supragingival plaques play substantial role in caries. Likewise, subgingival palques participate in periodontal disease progression.

Composition of dental plaque differs depending on site of adherence and plaque's maturation stage. It grows predominantly on dental surfaces that avoid mechanical cleaning – approximal surface between two teeth, fissures and pits of the tooth crowns, gingival crevices.

The process of plaque formation commences from adhesion of poorly soluble polymeric carbohydrates such as dextrans together with mucins and salivary proteins to dental enamel. Acid glycoproteins react with calcium ions of enamel whereas basic proteins bind to phosphates of hydroxyapatites. Primary biofilm is known as ***pellicle***.

Attachment of bacteria demonstrates rapid progression. By 5 minutes the number of microbes arises up to 10^5-10^6 of bacterial cells per 1 cm^2. Initial microbial bodies land within tooth pits and fissures; later they spread to smooth dental areas. Further microbial propagation and their exopolysaccharide excretion facilitate the growth of soft dental plaque.

Many bacterial cells can't attach firmly to clean dental surface but easily bind to primarily absorbed microbial layer. For instance, when coccoid flora surrounds embedded rod-like and filamentous bacteria, it produces mixed cellular clusters known as *corncob formations*.

The bacteria composing dental plaque can be divided into two large groups. The first comprises acidophilic agents able to propagate in acidic environment – lactobacilli, actinomycetes, peptococci, leptotrichia, corynebacteria and some others. The second one embraces bacteria with prominent proteolytic activity – veillonellas, fusiform bacteria, neisserias, vibrios, or spirochetes.

At initial steps of maturation the dental plaque has larger amounts of aerobic and facultatively anaerobic bacteria with dominating role of oral streptococci.

Oral viridance streptococci together with lactobacilli ferment sucrose resulting in overproduction of lactic acid and next sharp decline of local oral pH. Lactate can be further utilized by veillonellas, neisserias and other microbials accumulating more organic acids (eg. acetic, propionic or formic). All these changes influence microbial composition of dental plaque.

Exuberant consumption of sucrose and other simple carbohydrates from nutrients worsens the situation and intensifies enamel destruction, microbial retention and plaque maturation. In addition, elevated levels of carbohydrates in oral cavity lead to their polymerization by local microbiota. Synthesis of extracellular polysaccharides such as soluble or insoluble dextran and levan is typical for oral streptococci especially *S. mutans*. They facilitate microbial tooth adhesion and consolidate the matrix of microbial biofilm within dental plaque.

The synthesis of bacterial exopolysaccharides ceases at pH below 5,0.

Supragingival dental plaque predominantly harbors facultatively anaerobic gram-positive bacteria, mainly the broad spectra of streptococci and actinomycetes. Gram-negative representatives that pertain to *Veillonella*, *Bacteroides* and *Haemophilus* species are present constantly but in lower concentrations.

Similarly, subgingival dental plaque also confines the most common gram-positive microorganisms – streptococci and *Actinomyces spp.* Non-affected subgingival crevice carries moderate number of microbes; their total number varies from 10^3 to 10^6 cells per site.

Composition of bacterial plaques is different also on teeth of upper and lower jaws. A large proportion of streptococci and lactobacilli is present within dental plaques of upper jaw. Veillonellas and filamentous bacteria can be often found on teeth of mandibular bone.

Gram-positive cocci and rods prevail on approximal dental surfaces (between teeth) and within fissures. First day of plaque emergence is characterized by swift primary microbial colonization. After plaque maturation their microbial composition remains stable for a long time.

Next plaque progression is followed by lowering of its redox potential under the action of aerobic and facultatively anaerobic bacteria, thus engendering the growth of obligate anaerobic organisms. The dental plaque progressively accumulates bacteroids, porhyromonads, prevotellas, fusobacteria, leptotrichias and many others. Their metabolism results in alkaline byproducts (e.g., ammonia, urea, etc.) thereby elevating dental pH and dampening further plaque growth.

Sequential change of microbial communities, basic character of elderly plaque biofilm, accumulations of calcium and phosphates predispose to the formation of *dental stone* (*calculus*, or *tartar*). It begins to grow on dental surface especially in the area of gingival margin that impedes circulation of crevicular fluid.

Dental stone (***calculus***) is the solid formation tightly attached to dental crown and/or radix that is resulted from consolidation and calcification of contents of long-term dental plaque (degraded microbial bodies and polymeric matrix, inorganic matter, etc.)

Dental stones are also divided into ***supragingival*** and ***subgingival***. *Supragingival stones* can be ordinarily found nearby the openings of ducts of salivary glands or upon the lingual surface of lower molars. *Subgingival* attach to dental radices. This stimulates progression of dental pockets impacting gum detachment.

Overall, dental plaques and dental stones impair the normal self-cleaning of dental areas and promote the development of most common aggressive disorders – i.e., *caries* and *periodontitis*.

Efficient prophylaxis of these widespread dental diseases depends on the number of medical and hygienic measures for prevention and removal of dental plaques such as brash cleaning of teeth and dental flossing; the use of proper tooth pastes and powders that ensure plaque withdrawal.

Biological Role of Normal Oral Microbiota

Indigenous microflora of healthy oral cavity predominantly comprises commensal non-pathogenic microorganisms. It performs a lot of essential activities supporting normal body physiology. For instance, it creates biological barrier blocking invasion and propagation of pathogenic microorganisms and stimulates lymphoid tissue maturation thereby taking part in host defense against infectious agents. Likewise, the members of normal oral microbiota render antagonistic activity against multiple pathogenic species, which have the portal of entry in oral cavity. Autochthonous microflora participates in self-clearance of oral cavity. Similarly to many other representatives of human gastrointestinal tract, the resident oral bacteria supply the body with vitamins and amino acids produced by bacterial cells. Other metabolic end products of normal microflora can stimulate secretion of salivary glands and mucous membranes fostering permanent wash of oral cavity. This facilitates food intake, oral food digestion, chewing and swallowing.

Dysbiosis of Oral Cavity

Oral microbiota looks like explicitly complex, multi-component and multi-functioning assemble of microbial communities that is characterized by contemporaneous cross-linked interplays between aerobic, facultatively anaerobic and obligate anaerobic bacteria with their multiple species of gram-positive and gram-negative agents. Stable but fragile equilibrium established within oral microbiota through the years of long evolution maintains the healthy state of oral cavity.

The shift in this balance may deeply deregulate normal metabolism and function of oral ecosystem.

For instance, *indiscriminate use of antimicrobial agents* (antibiotics or antiseptics) especially with broad-spectrum of activity can easily provoke oral ***dysbiosis*** (or ***dysbacteriosis***). It promotes the damage of oral mucosa resulting in drug-related stomatitis, glossitis or other ailments. These disorders are caused predominantly by *Candida* fungi, or sometimes by enterococci and various gram-negative rods.

The most common etiological agent of oral dysbiosis is yeast-like dimorphic fungal species *Candida albicans*. These fungi demonstrate enhanced adhesive capacity to epithelial cells. Carious excavations create efficient ecological niche for candidal colonization and successful propagation. Under long-term therapy with antibiotics of broad spectrum or following secondary immunodeficiencies candidal overgrowth results in ***candidiasis***. Fungal hydrolytic enzymes (e.g., numerous proteases, neuraminidase and others) take active part in its pathogenesis.

The interaction between yeasts and oral mucosal membranes starts from fungal adhesion. Sucrose, maltose, glucose and other sugars contribute to tight microbial adherence.

Adhesive capacity of fungal cells alternates depending on microbial strain. It is responsible for virulence of certain candidal isolate. The most pathogenic *C. albicans* absorb upon human epithelium approximately 1.5 times faster than the members of other species. Antibacterial treatment promotes yeast adherence whereas complement activation through fungal mannans seriously dampens it.

When propagating, yeast-like fungi take part in dental enamel decay and caries progression. Similarly, they may cause mycotic stomatites and tonsillitis.

In most pathological cases the fungi of *Candida* genus are associated with other microorganisms of oral cavity (*mixed infection*). Their synergistic interplays ensue from fungal synthesis of numerous growth

factors (namely, vitamins) that stimulate propagation of many bacterial species, e.g., lactobacilli or actinomycetes. By contrast, lactobacilli generate large amounts of lactic acid; and acidification of local environment suppresses candidal growth.

Thus, ordinary fungal colonization doesn't expand into visible pathology. Nevertheless, in conditions mentioned above (extensive antibiotic treatment or local immunodeficiency) latent fungal infection transforms into clinical *candidiasis* (typical ***opportunistic infection***). Predominant clinical forms of candidiasis are local oral lesions, but in severe cases they grow into devastating generalized mycosis with multiple organ failure.

Chapter 7
MICROORGANISMS AND THE ENVIRONMENT. BASIC PRINCIPLES OF SANITARY MICROBIOLOGY

Microbial Distribution in Nature

Microbes are ubiquitous in nature, being distributed everywhere. They are found in the soil, water, air, in plants, animals, foodstuffs, various utensils, within human body and upon human skin or mucosal membranes.

Microbial ***ecology*** (Gk *oikos* – home, *logos* – science) studies *substantial complex relationships that connect microbial populations with their environment*.

All of microorganisms inhabiting a certain area or body compartment are regarded as ***microbial community***.

Biotope means *the place of habitation of the certain microbial population*.

Microbial community, biotope and their multiple specific interrelationships form ***ecosystem***.

The role that an organism plays in its particular ecosystem as well as the physical space it occupies is termed as microbial ***ecological niche***.

Ecovariant *is the isolate of a certain microorganism adapted for the habitation within definite ecological system*. Among various microbial isolates ***hospital ecovariants*** (or ***hospital strains***) are of great medical importance. Their ecological niche is formed in hospitals and clinics, so these strains are extremely resistant to many antibiotics and other antimicrobial drugs. They cannot be eliminated readily.

The study of microbial ecology creates the proper basis, which allows to get insights into the mechanisms of microbial parasitism as well as to elaborate the measures for the control of various infectious diseases.

There are certain common types of relationships among the microbes maintained within their microbial communities.

Long-term cooperative interactions established between microbial species are called ***symbiotic***, competitive – ***antagonistic***.

Symbiosis includes the diverse microbial interrelationships.

Neutralism means the mode of relations, where the bacteria don't influence on each other within microbial community.

Commensalism is the kind of symbiosis, where one species exploits another without harmful effect. Commensal bacteria are normal inhabitants of human body.

Mutualism is the beneficial co-existence of two or more species. For instance, nitrogen-fixing root nodule bacteria from *Rhizobium* genus live together with some leguminous species.

Synergism means intensifying of functions of bacteria during mutual cultivation or dwelling. In such a situation the cooperation of non-pathogenic and pathogenic bacterial species may lead to the emergence of infectious process. As an example, acute necrotizing gingivitis arises from the complex polymicrobial infection of oral cavity, which major causative agents are oral spirochetes and gram-negative anaerobic *Prevotella intermedia* species.

Satellitism is observed, when the by-products of one bacterial species activate the propagation of another microbial species (microbial *cross-feeding*). For instance, vitamins and growth factors produced by the yeasts stimulate the growth of *Bordetella pertussis*.

Parasitism is the complex of microbial interplays, where one organism exploits another with the harmful effect for the last. Typical parasites are bacteriophages – the viruses, affecting bacterial cells.

Similarly, competitive ***antagonistic relationships*** are also observed in any kind of complex microbial coexistence. As the result of their practical use, in biotechnology antagonistic bacteria are thoroughly selected and used for synthesis of antibiotics.

There are three main forms of antagonism: ***overt***, ***forced*** and ***violent***.

In case of ***overt antagonism*** the microbe-antagonist produces antibiotics independently on rival presence.

In situation of ***forced antagonism*** antibiotic production by some microbial population is triggered only if the rival appears within the biotope.

And ***violent antagonism*** is characterized by fact that both competitors don't produce antibiotics, but in conditions of poor cultivation one species uses another as the source of nutrition.

Mechanisms of antagonism include antibiotic synthesis, production of bacteriocins, exhaustion of nutrient media, acceleration of the rate of metabolism, pH and pO_2 changes, etc.

Sanitary Indicator Microorganisms and Their Characteristics

Microbes are ubiquitous in nature. Any type of ecosystem comprise a vast number of inherent microbial residents maintaining its integrity.

Every natural or artificial biotope temporarily or constantly contains microbes able to cause human diseases. Nevertheless, it is rather difficult to determine all of pathogenic agents in the environmental samples. The number of pathogenic species is enough high and their properties are extremely variable. Therefore, the methods for their identification and continious monitoring are highly diverse, someway laborious, time-consuming and thereby expensive. A new methods of massive parallel genetic identification of microorganisms are extensively designed now (e.g., DNA microarray technologies), but they still remain more the subject of research interests.

Instead of pathogenic bacteria so-called ***sanitary indicator microorganisms*** are tested and monitored. The elevation of the quantity of indicator microorganisms corresponds to the increased probability of pathogenic bacteria presence in the environment.

Sanitary ***indicator microorganisms*** possess several *common traits*:

a) they are constant inhabitants of human or animal body that is followed by their continuous discharge to the environment in considerable amounts;

b) they have to survive in the environment in terms comparable with pathogenic bacteria or longer;

c) lack of reproduction in the environment;

d) absence of propagation in another biological reservoir or host except human or animal body;

e) they should be assessed easily by appropriate and reliable laboratory methods of microbiological monitoring.

Any environmental medium is characterized by its particular ***indicator microorganisms***.

Besides, the sanitary quality of a certain environmental medium is assessed also by its overall microbial contents.

This *sanitary quality index* is known as ***total plate count*** (*total viable count*, or total microbial count) that is equal to the total number of microbial cells (colony forming units or CFU) present in 1 g or in 1 ml of the sample.

Microflora of Water

Microorganisms inhabit the water of all basins – from seas and oceans to lakes, rivers, streams, or bogs. They are spread everywhere and can be found even on the bottoms of ocean trenchs at depths up to 4000-9000 m.

The flora of rivers and lakes depends on water pollution and therefore from the quality of wastewater treatment and purification.

The representatives of many bacterial genera – *Pseudomonas* (e.g., *P. fluorescens*), *Aeromonas*, *Plesiomonas*, *Micrococcus* (*M. roseus*), *Nitrosomonas*, *Nitrobacter* and others – can be determined in water as the common aquatic microorganisms. Anaerobic bacteria are infrequently found in water, correlating with its pollution.

The degree of water contamination by various microorganisms is designated as ***saprobity***. It generally comprises the total amount of all the living matter present in water including animal and plant decay remnants.

There are three zones of saprobity depending on the degree of water pollution.

Polysaprobic zone is highly contaminated water, with a mass of organic substances and a few oxygen contents. The total count of microorganisms in 1 ml exceeds 1,000,000. Coliform bacteria and anaerobic bacteria dominate there.

Mesosaprobic zone is characterized by a moderate pollution of water that is followed by the mineralization of organic matter by active oxidation and nitrification. The total microbial count in 1 ml of water is in the range about 10^4-10^5 microbial cells. The number of coliform bacteria is greatly reduced.

Oligosaprobic zone corresponds to pure water. The total number of microorganisms is generally low, about 10 to 1000 microbial cells in 1 ml of water. The representatives of coliform bacteria are not determined.

The water is an appropriate medium for transmission of the diseases predominantly by ***fecal-oral route*** (***waterborne diseases***). The most common infections transmitted by water include the broad group of bacterial and viral enteric infections (salmonellosis, shigellosis, colienteritis, cholera, campylobacteriosis, hepatitis A and E) as well as leptospirosis, tularemia, amoebic dysentery, fungal infections.

Many pathogenic bacteria remain alive in water for a long time. For instance, shigellae survive in water for 7-9 days, salmonellas – about 3 months, *Vibrio cholera* and *El Tor* – for many months, *Francisella tularensis* – for about of 3 months, leptospirae – from several weeks up to 4-5 month.

There are two main parameters (indices) indicating water sanitary quality.

Primary one is ***total plate count*** (or *total viable count*) in 1 ml of water.

Another one is the ***quantity of fecal indicator microorganisms***. They have to be equal or less than their numbers established by regulation acts.

Total plate count of water *is the quantity of mesophilic chemoorganotrophic bacteria in 1 ml of water capable of producing colonies after incubation at 37^{o}C for 24 h.* It should be less than 50 colony-forming units (CFU) per 1 ml (cm^3) for tap water. In that case tap water is considered as clean satisfying sanitary regulations.

In the well water and in open reservoirs the amount of microbes in 1 ml should not exceed 1000.

The test for total microbial count determination in tap water is performed as follows. Tap is flamed, and then the water is opened and flows for 5 minutes. Then 1 ml of water is taken, poured into sterile Petri dish and mixed with 6-8 ml of melted and cooled up to 45^{o}C meat peptone agar. After pour plating agar is hardened and the probe is incubated in thermostat at 37^{o}C for 24 h. Then the total quantity of colonies is counted.

Indicator microorganisms of water are evaluated by determination of *E. coli* and its variants (so-called ***coliform bacteria***). They reflect the possibility of fecal pollution of water. The coliform bacteria comprise the members of *Enterobacteriaceae* family from *Escherichia, Citrobacter, Enterobacter, Klebsiella* genera. They are gram-negative rods without spores, oxydase negative, fermenting lactose and mannitol to acid and gas products at 37^{o}C in 24 h. These bacteria are discharged to the environment with feces from humans or animals.

Among total coliform bacteria there are ***thermotolerant bacteria***, *fermenting carbohydrates at 44^{o}C for 24 h*. These bacteria indicate fresh fecal environmental pollution.

Standards for tap water include the ***count of total coliform bacteria*** and ***thermotolerant bacteria*** in 100 ml of water. They should be absent in 300 ml of examined water probe.

Due to epidemiological situation some additional parameters of water quality (the quantity of ***coli phages***, ***enteroviruses***, ***C. perfringens spores***) are estimated. These agents must be absent also.

Taking into account the enormous epidemiological role of water in relation to enteric infections, the tests for the assessment of indicator bacteria in water must be rapid, not laborious and highly reliable.

There are two basic testing methods for determination of quantity of fecal indicator bacteria in water.

First one is the ***membrane filtration*** method that is performed in several steps.

Three 100 ml portions of water are filtered through 3 separate nylon filters placed in sterile conditions into the funnel manifold (apparatus for the membrane filtration). The filters are removed and put onto *Endo agar* or similar medium. After incubation at 37°C for 24 h the quantity of *red lactose-positive colonies* is evaluated. If the growth of lactose-positive colonies is absent, the test means negative and the quality of water corresponds to normality. In opposite case the investigation is continued. After counting of lactose-positive colonies the gram-stained slides are prepared and examined (for colibacteria *gram-negative rods* should be revealed). Oxydase test is performed that is *negative* for coli-bacteria. Then the colony sample is inoculated into semi-solid lactose-peptone media for incubation at 37°C within 24 h. Gas and acid production is detected and the *conclusion about quantity of total coliform bacteria is made*. It indicates the fecal water pollution regardless of its terms.

For identification of ***fresh fecal water pollution*** the quantity of *thermotolerant bacteria* is assessed. Additional examination includes inoculation of culture into semi-solid lactose-peptone media for incubation at 44°C within 24 h. If gas and acid production due to lactose fermentation is revealed, the conclusion about thermotolerant coli-bacteria (*E. coli*) presence is made, indicating *fresh fecal water pollution*.

Titration method is used for water testing in case of membrane filtration method inaccessibility or in case of opaque water with many suspended particles. It is based on lactose-peptone medium fermentation similar with previous method.

Microflora of Soil

Soil as the superficial land layer is the habitat of large amounts of plant and animal species as well as myriads of microorganisms organized into complex microbial communities.

All kinds of microbial representatives have the place of dwelling in soil – bacteria, fungi, viruses, or protozoans.

The greatest amount of microbial cells is present at 10-30 cm of soil depth. Here the number of microorganisms per 1 g of soil (***soil microbial counts***) is usually in the range from $5\text{-}10*10^6$ to $1*10^9$ depending mainly on the soil type.

Cultivated soil contains much more microorganisms (up to $5*10^9$ cells per gram) than the soil of fallow lands. In the soil area around plant roots known as ***rhizosphere*** the total number of microbes is closer to 10 billion

per gram. As the result, it has been estimated that the ploughed land harbors more than 5 tons of microbial mass per 1 hectare.

Chernozem or black soil contains billions of microbial bodies per 1 gram, peaty and forest soils are also rich of microbial cells whereas clayey podsols and loose sands harbor significantly less amounts of microbes.

Overall, soil microbial counts strongly depend on soil structure, water contents, available nutrients, levels of aeration, and intensity of pollution with animal or human wastes.

Moving to the soil depth, the total number of microbes declines sharply. Only sporadic microbes are found at 2-4 meters deep. But in underground water, oil wells, or coal accumulations single microbes can be detected at depth of tens meters.

Soil bacteria pertain to numerous bacterial orders – *Actinomycetales*, *Pseudomonadales*, *Nitrosomonadales*, *Enterobacteriales*, *Rhizobiales*, *Bacillales*, *Clostridiales*. The members of two latter orders produce spores that stay in soil for decades.

Fotosynthetic microbials of phylum *Cyanobacteria* and moderate amounts of microscopical algae can be determined in soil as well.

Besides bacterial agents, numerous ***fungi*** (more than 100 species) are found in soil as the resident habitants.

Soil protozoans comprise amoebas and the number of flagellated representatives inhabiting the outmost layers of soil with sufficient aeration and humidity.

A plethora of ***viral agents*** is also present in soil following their natural hosts – plant and animal cells, bacteria, fungi and protozoans. They maintain the balance among the diverse microbial communities limiting their uncontrolled propagation. On the other hand, viruses (e.g., bacteriophages) create the conditions for exchange of genetic material supporting lateral gene transfer between soil-dwelling microbial species.

Resident microflora plays a tremendous role in soil metabolism and maintenance of ***soil fertility***.

Soil autotrophs (cyanobacteria, nitrosomonads, nitrobacter, chlorobium) produce organic matter from carbon dioxide. And vice versa, heterotrophic bacteria (e.g., actinomycetes, pseudomonads, bacilli) and fungi intensively decompose the remnants of plant and animal cells. They utilize lignin, cellulose, pectin and other biopolymers. All these microorganisms participate in ***humus*** formation thereby enhancing substantially the fertility of soil and fostering soil self-clearing.

The activity of anaerobic bacteria (e.g., clostridia) results in putrefaction of degrading organic substances.

In the same vein, soil microorganisms are totally implicated into the ***global biogeochemical cycling*** of essential ***elements*** such us nitrogen, carbon, sulfur or iron.

For instance, a lot of microbial agents (e.g., pseudomonads and bacilli) participate in ***ammonification*** of amino acids resulting in ammonia production; other bacteria (e.g., *Nitrosomonas* and *Nitrobacter* species) catalyze ***nitrification*** of ammonia into nitrates.

Furthermore, multiple bacterial genera present in soil (agrobacteria, flavobacteria, pseudomonads, bacilli, vibrios and others) perform ***denitrification***, converting nitrates into gaseous nitrogen.

And finally, certain soil bacteria are capable of direct ***nitrogen fixation*** converting molecular nitrogen into ammonia. The members from *Rhizobium* genus exert nitrogen fixation in symbiosis with various leguminous plant species, whereas clostridia and azotobacter don't need symbiotic support for the reaction. This chemical transformation has the positive impact on soil fertility.

Some microbial agents, e.g., thiobacilli, convert sulfur into sulfates, and other bacteria reduce them into hydrogen sulfide.

At the same time the soil serves as the reservoir that may hold numerous pathogenic microorganisms discharged from their animal or human hosts.

In case of poor sanitation the most common is fecal pollution of the soil. In these situations the soil contains pathogenic enterobacteria (salmonellae, shigellae and others) spread by fecal-oral route of disease transmission. Likewise, the soil may harbor microorganisms transmitted with dust by air-borne route (e.g., *M. tuberculosis*) or by direct contact (e.g., the agent of tularemia).

The viability of pathogenic microbes in soil is greatly variable. In general, the soil is not the beneficial medium for non-sporeforming bacteria albeit they may stay long there in special conditions.

As an example, mid survival time for *Salmonella enterica* var. Typhi is about 2-3 weeks, but its maximal survival period is near 12 months. Similarly, for shigellae these periods are 1-5 weeks and 9 months, for *Vibrio cholerae* – 1-2 weeks and 4 months, for *M. tuberculosis* – 13 weeks and 7 months, for brucellae – 0.5-3 weeks and 2 months.

By contrast, the spores of soil-dwelling bacilli and clostridiae can survive in soil indefinitely long, at least for several decades. Thus, the contamination of tissues with soil can lead to severe wound clostridial infections like tetanus or gas gangrene, as well as it predisposes to anthrax in case of presence of *B. anthracis* spores.

As the soil is the natural habitat for many types of pathogenic fungi and actinomycetes, this maintains conditions for the development of actinomycosis and certain fungal infections (e.g., aspergillosis or various systemic mycoses).

In the same vein, soil is an important part in transmission of protozoan infections (e.g. leishmaniasis) and helminthic invasions (ascaridosis, toxocariasis, taeniasis, ancylostomiasis and many others).

Taking into account the substantial impact of soil on the communicability of human infections, the continuous monitoring of soil sanitary state is maintained with special emphasis on the control of enteric infections transmitted by fecal-oral route.

Biological contamination of soil is evaluated by assessment of quantity of indicator bacteria and/or by direct determination of pathogenic bacteria in soil.

Similar to water sanitary testing, ***indicator microorganisms of soil*** comprise ***total coliform bacteria*** (*E. coli* and other members of *Enterobacteriaceae* family) and ***enterococci***.

Total coliform bacteria are determined by ***titration*** method, ***membrane filtration*** method, and by ***direct inoculation*** of various dilutions of soil specimens into lactose-containing agar media (e.g., Endo agar).

Enterococci are determined by the same methods but with special media for their culture.

Further assessment of soil sanitary conditions includes quantification of ***coli phages***, ***enteroviruses***, and ***spores*** of *C. perfringens*.

Finally, for direct determination of microbial ***pathogenic species*** in soil the members of *Salmonella* and *Shigella* genera are detected. In this case the soil specimens are inoculated into the selective media for their culture. After primary isolation, the bacteria are further identified by the number of serological, biochemical and molecular genetic tests.

As the result, *the soil is regarded as clean* without sanitary limitations if the total number of coliform bacteria is ***less than 10 cells per 1 g of soil*** specimen, and pathogenic *Salmonella* and *Shigella* species as well as enterococci and enteroviruses are not determined.

The excessive amounts of coliform bacteria (10 and more per 1 g of soil), the presence of enterococci and/or enteric pathogenic bacteria indicate fresh fecal pollution of soil and elevated risk of enteric infections.

Additional testing of soil microbial load includes the determination of ***soil microbial counts***. It is equal to the total number of microorganisms

present in 1 g of soil capable of forming colonies after the incubation at 28-30^{o}C for 72 h.

The quantity of actinomycetes and fungi per 1 g of soil can be determined as well.

As all of these parameters are highly variable, the obtained results should be compared with the data characteristic for "clean soil" samples.

Microflora of Air

The presence of microorganisms in the air is inconstant. It ensues from many factors: the locality of the area, air physical characteristics (temperature, humidity and air movement), the degree of air pollution with industrial and agricultural wastes, air contamination from the soil and water, the amounts of rainfalls, etc. Aerosol particles (dust, smoke, water droplets) adsorb many microorganisms.

Air microflora is composed of the vast number of species entered there from the soil, plants, animal or human bodies. Numerous saprophytic bacteria like micrococci, sarcinae, various bacilli (e.g., *B. cereus*, *B. subtilis*) and fungi (moulds, yeasts), actinomycetes are often determined in the air.

The total number of microbes in the air is greatly variable in the range from single cells to many thousands per 1 m^3. As an example, the air of polar regions harbors only several bacteria per 1 m^3. On the contrary, in the large cities the air might be highly polluted. In the air of coniferous forests there are only few microbes mainly because of the production of volatile phytoncides that are the potent biocides with high antimicrobial activity. Also the bacteria render poor growth in the air upon the oceans, upon snow-covered lands, in the high mountains, etc.

Actually, the air is not a favorable medium for microbial habitaion. The lack of nutrients, desiccation, the microbicidal activity of sunlight create deleterious effects against bacteria, and most of them lose their viability. Nevertheless, despite the rather short time of microbial presence in the air, pathogenic microorganisms are able to infect susceptible persons. They spread by ***air-borne route***, thereby causing outbreaks and epidemics of *respiratory diseases*.

Airflows transfer microbes by *aerosol* with dust particles and droplets. A patient can discharge a droplet aerosol with pathogenic bacteria into the surrounding environment within a radius of 1.0-1.5 m and even more.

The density of microbial aerosol is related with the viscosity of mucosal secretions produced by respiratory tract. A less viscous liquid secretion is spread in the smallest droplets (1-10 μm) and may stay suspended in the air for hours or even days. Larger droplets of 100-2000 μm in size can be expelled to a distances up to 2-3 m but rapidly undergo sedimentation.

The causative agents of influenza, measles, rubella, and other viral acute respiratory infections; bacterial respiratory illnesses, e.g., tuberculosis, diphtheria, meningococcal infections, whooping cough, scarlet fever and many other diseases can be spread by microbial aerosol generated from sputum and other discharges after speaking, coughing, or sneezing.

The total amount of microbes is strictly controlled in the air of industrial sites such us manufacturing plants with their multiple production lines, especially in the fields of electronics, food industry, biotechnology, and pharmaceutical industry.

In the air of living rooms the number of microbes is strongly dependent on sanitary hygienic conditions of the house. In case of poor ventilation, insufficient cleaning, or overcrowding the total microbial load of the air rises sharply.

The microbial content of the air of health care facilities (hospitals, clinics, ambulatory centers) is also the subject of strict sanitary control. For instance, in the surgical operating rooms (operation theaters) the total airborne microbial count before the operation must be less than 500 cells per 1 m^3 of the air, and after the operations not more than 1000. In addition, pathogenic hemolytic staphylococci and streptococci should be not detected there.

For patients with severe immunosuppression (post-chemotherapy cancer patients or allograft recipients) the cleanrooms are organized, where the number of microbes is greatly reduced by air filtration.

Microbiological testing of air is performed to control the number and quality of air microflora.

The laboratory determination of *airborne* ***total microbial count*** comprises two main groups of methods – ***aspiration*** and ***sedimentation tests***.

Simplest is the ***sedimentation method***, where sterile opened Petri dishes with MPA are placed in different points of the room. After complete sedimentation of air microbes within 5-30 minutes depending on method modifications, the dishes are closed and placed for incubation into thermostat at 37^{o}C for 24 h.

The grown colonies are counted and total microbial quantity is calculated by special formulas.

For a more accurate assessment of air microbial contents a number of special instruments and tools is used. In ***aspiration method*** the air is pumped through the apparatus containing opened Petri dish with nutrient meduim.

Sanitary ***indicator microorganisms of air*** comprise hemolytic and viridans streptococci and pathogenic staphylococci (*S. aureus*). They are tested by special microbiological methods for their identification.

For the purpose of prophylaxis of air microbial pollution a number of protective methods is used that diminishes the amount of air-borne dust particles with microorganisms. The air of wards, operating theaters or laboratory rooms is decontaminated by UV-irradiation, the sputum and other discharges are disinfected, bacterial filters are installed into ventilation systems.

Chapter 8

ANTIMICROBIAL MEASURES: STERILIZATION, ANTISEPSIS, DISINFECTION, AND ASEPSIS

Antimicrobial Measures: Sterilization

Sterilization, disinfection, antisepsis, asepsis and chemotherapy refer to *antimicrobial measures, which cause a direct damage of infectious agents*.

Sterilization *is a complex of physical and chemical methods of* ***complete inactivation*** *of all vegetative and dormant forms* (*e.g., spores*) *of any kind of microbial agents*.

Prevention of body microbial contamination during various medical manipulations is the ***main goal of sterilization*** in medicine.

The sterilization is applied to the all kinds of medical devices and substances – medical instruments, drugs, dressing and stitch materials, linen, culture media, laboratory utensils, etc.

In microbiological practice different sterilization methods are employed for the maintenance of aseptic conditions in laboratory work, protection of culture media and cell cultures from extraneous microbial contamination, for prevention of microbial biodegradation of drugs or laboratory reagents.

All sterilization techniques can be divided into *physical* and *chemical* methods.

Physical methods of sterilization comprise a broad number of procedures based on various physical principles for sterilization – sterilization by ***heating***, ***high pressure***, ***mechanical*** sterilization (microbial *filtration*), sterilization by ***irradiation***.

One of most reliable methods of sterilization is ***autoclaving***. The sterilizing operation within autoclave is provided by contact of a saturated overheated steam under elevated pressure with sterilized objects that results in rise of temperature of sterilized things.

Depending on sterilized materials, the temperature of a saturated steam may be in the interval 110^{o}C up to 138^{o}C with additional pressure 0,4 up to 2,5 atmospheres and exposition time 30 to 60 minutes. Various nutrient media, isotonic sodium chloride solution, distilled water, the textile products are usually sterilized at 1 additional atmosphere at 121^{o}C within 15-30 minutes.

Heating with air in *hot air ovens* at 180^{o}C and exposure time for 60 min is highly effective sterilizing measure, but it may cause the decay of labile substances. The objects, poorly permeable for a vapor, but resistant to heat (glass, ointments, hydrophobic materials) might be sterilized by this method.

Flaming is a common method for sterilization in microbiology, primarily, for sterilization of wire loops, or outlets of the test tubes.

Incineration is used for destruction of *biohazardous wastes*.

The *fractional sterilization* (or ***tyndallization***) at 56-70^{0}C for 1 hour within 5 days is used for media or medical substances with heat-labile contents (proteins, vitamins, etc.) During the intervals between sterilization events the objects are placed into thermostat at 37^{0}C for overnight incubation. In this case the spores, still remaining in sterilized medium, overgrow, and newly germinated vegetative cells will be destroyed at next sterilization step.

Pasteurisation is not regarded as a method of complete sterilization. It is employed for partial rapid decontamination of milk, juices, wine, beer and other products. It uses *high-temperature, short-time treatment* (at 72^{0}C for 15 sec, or at 63^{0}C for 30 min) with next cooling to 4^{o}C.

In combination with aseptic packaging the pasteurized milk can be stored in refrigerators for 2-3 weeks.

The ***sterilization of heat-labile substances*** (vitamins, antibodies and immune sera, biological products, or culture media) is achieved by ***filtration through bacterial filters*** with controlled standard porosity. Usually the synthetic filters with 0.1-0.45 μm of pore sizes (cellulose acetate, nylone, polytetrafluoroethylene (PTFE) or others) are applied for filtration. These filters efficiently trap the bacteria and a large number of viruses, albeit the viruses of small sizes as well as bacterial toxins can pass through these filters without significant retention.

Sterilization by irradiation uses radiation of various wavelengths. *Ultraviolet germicidal irradiation* (***UV-irradiation***) is performed with the short-wavelength UV-light (about 260 nm) that damages microbial DNA. It is broadly employed for air sterilization of wards and rooms in medical health care units.

Heat-resistant instruments can be sterilized by ***infrared radiation*** (wavelength of 700-1000 nm) that inactivates microorganisms by heat generation.

In industrial conditions ***ionizing gamma irradiation*** is used for sterilization of packed dressings, plastic disposable syringes, systems for blood transfusions, plastic Petri dishes, vitamins, hormones antibiotics, etc.

Radioactive isotopes Co^{60} or Cs^{137} are introduced as the sources of gamma radiation that irreversibly damages DNA of microorganisms.

For ***chemical sterilization*** various chemical substances are available – ethylene oxide gas, ozone, formaldehyde, glutaraldehyde, hydrogen peroxide, peracetic acid, ethanol and some others.

Ethylene oxide treatment is one of the most efficient methods of chemical sterilization. It inactivates all viable forms of microorganisms including bacterial spores, viruses, fungi. Ethylene oxide easily diffuse through the package covers, thereby about 50% of manufactured medical disposable devices are sterilized by this method.

Ozone as sterilant gas is applied for sterilization and decontamination of air and water, for instance, for treatment of water of swimming pools.

Peracetic acid and ***glutaraldehyde*** can be applied for sterilization of fiber optics, e.g. endoscopes.

For the control of the efficacy of sterilization various meter devices are used (thermometers, manometers) as well as thermochemical indicators with known melting points of controlling substances (urea, benzoic acid and others). For biological control of sterilization the strips with spores of heat-resistant bacteria *Geobacillus stearothermophilus* are placed into containers with sterilizing objects. These spores can withstand short-term exposure to the temperature of $121^{o}C$.

Antisepsis

Antisepsis is *a complex of preventive measures, which allows to suppress microbial growth and dissemination upon intact or injured skin, mucosal tissues, wounds, and within body's compartments.*

The main group of ***antiseptics*** (or ***antiseptic drugs***) is of chemical origin. Biological (e.g., bacteriophages), physical and mechanical factors (surgical treatment, lavage, drainage, absorption) augment the favorable effects of antiseptics.

Together with other chemical antimicrobial compounds (*disinfectants* and *antibiotics*) antiseptics are referred to as ***biocides***.

The main requirements to antiseptics include:

– high antimicrobial activity with tolerance for skin and mucosal tissues;

– the absence of irritative, toxic, allergic, mutagenic, carcinogenic, or teratogenic effects;

– antiseptics should be readily dissolved in lipids and poorly or moderately – in water to block drug absorption by internal host tissues, albeit promoting their accumulation inside the skin;

– they must confine infectious agent within the wound, thereby preventing its penetration into lymph and blood;

– antiseptics should block the microbial adhesion, suppress bacterial virulence factors, and synergize with the action of antibiotics and physical antimicrobial factors.

All antiseptics are divided into following classes due to their chemical structure:

1. detergents (anionic and cationic, e.g. quaternary ammonium compounds like benzalkonium chloride, miramistin);
2. halogen-releasing agents (chlorine-, bromine-, iodine-containing antimicrobials, e.g. sodium hypochlorite, iodine);
3. biguanides (chlorhexidine);
4. oxidizing agents (H_2O_2, peracetic acid, $KMnO_4$);
5. aldehydes (formaldehyde, glutaraldehyde);
6. metal-containing compounds (silver nitrate and other salts, alloys or organic complexes of Ag and Cu);
7. phenols (triclosan, oxyquinoline);
8. alcohols (ethanol);
9. acids (benzoic, salicylic, boric and others);
10. alkali (sodium hydrocarbonate);
11. sulfonamides (sulfacyl-sodium or sulfacetamide);
12. dyes (brilliant green, methylene blue, etc.);
13. phytoncides (e.g., chlorophyllipt);
14. antibiotics (tetracyclines, aminoglycosides, neomycin, etc.)

Also they are discernible by their mode of action.

The destructive antiseptics cause denaturation and destruction of biopolymers (proteins, lipids, or DNA) within microbial cells. This activity is essential for aldehydes, halogens, metal salts, alcohols, phenols, acids and alkali, etc.

Oxidizing antiseptics (H_2O_2 and peracetic acid, halogen-releasing agents – sodium hypochlorite) break down microbial structures by generation of highly active free radicals.

Membrane attacking antimicrobials affect the permeability of microbial membranes (e.g., detergents, chlorhexidine).

Anti-metabolites and ***enzyme-inhibiting antiseptics*** block bacterial enzymatic systems (e.g., sulfonamides, silver nitrate).

From the most efficient antiseptics are halogen-releasing agents and oxidizers (sodium hypochlorite, peracetic acid), quaternary ammonium compounds, chlorhexidine.

Disinfection

The disinfection *is a complex of antimicrobial measures directed to eradication of microbial species present on various non-living* (*inanimate*) *objects of external environment by means of biocidal chemical substances* (***disinfectants***) *or by physical methods.*

The ***main goal of disinfection*** is to interrupt the epidemiological chain – to prevent the transmission of pathogenic agents from the source of infection to susceptible persons through the objects and factors of external environment.

The basic methods of disinfection harness highly active antimicrobial chemicals (***disinfictants***) to eliminate microbial cells from the external objects.

The main groups of disinfectants are similar to those of antiseptics.

Among them are ***oxidizing agents*** including ***halides*** (chlorine-containing substances – sodium hypochlorite, chloramine, bleach, etc.), and other *oxidants* (H_2O_2 or ozone), ***aldehydes*** (formaldehyde), ***phenols*** and ***alcohols*** (isopropanol, ethanol), ***quaternary ammonium compounds*** (benzalkonium chloride), metal salts (Ag cations or Cu alloys).

They can be used in combination with physical measures, e.g. UV-irradiation, or heating.

Three main kinds of disinfection exist: ***preventive***, ***current***, and ***final***.

Preventive disinfection is performed in the settings and sites of potential rapid spread of infections. It should be done continiously and uninterruptedly regardless of the source of infection presence. The measures of preventive disinfection include decontamination of various wastes, sewages and garbage, disinfection in public dining setting, railway stations, swimming pools and baths, as well as drinking water cleaning, milk pasteurization, foodstuffs preservation, and so on.

In hospitals, clinics and other health care settings the ***current disinfection*** is employed to reduce significantly the total amount of contaminating microbials. This disinfection measures should be carried out every day to maintain proper antimicrobial conditions in the hospital.

The purpose of ***final disinfection*** is to neutralize microorganisms in the infection focus after patient's transportation, hospitalization, or death hence to interrupt further infection transmission.

The most reliable control of disinfection quality is performed by bacteriological tests.

Asepsis

The asepsis *is an integral complex of all antimicrobial measures for prevention of microbial contamination of any medically relevant object, including instruments, dressings, work clothings, medicines, body's tissues and organs, wounds, etc.*

The main goal of asepsis is to protect the patient from any unwanted microbial burden that may aggravate patient's health status.

Asepsis comprises different methods of sterilization, mechanical and chemical cleaning, disinfection, antisepsis, isolation with barrier nursing, transmission-based precautions, etc. This approach is maintained in all health care settings being realized within standard clinical procedures, e.g. patient's examination, surgical and other invasive manipulations, drug infusions, production of sterile medicines and so on.

In microbiological practice asepsis presumes manipulations with the sterile instruments and in sterile conditions to exclude microbial contamination; the prevention of contamination of the specimen during its delivery to the laboratory; usage of sterile cultural media; laboratory work within laminar flow cabinets with sterile air, hazardous biowastes management, etc.

Chapter 9
CHEMOTHERAPY. ANTIBIOTICS

Antimicrobial Chemotherapy and Chemoprophylaxis of Infectious Diseases

Various antimicrobial substances that affect pathogenic microorganisms are widely used for the treatment of patients with infectious diseases and in some cases for the disease prophylaxis.

Antimicrobial chemotherapy *is the treatment of bacterial, viral, fungal and protozoan infections with chemical antimicrobial agents.*

The safety and efficacy of any antimicrobial drug can be characterized by its ***therapeutic index*** (also known as *therapeutic ratio* or *therapeutic window*).

It is the most likely expressed as *the highest dose a patient can tolerate without toxic effects* **divided by** *the dose required to control the infection* (therefore, *produces the desired efficacy*).

The chemical drug is suitable for medical applications, if its therapeutic ratio is ***not less than 3***.

Antimicrobial agents, which are used for treatment and prophylaxis of infections in humans and animals, are divided into two main groups – ***antiseptics*** and ***antibiotics***.

Antibiotics

Antibiotics are chemical drugs of natural, semi-synthetic, or synthetic origin, which in minimal concentrations inhibit the replication or cause the death of susceptible microbial agents in inner compartments of the body.

Discovery of antibiotics occurred in 1928-1929, when A. Fleming has demonstrated the obvious antibacterial action of *Penicillium notatum* fungal culture. In 1940 H. Florey and E. Chain have obtained stable penicillin product (its sodium salt). Later in 1943 A. Schatz and S. Waksman discovered new antibiotic streptomycin. Since that time a tremendous number of antibiotics was described, investigated and applied for different medical goals.

Antibiotics must be in accordance with several ***requirements***:

1. High antimicrobial activity and selectivity in doses, non-toxic for the patient.

2. Efficient therapeutic action in tissues and organs, low level of inactivation by tissue proteins and enzymes.

3. Absence or slow development of side effects.

4. Long period of metabolism (prolonged effect).

5. Slow growth of microbial resistance to the antibiotic.

6. High effectiveness of the drug with the low cost of therapy.

7. The drug must be compliant for different practical applications and stable in storage.

Unfortunately, no one of known antibiotics satisfies completely all these requirements.

Classification of Antibiotics

Antibiotics are classified according to their ***origin***.

Antibiotics of ***natural origin*** are obtained from various sources.

Vast number of antimicrobial agents is produced by ***actinomycetes***. ***Streptomycin*** is obtained from *S. griseus*, ***chloramphenicol*** is derived from the cultural fluid of a strain of *S. venezuelae*, ***tetracycline*** is produced by *S. aureofaciens*, ***erythromycin*** is derived from *S. erythreus*, ***nystatin*** has been extracted from the culture of *S. noursei.* ***Kanamycin*** is produced by *S. kanamycetius.* ***Amphotericins*** (***A*** and ***B***) are antimycotic antibiotics obtained from *S. nodosum.*

Antibiotics synthesized by other ***bacteria*** comprise ***gramicidin*** (derived from the culture of *Bacillus brevis*), ***polymyxins B***, ***E*** or ***M*** from *Paenibacillus polymyxa* and some others.

Fungal antibiotics are of wide use**.** ***Penicillin*** is produced by fungi *Penicillium notatum* or *Penicillium chrysogenum*.

Some antimicrobial substances are obtained from ***plants***. This broad group of antimicrobials is termed ***phytoncides***.

Finally, some substances of ***animal origin*** can possess antimicrobial activity. For instance, enzyme ***lysozyme*** is capable of hydrolyzing bacterial cell walls.

Semisynthetic and ***synthetic antibiotics*** comprise a great number of modern drugs.

Different semisynthetic penicillins are obtained on the basis of penicillin nucleus, *6-aminopenicillanic acid*, by substitution of the lateral radicals (***methicillin***, ***oxacillin***, ***dioxacillin***, ***ampicillin***, etc.). Similarly, modern ***cephalosporins*** are synthesized on the basis of *7-amino-*

cephalosporanic acid, the nucleus of cephalosporin (***ceftazidime***, ***cefotaxime***, ***cefepime***, etc.)

At last, many antibiotics are the products solely of ***chemical synthesis***. Among them are ***isoniazid***, ***nalidixic acid***, ***cycloserine***, ***pirazinamide***, etc. The synthetic drugs are ***sulphonamides***.

According to the ***character of action***, antibiotics are divided into ***bactericidal*** and ***bacteriostatic***. Antimicrobial drugs are considered to be "***-cidal***" if they kill affected microorganisms. If antimicrobials only inhibit the growth of susceptible bacteria they are called "***-static***". Growth inhibition results in microbial killing by host immune system.

According to ***spectrum of action*** antibiotics are divided into drugs with ***broad spectrum of action*** (affecting both gram-positive and gram negative bacteria, rickettsiae, chlamydiae etc.) and with ***narrow spectrum of action*** (e.g., inhibiting only particular group of gram-positive or other microbes).

The Mechanisms of Antibiotics Action

Antibiotics produce various deleterious effects against microbial cells. These effects are based on specific molecular mechanisms of antibiotic action. Among them are:

1. inhibition of cell wall synthesis;
2. impairment of the normal function of cell membrane;
3. inhibition of protein synthesis;
4. impairment of nucleic acid synthesis.

Antibiotics, Inhibiting Cell Wall Synthesis

All ***beta-lactam*** drugs (***penicillins*** and ***cephalosporins***) are strong and highly selective inhibitors of the synthesis of bacterial cell wall. *They are active against growing and propagating bacteria*.

The initial step of their action is the specific binding of these antibiotics to ***penicillin-binding proteins*** (***PBP***). About ten of different PBPs are known. Some of them reveal ***transpeptidation enzyme*** activity. PBP synthesis is controlled by nucleoid; therefore, the mutations may change PBP affinity for beta-lactams.

Beta-lactam binding leads to the termination of transpeptidation reaction, resulting in deep inhibition of peptidoglycan synthesis. The molecular mechanism of blockade of transpeptidation enzymes by beta-lactams ensues from the structural similarity of these antibiotics with peptide acyl-D-alanyl-D-alanine moiety. Inhibition of transpeptidation is followed by lytic enzyme activation with subsequent cell lysis. Thus, beta-lactams are ***bactericidal antibiotics***. Also the bacterial cells with impaired cell wall (protoplasts, spheroplasts) are abnormally sensitive to phagocytosis.

Penicillins and ***cephalosporins*** are of the most potent antibiotics. Beta-lactams possess very weak direct toxicity comparing with other drugs, but they can readily provoke hypersensitivity with allergic reactions. All penicillins render cross-sensitization and cross-reactivity.

Carbapenems, a new modern group of highly active beta-lactams, are devoid of many side effects of penicillins and cephalosporins. ***Imipenem*** and ***meropenem*** pertain to this drug group. They develop strong activity against many gram-negative and gram-positive bacteria, as well as against anaerobes.

Resistance to beta-lactam antibiotics arises mainly from the microbial synthesis of *penicillin-* or *cephalosporin-degrading enzymes* (***beta-lactamases***). They break down the bonds within the beta-lactam ring conferring microbial resistance to beta-lactams.

Extended spectrum beta-lactamases additionally degrade third-generation cephalosporins (ceftazidime, cefotaxime) or monobactams.

Zn-containing *metallo-beta-lactamases* are capable of destroying carbapenems.

Clavulanic acid, ***sulbactam*** and ***tazobactam*** are *irreversible beta-lactamase inhibitors* that block enzyme activity. Combined antibiotic antimicrobial agents (e.g., amoxycillin+clavulanic acid) overcome beta-lactamase resistance showing high activity against beta-lactamase-producing bacteria.

β-Lactamase production is usually related with plasmid control. Nevertheless, the serious threat for public health has arisen from the strains of ***methicillin resistant Staphylococcus aureus*** (or ***MRSA***) The strains of MRSA originated from chromosome-dependent alteration of staphylococcal ***penicillin-binding proteins*** (***PBP***). These bacteria produce modified protein ***PBP2a*** with low affinity to beta-lactam antibiotics. It is encoded by chromosomal gene ***mecA***.

It was found that staphylococcal unresponsiveness to methicillin confers their resistance to almost all of beta-lactams. Last decades MRSA

have become a tremendous problem for health care settings as they generate numerous life-threatening infections resistant to beta-lactam therapy.

Some other drugs, including ***glycopeptides*** *vancomycin* and *teicoplanin* as well as ***bacitracin*** and ***novobiocin***, inhibit early steps in the biosynthesis of peptidoglycan. Since these steps are reproduced inside the cytoplasmic membrane, these drugs must initially penetrate the bacterial envelope. As an example, ***vancomycin*** is highly efficient, but against gram-postive bacteria only. It remains as the a drug of last resort for treatment of resistant gram-positive bacteria, e.g., MRSA strains.

Antimicrobial Action by Cell Membrane Impairment

Various antibiotics (***amphotericin B***, ***nystatin*** and other ***polyenes***, ***polymyxins***, etc.) affect microbial cytoplasmic membrane. If cytoplasmic membrane becomes impaired, the cell is damaged due to membrane disruption followed by macromolecule and ion leakage.

Polymyxins affect gram-negative bacteria, and polyenes act against fungi.

Colistin (or polymyxin E) is produced by *Paenibacillus polymyxa* being composed of cyclic polypeptides. In certain clinical situations it is the drug of last resort for treatment of infections, caused by multidrug resistant gram-negative bacteria *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.

Polyenes bind to sterols, which are present in the fungal cell membrane but absent in the bacterial cells. Therefore, polymyxins are inactive against fungi, whereas polyenes are non-efficient against bacteria.

Antimicrobial Action by Protein Synthesis Inhibition

It has been found that a great variety of antibiotics inhibit protein synthesis in bacteria.

Bacteria possess 70S ribosomes, and conversely, mammalian cells use 80S ribosomes. Structural differences provide selective inhibition of bacterial protein synthesis without impairment of host ribosomal apparatus.

Aminoglycosides

Aminoglycosides (streptomycin, ***gentamycin***, ***amikacin*** and others) attach to 30S subunit of bacterial ribosome. Conversion in protein synthesis initiation site leads to incorrect amino acid insertion into newly polymerized protein. Also aminoglycosides hamper polysomes formation.

The effect is irreversible, that's why aminoglycosides show *bactericidal* effect.

Tetracyclines

Tetracyclines as well as aminoglycosides bind to the 30S subunit of microbial ribosomes. Tetracyclines (***tetracycline*** itself, ***doxycycline*** and others) inhibit protein synthesis preventing aminoacyl-tRNA attachment to ribosome. Tetracycline antibiotics possess *bacteriostatic* activity but have a broad spectrum of action.

The antibiotics from a new class of ***glycylcyclines*** are the derivatives of tetracyclines. The member of this promising group ***tigecycline*** demonstrates the remarkable efficacy especially against some antibiotic-resistant bacteria, such as *Staphylococcus aureus*, *E. coli* or *Acinetobacter baumannii*.

Chloramphenicol

Chloramphenicol interacts with the 50S subunit of the ribosome. It blocks binding of new amino acids to the peptide chain due to peptidyl transferase inhibition. It is mostly *bacteriostatic* antibiotic.

Macrolides and azalides

Erythromycin is the basic antibiotic in ***macrolides*** group. ***Azalides*** comprise more advanced drugs (e.g., ***azithromycin***). These drugs bind to 23S rRNA in the 50S subunit of bacterial ribosome. Probably they impair amino-acyl translocation in protein synthesis. Azalides develop *bactericidal* activity.

Lincomycins

Lincomycin and its derivative ***clindamycin*** are similar in action with macrolides. They attach to the 50S subunit of bacterial ribosomes blocking amino-acyl residue translocation.

Oxazolidinones (***linezolid***)

Linezolid is the sythetic antibiotic that reacts with 50S subunit of bacterial ribosome within the individual specific binding site. It is active

only against gram-positive bacteria. Linezolid is used for treatment of infections caused by highly resistant microbials, such as MRSA and vancomycin-resistant enterococci (VRE).

Antimicrobial Action by Nucleic Acid Synthesis Inhibition

Substantial part of modern antibiotics acts as nucleic acid synthesis inhibitors. Among them are ***fluoroquinolones***, ***rifampicin*** (***rifampin***), ***sulfonamides*** and ***trimethoprim***, and some others.

Fluoroquinolones are fluorinated derivatives of ***nalidixic acid***. Nalidixic acid does not possess the potent systemic antibacterial effect, being used mainly as urinary antiseptic drug. Newly synthesized fluoroquinolones (***ciprofloxacin***, ***ofloxacin***, ***norfloxacin***, ***levofloxacin*** and many others) appear to develop remarkable bactericidal activity and low toxicity.

Their mode of action includes the inhibition of bacterial *DNA gyrase* and *topoisomerase* that are essential for bacterial DNA replication.

Rifampicin (or ***rifampin***) antibiotic suppresses bacterial propagation due to irreversible inhibition of bacterial *DNA-dependent RNA polymerase*. This way it dampens bacterial RNA synthesis. Rifampicin develops strong bactericidal effect. It is able to enter phagocytes and other host cells; thus it can kill intracellular microorganisms. It is the first line drug for treatment of tuberculosis.

Sulfonamides and Trimethoprim

Sulfonamides were the first effective antimicrobial drugs, which have been discovered by G. Domagk in 1935.

They were proven to act as competitive analogs of *p-aminobenzoic acid* (***PABA***) – important precursor for folic acid synthesis. Folic acid is involved further to the synthesis of nucleic acids. Sulfonamides interact with the enzyme *dihydropteroate synthetase* impairing PABA metabolism.

Therefore, non-reactive analogs of folic acid appear, nucleic acid synthesis is made difficult, and bacterial cell growth terminates. Being administered alone, sulfonamides show bacteriostatic activity.

Trimethoprim substantially enhances sulfonamide action. It blocks the enzyme *dihydrofolic acid reductase*, which reduces *dihydrofolic* to *tetrahydrofolic acid* necessary for purine nucleotide synthesis.

Sulfonamides and trimethoprim perform two-step sequential inhibition of folic acid synthesis demonstrating the synergism of activity. Sulfonamide drug ***sulfometoxazole*** in combination with *trimethoprim* result in highly active antimicrobial drug ***co-trimoxazole*** (or ***biseptol***), which develops bactericidal effect. This combined drug is efficient in diverse infectious pathology – pneumocystis pneumonia in AIDS, enteric infections, urinary tract infections, malaria, and other diseases.

Development of Drug Resistance

Drug resistance is maintained by *genetic* (genotypic) and corresponding *phenotypic* microbial mechanisms. Also various environmental and host-derived *non-genetic factors* actively influence on microbial drug resistance.

Non-genetic factors provide *isolation* of microbe from antibiotic action. Bacteria may propagate in tissues inaccessible for antibiotics. Also bacteria can minimize metabolic processes (persistent *dormant state*) whereas some antibiotic can influence only dividing active bacteria. Finally, bacteria can persist *within the cells*, being preserved from antibiotics that are not able to enter the host cells. The example is intracellular salmonella resistance to aminoglycosides: these drugs cannot penetrate the cell membrane.

Genetic Drug Resistance

Two main types of genetic drug resistance are ***chromosomal resistance*** and ***extrachromosomal resistance***.

Chromosomal resistance evolves under the selective pressure of antibiotics that affect bacterial population. Bacterial nucleoid mutations appear, and the selected bacterial clone becomes resistant to administered drug. This is true for rifampicin resistance in tuberculosis treatment or beta-lactam resistance due to PBP-mutations. In latter case mutations abolish synthesis of some sensitive penicillin receptors (PBPs), thereby missing antimicrobial penicillin action.

Extrachromosomal resistance is encoded by ***plasmids*** and ***episomes*** – additional bacterial genetic elements. Also temperate bacteriophages can carry genes of antibiotic resistance.

R-plasmids contain genes providing ***mono***- or ***multiresistance*** to different antibiotics and sometimes to heavy metals. They can encode antibiotic-degrading enzymes, capable of destroying the antimicrobial drugs (***beta-lactamases*** for penicillin and cephalosporin hydrolysis, ***acetyltransferase*** for chloramphenicol inactivation, enzymes that acylate different aminoglycosides, etc.)

This variant of resistance is under continuous evolutionary control. For instance, one group of beta-lactamases is able not only to destroy penicillins and cephalosporins of first generations but also to hydrolyze 3rd generation cephalosporins (cefotaxime, ceftazidime, ceftriaxone, etc.) or monobactams (aztreonam). These enzymes are called ***extended-spectrum beta-lactamases***. A substantial amount of gram-negative bacteria are known to produce them (*E. coli*, *K. pneumoniae* etc.)

Phenotypic Resistance to Antibiotics – Basic Mechanisms

Expression of microbial genes for antibiotic resistance results in development of numerous powerful mechanisms that demolish the activity of antimicrobial agents. They comprise highly active and versatile biochemical pathways.

1. Microorganisms can produce enzymes or other substances that inactivate the active forms of antibiotics.
2. Microorganisms can change the structure of targets for antibiotics.
3. Bacteria can accelerate the synthesis of target molecules thus overcoming their inhibition by antibiotics.
4. Bacteria can shunt affected metabolic pathway thereby bypassing metabolic reactions, blocked by antibiotic.
5. Bacteria can synthesize another isoform of enzyme, which is active, but not influenced with antibiotic.
6. Microorganisms can enhance the efflux of antibiotics.
7. Drug entry to the cell can be altered due to bacterial membrane permeability changes.

Prevention of Microbial Antibiotic Resistance

The global progression and high levels of resistance to antimicrobial agents have become a tremendous problem of public health in XXI century. Now more than 700,000 patients annually die from infections,

caused by antibiotic-resistance strains. If the situation remains unchanged, further estimations predict by 2050 the growth of fatality cases up to 10 mln every year.

In order to restrict the development of microbial resistance several measures must be kept in antibiotic treatment.

Primarily, it is necessary to avoid indiscriminate antibiotic treatment. Antibiotics should be prescribed only in case of infection of bacterial nature. They should be administered in sufficient high doses to inhibit primary microbial population and first-step mutants. If it is necessary, synergistic drug combination is to be used (e.g., polychemotherapy in tuberculosis treatment).

It can be usefull to limit a broad antibiotic administration for veterinary purposes in order to prevent resistant strain selection.

At last, antibiotic treatment requires continuous monitoring of microbial drug resistance.

Side and Undesirable Effects of Antibiotic Use

Side effects of antibiotics mean *harmful and unfavorable actions of antibiotics against human body during the course of treatment*.

Among them are:

– emergence of allergic and pseudoallergic reactions;

– drug resistance development;

– undesirable reactions due to microbe suppression and degradation (endotoxic shock; dysbiosis and superinfection, antibiotic-associated diarrhea and colitis, caused by *Clostridium difficile*);

– toxic effects from antibiotics (photosensitization, drug-induced hepatites, psychotic reactions, etc.);

– progression of secondary immunodeficiency.

Indiscriminate use of antibiotics may also cause different undesirable effects on population level.

For instance, mass administration of antibiotics can result in allergic and toxic disease spread among the human population (hypersensitivity, blood disorders, drug-mediated hepatites, etc.)

Suppression of the normal flora of the body provides the high incidence of dysbiosis in the population.

Uncontrolled antibiotic use can hide serious infection without its eradication. It is extremely important in tuberculosis treatment, where

irregular or insufficient administration of antimycobacterial drugs provokes continuous transmission of drug-resistant disease.

Finally, indiscriminate use of antibiotics elevates drug resistance within microbial communities. It is most evident for hospital microbial variants, where the majority of strains develops multidrug resistance under antibiotic pressure.

Antimicrobial Susceptibility Testing

Laboratory testing for antibiotic resistance is performed for several reasons.

The *main goal of antibiotic susceptibility testing* is to ***predict the clinical success or failure*** of the infection treatment with this antibiotic.

Furthermore, susceptibility testing provides drug resistance monitoring in the population. It makes possible to determine the resistance of the microbial species to various groups of antibiotics.

In clinical practice susceptibility testing is especially valuable, if the isolated strain is supposed to be initially resistant, or it can cause rapid severe health disorder in case of delay of specific treatment. Also it is useful for monitoring of specific treatment course for chronic infections that require the long course of antibiotic therapy.

The relationships between a certain microbial strain and the particular antibiotic drug are characterized by ***minimum inhibitory concentration*** (***MIC***) *of antibiotic*. It is equal to the ***lowest concentration of the antibiotic*** that *inhibits a visible growth of tested microorganism* in standard experimental conditions.

Another widely used index of microbial susceptibility is ***minimum bactericidal concentration*** (***MBC***) *of antibiotic*, which *provide complete death of all bacteria in standard experimental conditions*.

MBC can be equal or greater than MIC and cause the death at least of 99,9% of tested bacteria.

Having determined MIC of antibiotic, the ***conclusion about the resistance*** of investigated bacterial culture is made. Depending on MIC value, the isolated bacterial strain is categorized as "***susceptible***", "***intermediate***" or "***resistant***" to this antibiotic.

The interpretive criteria for MIC values are worked out by Clinical and Laboratory Standards Institute (CLSI, USA) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).

According to the actual ISO standards, the clinical grades of resistance, based on MIC determination, are characterized as follows:

"Bacterial strain is ***susceptible*** if inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with a *high likelihood of* ***therapeutic success***";

"bacterial strain is ***intermediate*** if inhibited *in vitro* by a concentration of an antimicrobial agent that is associated with ***uncertain therapeutic effect***";

"bacterial strain is ***resistant*** if inhibited in vitro by a concentration of an antimicrobial agent that is associated with a *high likelihood of* ***therapeutic failure***".

In practice of clinical laboratories microbial susceptibility to antibiotics is assessed by ***agar diffusion tests*** and by ***serial dilution methods*** in liquid or solid media.

Disk Diffusion Test

Disk diffusion test (or ***Kirby-Bauer method***) is the most widespread variant of antibiotic susceptibility determination. Its technique includes several consecutive steps.

At first, the test microbial culture is pour plated onto the solid medium, providing optimal microbial growth.

Next step the filter paper disks with standard quantity of certain antibiotic are placed on the medium surface.

After overnight incubation the ***diameter of growth inhibition zone*** around the disk is measured. Diameter is proportional to inhibitory power of the drug for investigated bacterial culture.

This method is influenced strongly by the nature of the solid medium, its diffusion permeability for antibiotics, drug molecular size and stability, etc. Nonetheless, in standard experimental conditions the method was shown to give reliable and reproducible results.

Growth inhibition zone diameter is compared with standard data obtained previously for particular antibiotic and given bacterial species. Standard data has been determined by comparing of results of diffusion and dilution testing methods (see below).

The culture is considered to be ***susceptible*** to particular antibiotic, if the testing strain growth is inhibited by antibiotic concentration, which corresponds to the average therapeutic dose for the drug.

Resistant microbial culture is not inhibited even by maximal tolerated dose of certain antibiotics.

Another version of diffusion susceptibility testing allows to determine *MIC of antibiotic* for tested microbial culture.

The method called ***E-test*** can be more suitable in some cases and may produce more precise results.

In this method a narrow strip of polymer carrier containing descending concentration gradient of the antibiotic is placed upon solid medium after microbial inoculation. Inhibition of microbial growth appears only where antibiotic concentration in the strip exceeds MIC.

Being rather simple, this diffusion technique makes possible the direct determination of MIC, because different antibiotic concentrations are designated on the strip surface.

Dilution Tests Methods

Two basic variants of ***dilution methods*** include *titration of antibiotic* in ***liquid*** or ***solid*** *nutrient medium*.

For titration in ***liquid medium*** the sequential dilutions of the antibiotics are performed in appropriate broth medium (e.g., *Mueller-Hinton broth*). Then the standard concentration of tested culture is inoculated.

After overnight incubation the growth inhibition is evaluated.

The ***end point*** (i.e., ***MIC***) is determined as the *last dilution of the antibiotic, which is still able to inhibit the visible growth of the tested bacteria*. Now various microdilution methods are broadly used for rapid quantitative measurement of microbial resistance.

Agar dilution susceptibility tests are similar with liquid dilution methods, but the antibiotic is placed in various definite concentrations into several Petri dishes with agar. Simultaneous estimation of the resistance of many microbial strains is possible, though the method is more laborious and time-consuming.

Chapter 10

INFECTIOUS PROCESS. CHARACTERISTICS OF INFECTIOUS DISEASES. PATHOGENICITY AND VIRULENCE. BACTERIAL VIRULENCE FACTORS

Infection (or Infectious Process). Conditions for Infectious Process Emergence and Progression

Infection (or ***infectious process***) *is the complex pathological process has been evolved as the result of multiple interactions between the virulent bacteria and the susceptible host that is followed by tissue damage, organ dysfunction and subsequent stimulation of immune response and other adaptive reactions.*

Three main ***conditions*** are necessary for infectious process emergence.

First is the *virulent* ***causative agent*** *presence*; the second – *pathogen's* ***ability to penetrate*** *and invade the body*; and third one is the ***host susceptibility*** *to certain pathogen*.

The intensity of infectious process is promoted by three above-mentioned conditions. First condition is based on causative agent virulence and its dose; the second depends on the efficacy of tissue defensive barriers, and the third – on immune and other adaptive system activities.

Place of pathogen adherence and penetration is known as ***portal of entry*** for pathogenic bacteria.

According to their ability to cause infectious process all of microorganisms are divided into three main groups: ***obligate pathogenic***, ***facultatively pathogenic*** and ***non-pathogenic*** or ***saprophytic microorganisms***.

Obligate pathogenic microorganisms possess highly aggressive virulence factors and in most of cases induce the infectious diseases as the result of initial susceptibility of the majority of human hosts (plague yersiniae, anthrax agents, tetanus and botulism clostridia, etc.)

Facultatively pathogenic microorganisms can trigger infectious diseases under the conditions of host defense insufficiency, e.g. in *immunocompromised* patients (***opportunistic pathogens***), and when inoculated in high doses (staphylo- and streptococci, pseudomonads, klebsiellae and many other enteric bacteria, various fungi, etc.)

Non-pathogenic or ***saprophytic microbes*** usually don't cause diseases. Many of them are the normal habitants of human body. They may

play a role of "accidental" pathogens for humans, as they trigger some kind of infectious process only occasionally (i.e., with very low or negligible likelihood).

Characteristics of Infectious Diseases

There are two main forms of infectious process: ***infectious disease*** and ***microbial carrier state*** (***microbial carriage***).

Infectious disease *is the clinically manifested form of infectious process, its extreme degree of manifestation*.

Infectious diseases comprise *a great group of disorders caused by pathogenic bacteria, viruses, fungi, and protozoans*.

Thus, infectious illness is caused by live ***causative agent***.

In majority of cases these diseases are *contagious*, have a *latent period* of certain length and in most of cases followed by specific host response against the invaded pathogen with *production of immunity*.

Typical infectious disease has well-defined *common periods* in its course.

Incubation period lasts from the moment of infection to the onset of first clinical symptoms of the disease.

The duration of incubation period depends on the dose and virulence of the pathogen, immune system state and on the influence of environmental factors. It ranges from several hours (in influenza, cholera, etc.) to months and even years (hepatitis B, leprosy).

In some diseases the ***prodromal period*** follows the incubation period, where the non-specific symptoms similar in many diseases are manifested (fatigue, weakness, malaise, headache, dissiness, subfebrile temperature, loss of appetite, etc.)

The period of clinical manifestations (***height of the disease***) is characterized by the highest clinical manifestations of the ailment. The most typical features of the infectious disease height are fever, functional and organic disturbances in the affected system or organ (respiratory failure, digestive and urinary tract mulfunctions, or CNS disorders). This stage comes gradually to the ***outcome period***, where the disease is usually finished by ***convalescence*** (*recovery*). Other but unfavorable results of the infectious disease course include the development of ***chronic disease***, ***carrier state*** establishment, or ***lethal outcome***.

Various Forms of Infection

According to the origin of infection there are ***exogenous*** and ***endogenous*** infections. ***Exogenous*** infection appears from the external source of infection, while ***endogenous*** evolves after activation of internal infection.

Localization and the capacity of spreading determine ***local*** or ***generalized*** forms of infection. In latter case the agent spreads from the initial site throughout the whole body.

If the bacteria enter the blood stream, it may stay there for some time. This state is known as ***bacteremia*** (in case of viral diseases – as ***viremia***). Transitory bacteremia occurs in enteric fever, rickettsioses, tularemia and other diseases.

In case of immune system dysfunction and infection severity bacteremia is followed by further microbial dissemination. This complication is known as ***sepsis*** or ***septicemia***, where the bacteria are able to propagate within the blood. Sepsis is characterized by dissemination of pathogenic microbes and bacterial toxins over the body in parallel with the activation of ***systemic inflammatory response*** due to massive pro-inflammatory cytokine synthesis.

Finally the septic process damages almost all tissues. It is accompanied by the production of inflammatory and purulent foci in different organs and tissues.

Taking into account the great deleterious effects of pro-inflammatory cytokines in sepsis, the disease is regarded now as ***systemic inflammatory response syndrome***, emphasizing the tremendous role of host immune reactions in its pathogenesis.

Numerous bacteria are able to produce exotoxins (causative agents of diphtheria, tetanus, gas gangrene etc), which spread with blood flow. This state is named ***toxemia***.

According to the duration of their course the infections are divided into ***acute***, ***protracted*** and ***chronic***.

Acute infections are followed by the sudden onset and a comparatively short time of their course (usually *less than 1 month*). Among them are influenza, acute respiratory viral infections, measles, typhoid fever and many others. Diseases with ***chronic*** (elapsing *more than 3-6 months*) or ***protracted*** courses are lingering (HIV, tuberculosis, syphilis, brucellosis etc).

According to their manifestations, ***typical***, ***atypical***, ***abortive***, ***latent*** and ***inapparent*** infections can be outlined.

Typical infection demonstrates all the symptoms of the disease; ***atypical*** forms are characterized by some unusual infection course.

Abortive infection undergoes abrupt interruption after typical disease onset. It usually occurs due to the rapid activation of intensive immune response or by administration of highly efficient specific treatment.

A large number of infections may be hidden or without obvious clinical manifestations (***latent infections***). Nevertheless, the microbial pathogen remains slowly propagating here, and the infection can undergo transformation into the typical form under different internal or external stimuli.

Asymptomatic form of infection was named ***inapparent***, where the clinical symptoms of the disease are not detemined usually because of efficient control of the infection by immune system.

Persistence is the special state of infectious agent preservation in the host. The activity of pathogen is minimal, and it may stay in the human body for a long period of time, maintaining the infection.

Mixed infection means the combined infectious process caused by more than one microbial species. Opposite case is named ***monoinfection***.

Sometimes primary infectious agent suppresses greatly the local or general immune reactions or impairs the metabolism of the host. In that case the organism becomes susceptible to other microbial pathogens. This is known as ***secondary infection***. Such an example here is the development of bacterial pneumonia after the primary influenza attack.

Reinfection is a repeated infection caused by the same microbial species after the complete recovering from the previous case of the same infection. It is possible, when the sufficient anti-infectious immunity is not formed (gonorrhoea, helicobacteriosis, and other infections).

Relapse is the return of symptoms of the infectious disease, which ensues from the incomplete recovery of the patient (in case of relapsing fever, Brill-Zinsser disease, etc.)

Superinfection means the additional infection of the host with the same microbial species, where the previous infection has not ended yet. For instance, superinfection occurs in syphilis – the patient in the period of tertiary syphilis can be superinfected with *T. pallidum* again with the development of secondary syphilis symptoms.

Microbial Carrier State (Microbial Carriage)

Sometimes the unstable balance between the immune system and the pathogen is established as the outcome of infectious process.

Infectious agent can escape total elimination from the host by the number of special mechanisms. It may inhabit the isolated compartments of human body (e.g., the gallbladder for S. Typhi) or can stay intracellularly (rickettsiae or chlamydiae).

Microbial persistence is not followed here by disease manifestation, and the patient becomes ***the carrier*** of the causative agent.

The carrier *is an apparently healthy individual, who harbors the pathogen and can spread it to other individuals*. High-grade post-infectious immunity prevents carrier state development (e.g., in measles, chickenpox, or rubella), while in some other diseases the carrier state is long (enteric fever, shigellosis, meningococcal infections, etc.).

Acute carrier state lasts up to 3 months, and the more protracted carrier state is considered as ***chronic***.

Epidemic Process. Conditions for the Emergence of Epidemic Process

Epidemic process *is the process of generation and spread of infectious diseases, which is maintained by continuous circulation of causative agents in the population*.

As well as for infectious process, three main conditions need to develop the epidemic process:

1) ***the source of infection***,

2) the mechanisms (***routes***) of the ***disease transmission***,

3) ***susceptible population*** affected by the causative agent.

Source of infection *is the place of natural habitation of the causative agent, its propagation and discharge into the environment*.

Live organisms are the main sources of infection (***sick humans*** or ***animals*** and ***microbial carriers***).

The ***reservoir of infection*** preserves the pathogen in the environment but usually the infectious agent cannot propagate in the reservoir.

There is a large complex of versatile ***mechanisms*** (or ***routes***) ***of transmission*** of infectious diseases. Among them are:

– ***fecal-oral route*** that comprises *alimentary* (food-borne), *water-borne*, *contact* (via fomites) and other possible routes;

– ***air-borne route*** (e.g., ***air-droplet*** or ***dust*** disease transmission);

– ***vector-borne route*** (requires different ***vectors*** – insects, arthropods, etc. – for successive transmission);

– ***contact route*** with the disease transmission by ***sexual intercourse*** or by ***direct contact***;

– ***artificial route*** based predominantly on *disease transmission by medical manipulations* (***iatrogenic route***);

– ***vertical*** transmission (from mother to fetus).

Multiple auxiliary ***vehicles*** support the certain routes of transmission. For instance, fecal-oral route can be realized through the dirty hands, absorbing pathogenic bacteria, or by flies, carrying infectious agents. Various *fomites* (objects, surfaces or substances) contaminated with microorganisms may carry the pathogens from one susceptible person to another. Similarly, air-borne route depends on air and dust flows, air conditioning state, etc.

According to the main localization of the causative agent all infections are divided into ***intestinal*** (affecting gastrointestinal tract with fecal-oral transmission); ***respiratory*** (of respiratory tract); ***blood*** infections (with primary localization of pathogen in blood due mainly to vector-borne disease transmission or by artificial route); ***infections of integument tissues*** (causative agents are localized on skin or mucosal tissues), "***vertical"*** infections with disease transmission from mother to fetus.

According to specific habitation of the causative agents infectious diseases are classified as ***anthroponoses***, ***zoonoses*** and ***sapronoses***.

If a pathogen affects only humans, the disease is accounted as ***anthroponosis***. The disease, peculiar to animals, is regarded as ***zoonosis***. The causative agents of zoonoses also may affect humans resulting in ***zooanthroponoses***.

And the last group comprises the number of diseases with substantial role of external environment in their spread (so-called ***sapronoses***). For instance, anthrax spores stay viable in the soil for many years before the infection emergence. This is true for most of sporogenic bacteria. Water is the environment of vibrio cholerae habitation, as well as for leptospirosis agent.

In general, disease distribution within some population varies strongly, depending on infectious agent nature and population susceptibility.

Infectious diseases may be ***sporadic*** (*separate cases of the disease, registered in some area during a certain period of time*). Usually sporadic reflects an ordinary level of morbidity. And there are no evident epidemiological links between sporadic disease cases.

Great rise of incidence of certain disease that affects the large territories is known as an ***epidemic*** (or *epizootic* in animals).

Disease ***outbreak*** is “a small epidemic” – *restricted by time and area sharp raise of the disease, where individual disease cases are closely related*.

And *when the epidemic covers unusually large territories or spreads over many countries or even continents*, it is called as ***pandemic***.

Finally, a special form of infectious diseases spread is known as ***endemic***. ***Endemic*** *is characterized by the disease retaining in some locality for a long period of time*. Sometimes it is restricted by vector habitat borders, or by geographical barriers. Nevertheless, the endemic is a potent threat of massive disease outbreak due to developed people communications – travelling, air flights, etc.

Pathogenicity and Virulence

All of bacteria differ in their individual ability to cause infectious process.

Pathogenicity *means the potential capacity of certain microbial agent to cause an infectious process in susceptible organism*.

Pathogenicity is the species inherited ***genetic feature***.

Unlike saprophytic bacteria, pathogenic microorganisms harbor ***pathogenicity genes*** that encode the vast number of *virulence factors* – toxin production, invasion enzyme synthesis, adhesin expression, effector protein synthesis, etc.

Pathogenicity genes in bacteria are usually organized into special genetic clusters known as ***pathogenicity islands***.

Typical structure of the pathogenicity island includes several ***virulence-linked genes*** (encoding toxins, secretion system structures, capsule synthesis, etc.) as well as the genetic elements responsible for the mobility of pathogenicity island (IS-elements, integrase genes, direct repeats sequences, and others).

In addition, genetic sequence of pathogenicity island is quite different from the basic sequence of microbial genome. For instance, pathogenicity islands can be distinguished from the other parts of nucleoid by (G+C) nucleotide content.

Therefore, pathogenicity islands are the transmissible genetic structures capable of spreading among the various groups of bacteria by lateral gene transfer. The acquisition of these genes endows the bacteria with pathogenic properties.

Virulence signifies *the degree of pathogenicity of the certain microbial strain*. It is the ***quantitative ability*** of the microbial agent to cause infectious disease (***phenotypic trait***).

Virulence results from the expression of pathogenicity genes with the synthesis of a great variety of ***virulence factors*** (toxins, adhesins, invasive enzymes, etc.)

To measure the virulence levels, various units of virulence have been proposed. In general, virulence is indicated "by case fatality rate and/or by the ability of microorganism to invade the tissues of the host".

One of the virulence units for measurement is ***dosis letalis minima*** (or ***DLM***). It corresponds to the *minimal quantity of live microorganisms, which causes the death of 95% of experimental animals in a certain period of time*.

Also the *absolute lethal dose* – ***dosis certa letalis*** (***Dcl***) – can be evaluated. *This dose causes the death of all 100% of the experimental animals*.

And more precise is ***median lethal dose*** or ***LD50*** – *the dose that is lethal to 50% of the infected experimental animals*.

The ***infectious dose*** (or ***ID***) of certain pathogenic agent indicates the *definite amount of pathogenic microorganisms that is enough to produce the infectious disease in standard experimental conditions*. It is expressed in units of ***ID50*** or ***ID95***.

It has been found that the infectious dose varies from less than 10 bacterial cells for enterohemorrhagic *E. coli* to 10^8-10^{11} microbial cells for El Tor cholera vibrios.

The ***potency of toxins*** is estimated by laboratory animal tests according to Dlm and LD50. For instance, 1 Dlm of the diphtheria toxin is equivalent to the minimal amount of toxin that after subcutaneous injection of guinea pigs kills them on the fourth day of the experiment.

Virulence is altered under the influence of the environment and by pressure of host defensive systems. It can be increased by the number of microbial passages through the susceptible experimental animals as well as by culturing on the special media enriched with growth factors.

On the other hand, virulence can be diminished by the great variety of factors (passages through resistant lines of laboratory animals, treatment with antimicrobial drugs, the action of disinfectants and other chemicals, cultivation in poor media, etc.)

More advanced methods include genetic engineering manipulations that may either increase or decrease the virulence.

Basic Virulence Factors of Bacteria

Every case of infection comprises a great set of specific reactions between the virulent microorganism and its susceptible host. They include selective interaction and attachment of bacteria to host cell receptors, membranes and tissues (***adhesion*** or ***adherence***), replication of pathogenic agents in the place of their attachment (***colonization***), their ability to overcome the cellular and tissue barriers (***penetration*** and ***invasion***), toxin production (***toxigenicity***), inactivation of host immune factors (***immunosuppression***), inactivation of antimicrobial agents (***antimicrobial resistance***).

All these reactions are stimulated by the powerful virulence factors, produced by microbial cells. As the result, highly virulent bacteria in minimal doses may cause severe diseases with lethal outcome.

Adhesion Factors

Adherence is the initial step for the infectious process. Then it is followed by microcolonies growth (***colonization***). After successful adherence and colonization most of bacteria produce ***microbial biofilm***. Biofilm is the aggregate of microorganisms, where the cells are embedded into a self-produced matrix of extracellular polymeric substances tightly attached to the adjacent tissues. Biofilmt creates a tough polymicrobial layer poorly permeable for antimicrobial agents.

All these kinds of interactions ensue from the synthesis of various virulence factors by pathogenic bacteria.

For instance, microbial cells express a number of specific ***adhesins*** – surface molecules that bind to the host cell receptors. In this vein, many bacteria (e.g., *E. coli*) have 1st type ***pili*** that bind to receptors containing D-mannose.

Likewise, group A streptococci carry ***fimbriae*** capable of binding to certain host cell receptors. Fimbriae contain lipoteichoic acid and M-protein.

Also the bacteria possess the vast number of ***lectins*** – adhesive proteins, responsible for binding to carbohydrate moiety of host membrane structures,

Cell Entry and Invasion into the Host Tissues

Invasion of the host's epithelium is essential for intitation of the infectious process. Some bacteria (*Salmonella spp., Shigella spp., Yersiniae spp*. and others) invade specific types of host epithelial cells by flagella-like structures, known as "***needle complex***" or ***injectisome***. Bacteria inject special *III type secretion* proteins into the host cells. This action induces the cytoskeleton actin remodelling with subsequent formation of vacuole. Bacteria are captured further by cell membrane protrusions and transferred into the cell. For shigellae at least three proteins (***invasion plasmid antigens***, ***Ipa***), IpaB, IpaC, and IpaD contribute to this process. Shigellae bind to integrin receptors upon the surface of M cells in Peyer's patches of human intestine.

L. monocytogenes is also able to stimulate its own engulfment by the host cells. Protein ***internalin*** plays a primary role in this process.

Legionella pneumophila infects pulmonary macrophages resulting in severe pneumonia. Macrophages capture the legionellae, but phagolysosome fusion is inhibited, and the bacteria propagate within the vesicle.

Capsule production also promotes bacterial escape from microbicidal phagocyte action thus maintaining microbial spread by phagocytes.

A great number of tissue degrading enzymes helps bacteria to invade. ***Hyaluronidases*** are the enzymes that hydrolyze hyaluronic acid, a major constituent of the ground substance of connective tissue. These enzymes are produced by many bacteria (e.g., staphylococci, streptococci, multipke anaerobic species, etc.) Hyaluronidase production spurs the microbial spread through the tissues.

Many bacteria (*Clostridia spp., Pseudomonas spp.,* and others) synthesize proteolytic enzymes ***collagenase*** and ***elastase***, which degrade collagen and elastin, the major proteins of fibrous connective tissue.

Bacterial Toxins

Two basic types of bacterial toxins are known: ***exotoxins*** and ***endotoxins***. They display striking differences in their structure and basic traits.

Exotoxins are heat-labile substances of ***protein nature***. They are actively secreted by living ***toxigenic*** cells, being produced both by gram-positive and gram-negative bacteria.

Also they are highly antigenic and stimulate the formation of high-titer ***antitoxins*** (antitoxic antibodies). Antitoxin is capable of toxin neutralization with great efficacy.

Treatment by formaldehyde leads to exotoxin conversion into *antigenic non-toxic* ***toxoid***, which is used for active immunization.

In most of cases exotoxins bind to ***specific receptors*** upon or within the host cells. This is related with highly ***specific mechanisms*** of their action.

And finally, exotoxin expression is often controlled by extra-chromosomal ***tox-genes*** of plasmids or bacteriophages.

Endotoxins are found predominantly in gram-negative bacteria. They are tightly integrated within the cell wall of gram-negative bacteria and *released after bacterial destruction*. Bacterial lipopolysaccharide (***LPS***) complexes are the main constituents of endotoxin, and ***lipid A*** is considered to be most responsible for the toxicity.

Similar toxic activity of gram-positive bacteria is maintained by lipoteichoic acids and peptidoglycan of their cell wall.

Endotoxins are heat stable, cannot be converted into toxoids; the synthesis of LPS is predominantly directed by chromosomal genes.

Pathological mechanism of LPS endotoxin action ensues from ***pro-inflammatory cytokine production*** by immune cells.

When released from destroyed cells, bacterial LPS specifically binds to host LPS-binding protein (LBP), circulating in blood. The arisen complex LPS-LBP interacts with ***CD14*** molecule expressed on the membranes of macrophages and dendritic cells.

Binding of LPS-LBP to CD14 activates macrophage *Toll-like receptor 4* (***TLR 4***) that is coupled with CD14 on macrophage membrane. The signal from TLR 4 is transmitted into the cell that leads to the activation of transcription factor ***NF-kB***. The activity of NF-kB stimulates expression of the vast number of pro-inflammatory cytokines by immune cells (macrophages, dendritic cells, T helpers of 1^{st} type, and many other regulatory and effector cells).

Exuberant secretion of pro-inflammatory cytokines of various families (IL-1, IL-2, IL-6, IL-12, IL-17, IL-18, α-TNF, γ-interferon, multiple chemokines, and other molecules) promotes ***systemic inflammatory response*** (or "*cytokine storm*"). This leads to systemic microcirculation damage followed by diffuse intravascular coagulation, fever, and shock with hypotension, resulting in impaired perfusion of brain, heart and kidneys (***multi-organ dysfunction syndrome***).

Unlike endotoxins, ***exotoxins*** possess highly variable ***specific mechanisms*** of action. They are summarized in the Table 4, where the main groups of exotoxins are presented.

Many exotoxins are composed of ***A*** and ***B*** *subunits*. ***B subunit*** mediates adherence of the toxin complex to the cell receptor and stimulates exotoxin entry into the host cells. ***Subunit A*** develops the toxic activity.

For instance, strains of *C. diphtheriae* can carry a temperate bacteriophage, which code for diphtheria toxin. These strains become toxigenic and cause the diphtheria. Native toxin molecule of MW 62,000 is enzymatically degraded into two fragments, A and B. Fragment B binds to specific host cell receptors and facilitates the penetration of fragment A into cytoplasm. Fragment A inhibits peptide chain elongation factor EF-2 by its ribosylation. Block of protein synthesis disrupts normal cellular physiological functions. Diphtheria toxin can be lethal in a dose of 40 ng.

The described virulence factor, as well as some other exotoxins, pertain to group of ***protein synthesis inhibitors***.

Membrane attacking toxins demonstrate another mode of action. They directly damage the membranes of target cells.

For instance, *C. perfringens* produces great number of toxins with necrotizing and hemolytic activity. ***Apha-toxin*** of *C. perfringens* is the enzyme lecithinase (phospholipase) that destroys cell membranes by lecithin hydrolysis.

Also membrane attacking toxins may develop pore-forming activity (***staphylococcal alpha-toxin***).

Striking example of exotoxins able to impair cellular metabolism by ***secondary messenger activaton*** is *V. cholerae* toxin. It is the ***enterotoxin*** with a molecular weight of 84,000.

The toxin contains two subunits, A and B. Subunit B is composed of five identical peptides and binds to ganglioside membrane receptors of intestinal epithelium. Subunit A penetrates cell membrane promoting ADP-ribosylation of G-proteins. This activates membrane guanylate and adenylate cyclases resulting in great increase of intracellular cAMP concentration. The latter stimulates secretion of chlorides into the small intestine with subsequent block of sodium and water reabsorption. As the result, massive life-threatening diarrhea develops (up to 10-20 liters per day).

Superantigen toxins show potent biological activity by stimulation of great number of T cells (more than 20% of total count). Superantigens have strong binding capacity to some common variable T cell receptor domain (Vb-variants). Consequently they activate great number of T-

lymphocytes that is followed by redundant production of pro-inflammatory cytokines (IL-1, IL-2 IL-6, IL-12, IL-18, α-TNF, γ-interferon, etc.) Cytokine liberation activates inflammatory reactions with severe tissue damage.

Numerous bacterial toxins (staphylococcal enterotoxins, streptococcal toxins, etc.) pertain to superantigens. Well-defined are staphylococcal ***toxic shock syndrome toxin*** (***TSST***), which causes ***toxic shock syndrome***, and ***pyrogenic exotoxins*** of group A beta-hemolytic streptococci The major clinical manifestations of above-mentioned disorders are secondary to the effects of pro-inflammatory cytokines (toxic shock, high fever, organ disfunction, etc.)

And there is a separate special group of ***toxins*** capable of ***specific protease activity***. Among them are extremely toxic ***neurotoxins***. Bacteria, producing neutotoxins, cause severe damage of central and peripheral nervous system. In particular, *C. botulinum* produces the most potent known neurotoxin. There are several distinct serotypes of toxin. Among them, types A, B, and E toxins cause the disease in humans. Botulotoxin is absorbed from the gut and binds to the receptors of presynaptic membranes of motor neurons of the spinal cord and cranial nerves. Intensive proteolysis of target proteins of the neurons blocks acetylcholine liberation within neuromuscular synapses that results in impairment of muscular contraction and paralysis.

Table 4.

Main bacterial toxins, their characteristics and mechanisms of action

Bacteria	**Toxin**	**Mechanism of action**	**Molecular target**	**Disease or disorder**
Membrane attacking				
Clostridium perfringens	Perfringolysin	«-»	Cholesterol	Gas gangrene
Listeria monocytogenes	O-listeriolysin	«-»	Cholesterol	Alimentary infections, meningitis
Staphylococcus aureus	Alfa-toxin	«-»	Cytoplasmic membrane	Abscesses, purulent infections, etc.
Streptococcus pneumoniae	Pneumolysin	«-»	Cholesterol	Pneumonia
Sreptococcus pyogenes	O-streptolysin	«-»	«-»	Tonsillitis. Scarlet fever

Protein synthesis inhibitors				
Corynebacterium diphtheriae	Diphtheria toxin	ADP ribosyl transferase	Elongation factor 2	Diphtheria
E. coli, Shigella dysenteriae	Shiga toxin Verotoxins	N-glycosidase	28S rPHK	Hemorrhagic colitis, hemolytic uremic syndrome
Pseudomonas aeruginosa	Exotoxin A	ADP ribosyl transferase	Elongation factor 2	Pneumonia
Secondary messengers activators				
E. coli	Thermolabile toxin	ADP ribosyl transferase	G-proteins	Diarrhea
E. coli	Thermostabile toxin	Activates guanylate cyclase	Guanylate cyclase receptor	Diarrhea
Bacillus anthracis	Edema factor	Adenylate cyclase	ATP	Anthrax
Bordetella pertussis	Pertussis toxin	ADP ribosyl transferase	G-proteins	Whooping cough
Clostridium botulinum	Toxin C2 Toxin C3	ADP ribosyl transferase «-»	G-actin G-proteins (regulators of cytoskeleton)	Botulism Botulism
Clostridium difficile	Toxin A Toxin B	Glycosyl-transferase	G-proteins (regulators of cytoskeleton)	Diarrhea. Pseudomembranous colitis
Vibrio cholerae	Cholerogen	ADP ribosyl transferase	G-proteins	Cholera
Superantigens				
S. aureus	Enterotoxins	Superantigens	T cell receptor and HLA II	Food poisoning
S. aureus	Exofoliatins	«-»	«-»	Scalded skin syndrome
S. aureus	Toxic shock syndrome toxin	«-»	«-»	Toxic shock syndrome
S. pyogenes	Pyrogenic exotoxins	«-»	«-»	Scarlet fever. Toxic shock syndrome
Proteases				
B. anthracis	Lethal factor	Metal protease	Mitogen-activated proteinkinase kinase (MAPKK)	Anthrax
C. botulinum	Neurotoxins A-G	Zn-containing protease	Synaptic proteins	Botulism
C. tetani	Tetanus toxin	Zn-containing protease	Synaptic proteins	Tetanus

MEDICAL IMMUNOLOGY

Chapter 11

IMMUNOLOGY AND IMMUNITY – BASIC DEFINITIONS. STRUCTURE OF IMMUNE SYSTEM. DIFFERENTIATION OF T- AND B-LYMPHOCYTE SUBSETS, THEIR FUNCTIONS

Immunity. Basic Types of Immune Response

Immunology is the science investigating ***immunity***.

Immunity *is the great set of reactions between* ***immune system*** *and particular specific active substances* (***antigens***).

Immune response plays an essential role in body protection against the tremendous variety of foreign agents. Nevertheless, it should be noted that the broad scope of primary defensive reactions is not directly mediated by the host immune system. These reactions are controlled by the general mechanisms of species *hereditary resistance* as well as by the common pathways of *non-specific resistance*.

Hereditary resistance presumes *the insusceptibility of members of certain species to the diseases that affect another species*.

The versatile mechanisms of hereditary resistance include the lack of specific receptors on the target cells required for adhesion and propagation of the causative agents, and conversely, the organization of the host metabolic pathways intolerable for certain microbial pathogens or the production of substances that naturally block the reproduction of pathogenic agents.

As an example of the species-inherited resistance is the insusceptibility of humans to many zoonotic diseases such as cattle plague or chicken cholera. Similarly, animals are not affected by many human infections like enteric fever, scarlet fever, syphilis, measles, etc.

The acquisition of hereditary resistance is the result of a long evolution of complex relationships between the host and pathogenic microorganisms. It hinges on the biological peculiarities of the certain species, which formed in the course of evolution and natural selection, species variation and continual adaptation to the environmental conditions.

Likewise, there is a vast number of reactions of ***non-specific host resistance*** that actively participate in body protection. They comprise the barrier functions of skin and mucosal tissues, body temperature reactions (fever), mucociliary clearance of pathogens by epithelial cells and mucosal secretions, microbicidal activities of tears, saliva, secretions of sweat and

sebaceous glands, acid contents of gastric juice, the elimination of pathogens with body excretions, and some other similar activities.

By contrast to non-specific resistance, immune response is triggered by specific foreign substances and agents.

According to the *mechanism of the development* the immunity is divided into two main types: ***innate*** and ***acquired*** (or ***adaptive***).

Both these types include ***cellular*** (maintained by specialized *immune cells*) and ***humoral*** immune reactions (the latter are promoted by *molecular soluble immune factors* present in *biological fluids* of the host).

Innate immunity encompasses immune cells and molecules have arisen *regardless of prior antigenic challenge*. Having been created as the first line of the immune defense, they are responsive against the broad groups of foreign agents and substances. Hence, the reactions of the innate immunity are ***largely non-specific***, or demonstrate low ***or limited specificity*** towards the most common structures that are shared by the vast number of pathogenic agents. This brings the innate immunity closer to the non-specific host resistance.

By contrast, cellular and humoral reactions of ***acquired*** (***adaptive***) ***immunity*** *need primary antigenic stimulation* being triggered by the exposure of the immune system to the specific antigen (***Ag***). Thus, ***acquired immunity*** demonstrates ***high specificity*** and ***selectivity*** in response against invaded foreign agents or structures.

Acquired immunity is commonly divided into ***natural*** and ***artificial immunity***.

Both can be further sub-divided into ***active*** and ***passive immunity***.

Acquired active immunity is maintained by active production of special defensive factors (various molecules and cells) by the body itself after antigenic exposure.

Acquired passive immunity depends on the external source of defensive factors delivered into the host by different ways.

Natural active immunity is acquired following manifested or latent *infectious disease* or infectious process.

Natural passive immunity is observed mainly in newborns and infants; it is acquired from mother through the placenta in the period of fetal development or maintained with mother's milk in breast feeding. The duration of passive immunity of the newborn is short. After about six months this immune state disappears and children become susceptible to many infections (measles, diphtheria, scarlet fever, etc.).

Artificial active immunity is triggered by ***active immunization*** (***vaccination*** with vaccines or toxoids).

In turn, ***artificial passive immunity*** is reproduced by *passive immunization* (administration of ***immune sera*** or ***antibodies***).

Each of immune reactions includes ***local*** and ***generalized immune response***.

Local response develops in different body compartments as the function of local specialized immune tissues or resident cells.

In ***generalized immune response*** all of the immune subsystems are activated.

According to the origin of foreign agent (***antigen***) that elicits specific immune reactions, immune response is divided into ***anti-infectious*** and ***non-infectious*** immunity.

Anti-infectious immunity is stimulated by various microbial antigens. There are several kinds of anti-infectious immunity.

Anti-bacterial immunity caused by bacteria may be ***sterile*** or ***non-sterile***. In case of ***sterile immunity*** the host immune response remains stable despite antigen complete elimination.

Non-sterile immunity is maintained by residual microbial cells retained in the body after the infection.

Anti-toxic immunity is induced against the toxins of bacteria, ***anti-viral*** – against viruses; ***anti-fungal*** is caused by different fungi, ***anti-parasitic*** – by various parasites.

Non-infectious immunity is directed against non-infectious antigens. Due to nature of the antigen it is divided as follows:

– ***autoimmunity*** comprises immune reactions provoked by self-antigens of the body; it arises because of the self-tolerance breakdown;

– ***transplantation immunity*** develops after allo- or xenotransplantations; it is mediated predominantly by the antigens of ***major histocompatibility complex*** (human leukocytes antigens or HLA in humans and H-2 antigens in mice);

– ***anti-tumor immunity*** arises against tumor antigens expressed on cytoplasmic membranes of cancer cells; these antigens emerge due to the genetic instability of tumor genome;

– ***reproductive immunity*** encompasses the reactions in "***fetus-mother***" system in the period of pregnancy and embryogenesis; in particular, they may develop against the fetal antigens, encoded by genes inherited from father.

Structure of Immune System

Immunity is the function of the immune system.

Immune system comprises *a great set of molecules, cells, tissues and organs that realize immune reactions*. It consists of numerous subsets working as united cooperative cross-linked community.

There are the following subsets within immmune system on the cellular and molecular levels of biological organization.

Lymphoid sub-system includes the cells synthesizing ***specific factors*** of immune defense – ***antibodies*** (***Abs***) and T cell ***antigen-binding receptors*** (***TCR***).

One more sub-system comprises ***humoral factors of innate*** (***non-specific***) ***immunity***.

Also there are:

– ***mononuclear phagocyte system*** (***MPS***),

– ***dendritic cell system***,

– ***granulocyte system*** (encompasses neutrophil, basophil and eosinophil leukocytes),

– ***natural killer*** (***NK***) ***cells system***,

– ***complement system***,

– ***platelet system***.

All of these sub-systems with exception of lymphoid one participate in immune reactions via *non-specific mode of action*. Lymphoid system comprising T and B cells produces *specific molecules* of immune response.

Cytokines

Complex reactions involved into the immune response include multiple processes of cell maturation and differentiation maintained by the broad networks of intercellular communications.

A great assemblage of highly variable regulatory molecules promotes closest interrelationships between immune cells. These mediators were named ***cytokines***.

Cytokines possess the number of common features.

1. There are low molecular weight secreted glicoproteins, usually of 15-25 kDa of molecular mass.

2. Cytokine action is transient and usually of short range. Unlike endocrine hormones, they normally display ***autocrine*** (self-directed effect) and ***paracrine*** action (affect the nearest cells).

3. Cytokines are highly potent, acting at piko- or femtomolar concentrations.

4. Every cytokine binds to its specific high affinity cell surface receptor. This event transmits the signal into the cell thus regulating cellular mRNA transcription and protein synthesis.

5. Cytokines often have multiple or ***pleiotropic*** effects, affecting great variety of cell types; the action of different cytokines reveal considerable overlapping and redundancy. They mediate cell growth, inflammation, immunity, cell differentiation and cell reparation.

Cytokines are classified into the next following sets.

1. ***Interleukins*** maintain interrelationships among the immune cells as well as interlinks between immune and non-immune systems (central nervous system, endocrine, digestive, respiratory systems, etc.)

2. ***Interferon*** family comprises various types of interferons with pleiotropic antiviral effects, immune modulation and complex cell regulatory actions.

3. ***Tumor necrosis factor (TNF)*** group consists of 2 forms of cytokines: ***TNF-α*** and TNF-β (or ***lymphotoxin***).

4. The group of ***colony stimulating factors*** comprises the set of related cytokines such as **GM-CSF** (granulocyte-macrophage-colony stimulating factor), **G-CSF** (granulocyte-colony stimulating factor) and some others.

5. The group of ***growth factor cytokines*** comprises ***transforming growth factors*** (***TGF***), endothelial growth factor (EGF), platelet-derived growth factor (PDGF), etc.

6. ***Chemokines*** comprise the numerous families of cytokines of various origins that regulate cellular directed movement along the chemokine concentration gradient. Chemokines can be also the members of other cytokine groups, e.g. IL-8.

Interleukins

Interleukins (***IL***) are the most potent cytokines maintaining balances within the immune system. To date, 36 interleukins are known.

IL-1 (alpha and beta) is produced by macrophages, dendritic cells and some other cell types; activates differentiation of T cells presumably into Th1 helper type stimulating cell-mediated immunity (***pro-inflammatory cytokine***); it activates hypothalamic receptors possessing strong pyrogenic activity with fever development. Also it stimulates other types of immune cells.

IL-2 is produced by T helper cells (predominantly of ***Th1*** type); it stimulates the large sets of immune cells (T- and B cells, monocytes, NK cells) maintaining cell proliferation and differentiation.

IL-3 is a potent ***hematopoietic factor***, produced by bone marrow cells and T cells. It stimulates bone marrow precursors of immunocytes, initiating their differentiation.

IL-4 is expressed by T helper-2 (***Th2***) cell subset; it stimulates early B cell proliferation, conversion of "naive" Th0-cells into Th2 type, supporting ***humoral immune response*** and allergic reactions with switching to IgE production.

IL-5 is secreted presumably by T cells (Th2) and stimulates eosinophil and basophil maturation, immunoglobulin synthesis; as the result, it takes part in allergy development.

IL-6 is ***pro-inflammatory cytokine*** with wide pleiotropic effects produced by different cell lines (T cells, macrophages, keratinocytes, endothelial cells, etc.); it stimulates B cell differentiation and promotes cellular inflammation.

IL-7 is another important ***hematopoietic factor***, produced by bone marrow stromal cells and some other cell types. It induces proliferation of early bone marrow precursors of immune cells, especially T-lymphocytes.

IL-8 is strong chemotaxis agent (***chemokine***) secreted by macrophages, endothelial and epithelial cells and other cell types. It stimulates directed migration of neutrophils and basophils, their adhesion and metabolic activation.

IL-10 is the cytokine with suppressive activity for cell immune reactions. It is considered to be produced by macrophages and by Treg or regulatory T cells. It blocks IL-1, IL-2, IL-6 and alpha-tumor necrosis factor synthesis (***anti-inflammatory cytokine***).

IL-12 is produced by macrophages and dendritic cells, activates differentiation of T cells into Th1 helper type, thus redirecting the immune response towards ***the cell-mediated immunity***. It is potent ***pro-inflammatory cytokine*** enforcing IL-2 and γ-interferon synthesis by T cells.

IL-13 is synthesized by T helper-2 (Th2) cells and mast cells; it stimulates B cells, activates allergic reactions with IgE production, stimulates mucus secretion by epithelial cells, suppresses inflammation (***anti-inflammatory cytokine***).

IL-17 is produced by special subpopulation of T helper cells (***Th17***); it demonstrates highly pleotropic effects and activates the broad set of immune cells (T cells, neutrophils, macrophages and some others), thereby promoting inflammation (***pro-inflammatory cytokine***); it also activates hematopoiesis of myeloid cell lines, endothelial and epithelial cells. On the other hand, IL-17 is capable of stimulating antibody synthesis.

IL-18 is secreted by macrophages, dendritic cells, keratinocytes; it is also potent ***pro-inflammatory cytokine*** that stimulates transition Th0 into Th1 helper type, accelerates synthesis of IL-2 and γ-interferon by Th1 cells, and activates NK cells.

IL-21 is produced by Th17 and Th2; it stimulates Th17 development, differentiation of B cells, maturation of NK cells.

IL-23 is expressed by macrophages and dendritic cells; its main function is the maintenance of Th17 helper cells and T memory cells.

IL-28 and ***IL-29*** are referred to as *lambda-interferons*.

Interferons

It is a family of broad-spectrum immunoregulatory and antiviral agents. They were first recognized by the phenomenon of viral interference, where the animals infected with one virus became resistant to the infection by a second unrelated virus.

According to their functions, three main types of interferons are determined.

Interferons of I type include the major families of ***α-interferons*** (***IFN-α***) and ***β-interferons*** (***IFN-β***), as well as several minor interferon groups (e.g., omega-interferon).

α-Interferon is produced by leukocytes, while fibroblasts and probably other cell types synthesize ***β-interferon***.

Cells start to express alpha- or beta-interferons being infected by a virus. Interferons arise in the extracellular fluid and bind to the specific receptors on the membranes of uninfected neighboring cells. The bound interferon renders its antiviral effect. Several cellular genes are found to derepress under α- and β-interferon activities allowing the synthesis of novel enzymes. One of them, a *protein kinase*, catalyzes the

phosphorylation of *initiation factor* and ribosomal proteins necessary for protein synthesis, thereby reducing greatly the viral mRNA translation. Another enzyme, *oligoadenylate synthase*, accelerates the formation of a short polymer of adenylic acid, which activates a latent endonuclease (RNAse L) that in turn degrades both viral and host mRNA.

Interferon of type II is known as ***γ-interferon*** (***IFN-γ***). It differs strongly from two above-mentioned interferons, exhibiting the traits of typical interleukin. Gamma-interferon is produced by T helper cells of 1 type; it stimulates different cell populations especially macrophages, NK cells, supports conversion of "naive" Th0-cells into Th1 type, thus maintaining ***cell-mediated inflammation***. Aso it enhances the expression of HLA antigens on the cell surface.

Interferons of type III include three ***lambda interferons*** (***IFN-λ***) as well as some other regulatory molecules. They demonstrate evident antiviral activity.

The interferons are proven to have a broad scope of actions beyond the control of viral infection. It is clear, for example, that interferon-induced enzymes may inhibit host cell division together with the blockade of viral replication. The interferons may also modulate the activity of other immune cells, e.g. natural killer cells.

Tumor Necrosis Factors

Tumor necrosis factor-alpha (or ***TNF-α***) and ***lymphotoxin*** (previously known as ***TNF-β***) are the distinct cytokines with non-equal activities.

TNF-α is produced by macrophages; it activates differentiation of Th0 cells into Th1 helpers (***cell-mediated immunity***). TNF-α is strong ***pro-inflammatory cytokine***, induced by different internal and external stimuli (e.g., by LPS of gram-negative bacteria). In addition, it develops cytotoxic activity, promotes apoptosis, tumor cells destruction.

Lymphotoxin is synthesized by T cells, demonstrating inflammatory, antiviral, and immunostimulatory activities. It stimulates apoptosis of tumor or virus-infected cells.

Growth Factor Cytokines

The group of ***growth factor cytokines*** among the other molecules comprises highly potent cytokine ***transforming growth factor-beta*** (***TGF-β***) that is produced by ***Treg*** or ***regulatory T cells*** and macrophages.

It is the major ***inhibitory cytokine*** suppressing proliferation of T and B cells, Th17 differentiation, and macrophage activation (***anti-inflammatory cytokine***).

CD Molecules

Interleukins interact with their specific receptors located within the cell membranes. Overall, the process of differentiation and proliferation of immune cells is followed by the change of specific composition of their membrane molecules.

All of these receptor molecules are ***immune phenotype markers*** of particular cell line, which appear at the certain stage of cell development.

These molecules were named as ***CD molecules*** or ***CD antigens*** (abbreviates from *clusters of differentiation* or *clusters of designation*). More than 370 of CD members are known to date.

The most important molecules with established functions are described below.

– **CD1** – expressed by cortical thymocytes, some dendritic cells; it takes part in lipid antigen recognition and presentation;

– **CD2** – common T cell marker, adhesion molecule, also expressed by NK cells, receptor for sheep red blood cells; provides T cells rosette formation;

– **CD3** – marker of all maturated T cells, supportive molecule for antigen-specific T cell-receptor (TCR), participates in signal transmission via T cell receptor;

– **CD4** – T helper marker, human immunodeficiency virus (HIV) cell receptor, takes part in recognition of the antigen in complex with HLA II-class molecules by T helper cells;

– **CD5** – marker of particular B cell subpopulations, albeit T cells also express this antigen;

– **CD8** – marker of cytotoxic T cells, participates in recognition of the antigen in complex with HLA I-class molecules by cytotoxic T cells;

– **CD14 –** expressed by monocyte-macrophage and granulocyte lineages, receptor for complex “bacterial lipopolysaccharide–

lipopolysaccharide-binding protein"; activation of the cells via CD14 leads to massive pro-inflammatory cytokine release;

– **CD16** – the antigen of neutrophils and NK cells; the low-affinity receptor to IgG antibodies (***Fcγ RIII***);

– **CD11/18** – leukocyte ***integrin***, presented on lymphoid and myeloid cells;

– **CD19-CD22** – B cell markers;

– **CD25** – IL-2 receptor chain on lymphocyte membranes;

– **CD28** – co-stimulatory molecule for successful activation of T cells; binds to its counterparts ***CD80*** and ***CD86*** upon the membranes of antigen-presenting cells;

– **CD32** (***Fcγ RII***) – born by monocytes and neutrophils; it is the medium-affinity receptor to IgG;

– **CD34** – early marker of hematopoietic stem cells;

– **CD35** – cellular receptor for C3b component of complement (CR1); it is expressed on many cell types (e.g., granulocytes and macrophages); it stimulates opsonization;

– **CD40** – marker of maturated B cells; interacts with T cells via binding to ***CD40L*** (***CD40 ligand*** or ***CD154***) in B cell antigen presentation and cell activation;

– **CD45** – leukocyte common antigen; its molecular variation **CD45RO** – marker of T memory cells; molecule **CD45RA** – marker of naive T cells;

– **CD54** – adhesin ***ICAM-1*** – *intercellular adhesion molecule 1*; it is the ligand for integrin CD11/CD18, being expressed on lymphoid cells, monocytes and other cell types;

– **CD56** – marker of natural killer (NK) cells; also present on some other cell types, including T cells;

– **CD62 –** ***selectin*** molecule family**,** ***CD62P*** – platelet selectin, ***CD62E*** – endothelial, ***CD62L*** – leukocyte selectin; selectins pertain to the group of specialized adhesion molecules;

– **CD64** – high-affinity receptor to IgG antibodies (***FcγRIII***), expressed on monocytes, neutrophils, etc.

– **CD95** (***Fas/Apo-receptor***) **–** expressed by thymocytes, T- and B cell subsets, interacts with ***Fas-ligand*** or ***CD178***, thereby activating programmed cell death (***apoptosis***);

– **CD152** – present on activated T lymphocytes; inhibitory co-stimulatory molecule of T cells that binds to ***CD80*** or ***CD86*** and dampens activated T cells;

– **CD158** – expressed on natural killer cells, killer inhibitor receptor (*KIR*); suppresses NK cells activation on interaction with class I HLA molecules;

– **CD159** – present on natural killer cells, promotes their activation on interaction with class I HLA molecules

Lymphoid System

Immunity is manifested on molecular, cellular, and body levels. Body's immune system is a total sum of lymphoid organs composed of ***central*** (***thymus*** and ***bone marrow***) and ***peripheral organs*** – *lymph nodes*, *spleen*, *lymphocytes* of peripheral blood, *mucosal-associated lymphoid tissue* (***MALT***) that comprises *gut-associated lymphoid tissue* (***GALT***) with *appendix*, *Peyer's patches* and solitary lymphoid follicles; *bronchial-associated lymphoid tissue* (***BALT***) and *nasal-associated lymphoid tissue* (***NALT***) of respiratory tract, *conjunctival-associated lymphoid tissue* or ***CALT***, etc.

All of blood cells are derived from hematopoietic stem cells. Fetal hematopoiesis is performed in fetal liver and bone marrow, in adults – in bone marrow only. Under the influence of different cytokines and growth factors all of blood cell precursors arise. They further become differentiated into mature cell populations.

There are 3 main populations (subsets) of lymphocytes – ***T cells***, ***B cells*** and ***NK cells*** (or *natural killer cells*). They originate from ***hematopoietic stem cells*** through the stage of ***common lymphoid progenitor cells***. Stem cells growth factors, IL-3 and IL-7 are the major cytokines involved in their maturation.

T- and ***B cells*** undergo ***antigen-independent differentiation*** and ***maturation*** in central lymphoid organs followed by ***antigen-dependent differentiation*** after their migrations into specific zones of peripheral lymphoid tissues.

T-Lymphocytes. T Cell Development and Differentiation

T cell antigen-binding membrane receptor or ***TCR*** is the structural and functional hallmark of the total T cell lineage. The level of maturation and membrane expression of TCR corresponds to the developmental stage of T lymphocytes.

There are two basic subsets of T cells bearing distinct TCR molecules. The majority of human T cells expresses membrane T cell receptor composed of alpha and beta chains (***αβ T cells***). The rest of T cells carries membrane TCR of gamma and delta chains (***γδ T cells***).

The representatives of ***αβ T cells*** settle in all lymphoid tissues and organs, whereas ***γδ T cells*** are present within mucosa of gastrointestinal tract or respiratory tract. Hence, ***γδ T cells*** play the primary protective role within superficial barrier tissues, thus preventing pathogen invasion.

Early stages of T cell development are followed by the migration of T cell precursors initially from the fetal liver and later after birth from the bone marrow toward the ***thymus***, where the maturation of T cells occurs. It should be noted that αβ T cells predominantly arise from the cells migrated from the bone marrow, whilst the most of γδ T cells originate from T cell precursors of the fetal liver.

Thymus is the lymphatic epithelial gland active in the fetal period and in childhood before puberty. Later it undergoes involution, but a part of lymphoepithelial tissue remains active for a long time.

Thymus is the place of ***antigen-independent differentiation*** of primary T cells generally termed as ***thymocytes***. When maturating, they gradually move from cortical to the medullar zones of thymus. Thymic hormones actively influence on the T cell maturation.

Membrane expression of TCR and certain number of membrane CD molecules reflect the steps of T cell development.

The earliest ***pro-T cells*** express recombinase Rag proteins that stimulate rearrangement of T cell receptor genes. Transition of these cells into ***pre-T cells*** is followed by expression of one chain (β or γ) of TCR and membrane CD1, CD2 and CD7 molecules. All these thymocytes initially demonstrate CD4 and CD8 negative phenotype (known as "***double-negative***" T cells).

Further differentiation of T cells leads to the membrane expression of complete two-chain TCR molecule with the addition of CD3. These cells also acquire membrane CD4 and CD8 molecules ("***double-positive***") T cells.

At this stage the whole number of thymocytes bearing membrane TCR undergo the process of *positive* and *negative selection*. It occurs by the specific contact of T cells with thymic epithelial cells. In this case thymic epitheliocytes present the host self antigens for maturating T cells in complex with the host HLA molecules of I or II class.

At first T cells bearing TCRs that ***weakly*** *react with the complex self Ag-self HLA molecule* on the membranes of thymic epithelial cells are

selected (***positive selection***). Other non-reacting thymocytes are eliminated by apoptosis. This ensures the recognition of foreign antigen by specific TCR only in the complex with the host HLA molecule (***double recognition***, ***HLA restriction***).

On the other hand, the remaining thymocytes that bear TCR with the *highest affinity* (binding capacity) to the self Ags are also eliminated by apoptosis (***negative selection***). The deletion of the most powerful autoreactive T cell clones creates the unresponsiveness to the body self antigens (***central tolerance***) thus preventing the emergence of autoimmune disorders.

When the T cell recognizes the antigen in complex with I class HLA, this requires additional binding to co-stimulatory CD8 molecule. And conversely, the recognition of an antigen in complex with II class HLA requires binding to the membrane co-stimulatory CD4 molecule.

The latter events lead to the transformation of initial double-positive T cells into ***single-positive*** "*naive*" (***CD4+***) ***helper*** or (***CD8+***) ***cytotoxic T cells***.

Single-positive T cells migrate toward the peripheral T-dependent zones of lymphoid tissues. When the contact with foreign antigen occurs, T lymphocytes initiate ***antigen-dependent differentiation***. This leads to the T cell conversion into their final subsets with specialized effector functions. The process of foreign antigen binding stimulates the selection and blast transformation of Ag-specific T cells that results in proliferation of Ag-specific T cell clones (***clonal expansion***) and formation of long-living memory T cells.

The most common T cell markers are TCR, CD2, CD3, and CD7 as well as CD4 for helper cells and CD8 for cytotoxic cells.

Normal quantity of common T cells is about of 60% (50-75%) from the whole blood lymphocytes population.

Subpopulations (Subsets) of T Lymphocytes

T helpers recognize the processed ***antigen*** *in complex with* ***HLA-II class*** molecules by their membrane TCR. As the result, ***T helpers*** stimulate proliferation and differentiation of T- and B cells.

There are 2 major T helper subsets distinguished by their functions – ***T helper 1*** and ***T helper 2*** (***Th1*** and ***Th2***). They produce the specific combinations of cytokines largely opposite in their activities.

Co-stimulatory ***CD4*** molecule of T helpers interacts with HLA-II class molecules in presentation of antigen to T cells.

Th1 secrete ***IL-2*** and ***γ-interferon***, thus stimulating ***cell-mediated immunity***. Th1 trigger the reactions, defending the host against the broad number of agents, including intracellular pathogens. They activate macrophages, dendritic cells, and cytotoxic lymphocytes thereby promoting ***inflammation***.

Th2 enhance the activity of B lymphocytes stimulating their transformation into plasma cells and the ***synthesis of antibodies***. They activate immunoglobulin isotype switching resulting in production of antibodies of all Ig classes including IgE.

Th2 produce ***IL-4***, IL-5, IL-6, IL-10, IL-13, IL-15 and therefore maintain ***humoral immune response***.

A special population of T helper cells ***Th17*** is activated by IL-21, IL-23, and TGF-β.

By production of ***IL-17***, IL-21, IL-22 they stimulate the extremely large number of immune and non-immune cells (T cells, neutrophils, macrophages, NK cells, B cells, epithelial and endothelial cells), resulting in progressive chronic infllammation, enhancement of phagocytosis and antibody synthesis, maturation of myeloid cells, autoimmune response.

Another T helper subset of ***follicular T helper cells*** $\boldsymbol{T_{FH}}$ arises from Th0 by the contact with antigen-presenting B cells in the follicles of lymph nodes. When activated, $\boldsymbol{T_{FH}}$ stimulate the transformation of follicular B lymphocytes into *long-living antibody-secreting plasma cells* and memory B cells.

Regulatory T cells or ***Treg*** are differentiated as natural immune ***suppressor*** cells. Molecular markers of this cell type are CD4 and CD25 that are co-expressed together upon cell membranes.

Also natural regulatory T cells contain the active form of specific transcriptional factor ***Foxp3***.

After the recognition of Ags presented by dendritic cells, ***Treg*** express co-stimulatory ***inhibitory molecule CD-152*** resulting in suppression of activity of antigen-presenting cells. In addition, they produce large amounts of inhibitory cytokines ***TGF-beta*** and ***IL-10*** that restrain the proliferation of various subsets of immune cells.

Overall, T helpers comprise up to 40-50% of lymphocytes.

T cytotoxic cells (or ***Tcs***) pertain to the T cell subset bearing ***CD8*** marker. They recognize the ***antigen*** *in complex with* ***HLA-I class*** molecules presented on the membranes of infected cells or cancer cells. Therefore, cytotoxic T cells eliminate ***intracellular pathogens*** (viruses or

bacteria) and participate in host immune surveillance by killing of malignant cells.

Co-stimulatory ***CD8*** molecule binds to HLA-I class antigens supporting antigen recognition by cytotoxic cells.

Activated (CD8+) T cells (***T killers***) bind to the antigens on the membranes of affected host cells and activate ***apoptosis*** or programmed death of target cells. To aim this, Tcs produce cytotoxic proteins ***perforin***, ***granzymes***, and ***granulysin***. Perforin acts as pore-forming toxin allowing granzymes to enter the cell and stimulate apoptosis via activation of caspases.

In addition, Tcs stimulate apoptosis by elevated expression of FasL that binds to apoptosis receptor CD95 Fas/Apo upon the membranes of target cells.

Memory T cells are long-living subpopulations of CD4+ and CD8+ cells arisen from activated T cells bearing antigen-specific TCR. Their lifespan lasts for more than 20 years. In case of next antigenic challenge they serve as progenitors for the burst emergence of antigen-specific T cell clones.

T Cell Receptor

T cell receptors (TCRs) belong to the superfamily of immunoglobulin molecules with basic domain structure.

TCR is expressed on T cell membranes. It is the heterodimeric molecule composed of ***alpha***- and ***beta-chains*** (with molecular weight about 40-50 kDa each) or more rarely of γ/δ-chains. T cells bearing γδ TCR version (about 1-5% of total lymphocyte count) take part in local immune reactions within the mucous tissues.

TCR is bound tightly to CD3 molecular complex on T cell membrane.

Every chain of TCR is composed of ***constant*** and ***variable*** globular ***domains***. Variable domain contains antigen-binding site of TCR. It recognizes the processed antigen only in the complex with HLA-I or II class molecules ("*double recognition*" or "*HLA restriction*" phenomenon).

There is a large quantity of V, D, and J gene segments (totally more than 150) that code for the variable portions of TCR chains. Each individual sequence of TCR binding site results from the *random recombination* (***rearrangement***) of certain genetic V, D, and J segments. This process generates the prominent variability of T cell specifities.

B Lymphocytes: the Development and Functions

B lymphocytes and antibody-secreting ***plasma cells*** are the basic cells of ***humoral immunity***.

Normal quantity of B cells in peripheral blood is about 25% (in the range of 18-30%) from total blood lymphocyte population.

B lymphocytes are the progenies of hematopoietic stem cells. Their early differentiation occurs in fetal liver and later in bone marrow, in adults – in bone marrow only.

B cells were designated according to the name of central lymphoid organ of humoral immunity in birds (*Fabricius' pouch* or in Latin "***b****ursa Fabricii*"). Central lymphoid organ in mammals for B lymphocytes production is bone marrow. It is generally ascertained that the nearest analogue of Fabricius' pouch in mammals are Peyer's patches in the intestine.

B lymphocytes begin to differentiate from *hematopoietic stem cell* through the stage of *common lymphoid progenitor cell*.

B cell precursors arise under the influence of growth cytokines (IL-3, IL-7, IL-4, IL-6 and some others).

As the final result of development, at least two distinct lineages of mature B cells are generated – *minor* ***B-1 subset*** and the majority of other B lymphocytes sometimes termed as ***B-2 subset***.

Most of ***B cells*** (e.g., B-2 subset) undergo ***antigen-independent differentiation*** in the bone marrow. It is next followed by their ***antigen-dependent differentiation*** after the migration of B cells into B-related zones of peripheral lymphoid tissues.

During pregnancy, B cells of fetus are generated primarily in fetal liver, after birth – in the bone marrow.

Early stage of B cell development or ***pro-B cell*** is characterized by activation of recombinase Rag proteins that stimulate rearrangement of immunoglobulin-encoding genes.

Subsequent transformation into ***pre-B cell*** is followed by membrane expression of IgM heavy chain (μ-chain) and membrane CD10 and CD19 molecules.

Next stage of development creates the ***immature B cells*** that carry complete IgM molecule on their surface (antibody *B cell receptor* or ***BCR***).

Immature B cells with BCR demonstrating *high binding capacity to the self antigens* undergo the process of ***receptor editing***. This is followed by reactivation of Rag recombinases of B cells resulting in the additional

rearrangement of BCR light chain. This leads to the decrease of BCR binding power against the self antigens.

In case of inefficient receptor editing, the clones of immature B cells retaining BCR with high affinity against the self structures are eliminated by *apoptosis* (***negative selection*** of autoreactive B cells).

The rest of B cells predominantly migrate to the spleen, where their transformation into *follicular* ***mature B cells*** occurs.

Mature B cells simultaneously express *two types of membrane BCR* of the same specificity – IgM and IgD receptor molecules. They extend their migration to other peripheral B-dependent zones of lymphoid system (within the spleen, lymph nodes, mucosal-associated lymphoid tissue of gastrointestinal and respiratory tracts, etc.)

In peripheral B-dependent zones mature B lymphocytes commence their ***antigen-dependent differentiation***. They lose surface IgD. After antigen binding to Ig receptor, B cells undergo ***blast transformation*** resulting in ***clonal expansion*** of antigen-specific B cell clones.

At the end of blast transformation B lymphocytes change into ***plasma cells***. The latter are secretory cells capable of producing antibodies of the same specificity but only of 1 from 5 available Ig classes (IgM, IgD, IgG, IgA, or IgE).

B cells require T cell help (***Th2***) for their appropriate differentiation. Th2 produce IL-4, IL-6 and other cytokines stimulating B cell development. ***Follicular helper cells*** drive the transformation of follicular B lymphocytes into *long-living antibody-secreting plasma cells* and memory B cells.

B cells express the number of differentiation markers like CD19, CD20, CD21, CD22, CD40, CD72, receptors to C3b-component of complement, etc.

Nevertheless, some antigens with high molecular weight and strong molecular charge (*T-independent antigens*) can directly stimulate B cells without T cell help. Among these antigens are bacterial polysaccharides and lipids, flagellin proteins, and other similar structures.

Most of B cells responding against T-independent antigens pertain to minor ***B-1 cell*** subpopulation, highly present in mucous tissues and body compartments.

B-1 cell subset arises earlier in ontogeny than conventional B lymphocytes. They originate from fetal liver-derived hematopoietic stem cells. In mucosal tissues they demonstrate self-renewal without support from the host bone marrow.

After transformation into plasma cells they produce *polyspecific* ***natural antibodies*** capable of binding to many microbial structures. Natural antibodies pertain mainly to ***IgM class*** and to less extent to certain IgG subclasses, e.g., IgG3. They create a first line of body defense against microbial pathogens. However, in some cases natural antibodies become responsible for autoimmune reactions as the result of their polyspecificity.

In addition, at least one half of *IgA-secreting cells* of gut mucosa is regarded as derived from B-1 cell subset.

Thus, B-1 subset is found to be the special B cell population supporting ***innate humoral immunity***.

The behavior, similar to B-1 cell subset, is characteristic also for so-called *marginal zone B cells*.

Natural Killer Cells (NK Cells)

It is a rather small cell population (5-15% of blood lymphocytes), containing the great number of azurophilic granules in cytoplasm. These large-size lymphocytes play a protective role of tremendous importance – ensure the lysis of cancer or virus-infected target cells regardless of their antigenic specifity (so-called ***non-immune cytolysis***). In addition, natural killers can destroy some bacteria and protozoans.

NK cells release a great variety of cytotoxic substances – ***perforin***, which resembles in action the membrane attack complex (MAC) of the complement, ***lymphotoxin*** (previously known as β-tumor necrosis factor), some special cytolytic enzymes, or ***granzymes*** that activate apoptosis. Also they induce cell death by direct contact activating apoptosis of target cells via CD95-Fas ligand interaction.

Membrane markers of NK cells are ***CD16*** and ***CD56***.

Infected cells become sensitive to natural killers owing to the impairment of HLA I-class expression on their surface. In normal conditions NK cells bear ***inhibitory receptors*** (like CD158), which through interaction with HLA I-class antigens of the host cells permanently suppress NK activation. After viral infection of the cell or its tumor transformation membrane HLA I-Ag expression alters, and HLA antigen conformation appears to be distorted. This provokes natural killer activation with subsequent lysis of infected cell.

In addition, NK cells were proven to have another type of surface molecules that directly initiate their killing activity – ***killer activation receptors***.

Natural killers fill the breach in the full-value immune defense: while T-cytotoxic lymphocytes attack infected cells after the specific recognition of their antigenic peptides in complex with HLA I-class antigens, NK cells destroy the target cells, devoid of their own “self” markers.

NKT Cells

Minor subpopulation of lymphoid cells bears membrane markers characteristic for T cells and natural killer (NK) cells. Hence, this lymphocyte subset is termed NKT cells. They express antigen-specific αβ T cell receptors together with CD56 marker on their membranes.

The main function of NKT lymphocytes is to recognize and bind to lipid endogenous and exogenous antigens (e.g., lipoproteins of microbial cells). These antigens are presented to NKT cells in complex with CD1 antigen. It has been found that CD 1 molecule has very similar structure with HLA I class antigens being capable of lipid binding.

Thus, NKT cells play substantial role in the reactions of innate immunity promoting elimination of microbial cells via the lipid components of their envelope (e.g., immunity against *M. tuberculosis*).

Chapter 12
BASIC MECHANISMS OF INNATE IMMUNE RESPONSE. PATHOGEN-BINDING RECEPTORS. PHAGOCYTES AND PHAGOCYTOSIS

Pathogen-binding Receptors of the Cells of Innate Immunity

It has become clear now that the innate immune response against the all groups of infectious agents (bacteria, fungi, viruses or protozoans) is largely dependent on primary interaction of the innate immune cells with highly conservative structural motifs of microbial cells or viruses termed as ***pathogen-associated molecular patterns*** (***PAMP***).

The most commion structures of PAMPs have been formed during the process of long microbial evolution. They appeared to be quite similar among the distinct microbial groups. This type of interaction between the innate immune cells and pathogenic agents was designated as ***pattern-based recognition***. It is essential for the innate immune response against the great variety of microbials.

In turn, the immune system recognizes microbial pathogenic patterns by several groups of specialized receptors. These receptors are phylogenetically ancient; their structure is similar in various animal species that stay on different steps of the evolutionary ladder. They were generally entitled as ***pattern-recognition receptors*** or ***PRRs***.

The first representatives of PRR family were discovered in drosophila fruit flies. They have got the names of *Toll receptors*. In drosophila flies Toll receptors are responsible for tissue diferentiation and organ morphogenesis. Besides, they were shown to take part in defensive reactions against some infectious pathogens (e.g., fungi).

In some time it was firmly established that similar receptors are expressed on the cells of highest animals including humans and other mammal species. According to the above mentioned case they were termed as ***Toll-like receptors*** or ***TLRs*** for short.

In addition, the members of other families of pattern-recognizing receptors were recently discovered in humans as well.

Toll-like Receptors in Humans and Their Functions

In humans more than 10 representatives of TLR family were described to date. They are expressed on membranes of many cells of innate immunity. The most significant role they play for *antigen-presenting cells* – dendritic cells, Langerhans cells, macrophages and others.

TLR-1 binds to ***lipopeptides*** of various bacterial groups.

TLR-2 recognizes the large number of pathogenic microbial patterns – ***lipoteichoic acids*** of the most of ***gram-positive bacteria***, borrelial and treponemal lipoproteins, lipoproteins of micobacteria; cell wall structures of neisseriae, listeriae, and fungi.

TLR-3 binds to ***double-stranded RNA***, thus promoting ***antiviral immunity***.

TLR-4 interacts with ***lipopolysaccharides*** (***LPS***) of ***gram-negative bacteria*** (e.g., enterobacterial representatives) and with heat-shock proteins.

TLR-5 reacts with bacterial ***flagellin*** (*microbial H-antigen*).

TLR-6 also binds to ***lipopeptides*** (e.g., in micoplasmas).

TLR-7 interacts with ***single-stranded RNA*** of ***viruses***.

TLR-8 binds to ***single-stranded RNA*** of ***viruses*** and ***bacterial RNA***.

TLR-9 recognizes ***bacterial*** and ***viral DNA***.

TLR-13 reacts with the sequence of ***bacterial ribosomal RNA***.

Besides TLR receptor family, which members commonly recognize pathogenic structures from the outside, other families of pattern-recognition receptors (e.g., *NOD* and *RLR*) are capable of binding microbial pathogens intracellularly after their invasion and degradation.

The functions of receptors of TLR family are extremely meaningful in the development of ***innate natural immune response***.

The basic role of TLRs is the activation of the cells of innate immunity after their primaty binding to the antigens.

Notably, antigen binding by TLRs on the membranes of ***dendritic*** antigen-presenting cells (***APC***) results in sharp rise of expression of ***co-stimulatory molecules*** by dendritic cells. This ensures further activation of antigen-specific T cells and their next proliferation. On the contrary, T cells, devoid of co-stimulation, become unresponsive to the certain antigen, falling into *anergy* state.

Moreover, primary binding of microbial pathogens to various types of TLR leads to the re-direction of the antimicrobial immunity towards ***cell-mediated*** or, alternatively, ***humoral*** immune response. Such a change of the immune response depends on the different cytokine profile that is secreted by dendritic cells after alternative TLR activation.

For instance, stimulation of ***APC*** (e.g, dendritic cells) ***via TLR-4*** elicits the production of pro-inflammatory cytokines (IL-1, IL-12, TNF-alpha and interferons) resulting in Th0 transition into Th1 with activation of ***cellular immunity***.

And conversely, stimulation of ***APC via TLR-2*** largely results in transformation of Th0 into Th2 followed by synthesis of IL-4 and IL-10 cytokines, suppression of inflammatory response and activation of ***humoral immunity*** with antibody secretion by plasma cells.

Besides the recognition of microbial structures, pattern-recognition receptors from TLR and other familes successfuly react with the host products of cell destruction and non-microbial inflammation. They were generally termed as ***damage-associated molecular patterns*** (***DAMPs***) or ***alarmins***. Their release is associated with the cell injury. The great number of intracelllular molecules as well as the components of extracellular matrix can play a role of alarmins (host nucleic acids and nucleotides, hyaluronan and proteoglycan fragments, defensins from leukocytes, and many others).

The signals from alarmins can also stimulate the cells of innate immunity resulting in inflammation.

Sometimes pathogen-associated and damage-associated molecular patterns are combined together as ***danger****-associated molecular patterns*, which signals stress the immune system activating the innate immune response.

Humoral Factors of Non-Specific (Innate) Immune Response

The system of ***humoral factors of innate immunity*** comprises the enzymes, soluble proteins and peptides that promote non-specific immune reactions in biological fluids of the host (in plasma, saliva and other mucous secretions, breasr milk, tears, etc.)

Strong antimicrobial effects are demonstrated by the numerous group of so-called ***acute phase proteins*** present in plasma. Their concentrations increase following inflammation.

Among them are ***C-reactive protein***, ***mannose-binding lectin***, ***alpha-2-macroglobulin***, ceruloplasmin, ferritin, fibrinogen, serum amyloid components, and some others.

Their common mechanisms of activity include *tight non-specific binding* to microbial cells with bacterial arrest in the site of inflammation, prevention of microbial adhesion, activation of phagocytosis (*opsonization*), *activation of complement system* via lectin and alternative pathways, *iron deprivation* of bacterial cells, inhibition of microbial enzymes and toxins.

C-reactive protein and serum amyloid components are the members of ***pentraxin*** protein family regarded as the special group of *pattern-recognition receptors*.

Similar antimicrobial activities are characteristic for ***fibronectin*** plasma protein.

Lactoferrin is the iron-binding glycoprotein of biological fluids. Its antimicrobial action is related with high *iron-binding capacity*. Iron-deprived bacteria stop their growth and reproduction.

Cationic enzyme ***lysozyme*** (or ***muramidase***) destroys glycosidic bonds between N-acetyl-glucosamine and N-acetyl-muramic acid within bacterial cell wall *peptidoglycan* (or murein). As the result, it causes the lysis of bacterial cells.

In addition, a great number of ***antimicrobial peptides*** of low molecular weight releases from the host cells into various biological fluids. Among them are ***defensins*** and ***cathelicidins*** from leukocytes, ***beta-lysins*** of platelets, ***histatins*** and ***cystatins*** from saliva and some others.

Mononuclear Phagocyte System

One of the most ancient form of the innate immunity is ***phagocytosis***. It is a defensive reaction entailing the capture and digestion of foreign particles (e.g. bacteria or remnants of disintegrated cells) by ***phagocytes***.

I.I. Metchnikoff first discovered in 1882 that amoeboid cells of the mesoderm in starfishes were able to engulf and digest various foreign particles. I.I. Metchnikoff subdivided the cells capable of phagocytosis into ***microphages*** and ***macrophages***.

Microphages include ***granular leukocytes***: ***neutrophils***, ***eosinophils*** and ***basophils***. Only neutrophils possess a marked ability for phagocytosis. Eosinophils and basophils are characterized by relatively weak phagocytic activity.

Macrophages comprise the large set of specialized cells. They are consolidated into ***mononuclear phagocyte system***. It includes mobile cells (blood monocytes, phagocytes of the lymph nodes and spleen, connective tissue hystiocytes, etc.) or non-mobile elements (the resident macrophages of the lymphatic tissue and spleen, endotheliocytes of the blood vessels, liver resident macrophages or Kupffer stellate cells, mesangial macrophages, alveolar macrophages, glial macrophages, osteoclasts, etc.)

Myeloid monocyte precursors differentiate in bone marrow into promonocytes and then into mature monocytes. The latter come into blood flow. This circulating cell population migrates throughout the capillary wall into tissues and organs and transforms into resident macrophages

Molecular differentiation markers of macrophages are CD14, CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), receptors to C3b-component of complement (CD35) and cytokine receptors (to IL-2, IL-4, γ-IFN, etc.)

Macrophages possess 3 main biological functions: ***phagocytosis***, ***antigen presentation***, and ***cytokine secretion***.

Phagocytosis

The process of phagocytosis consists of 5 main stages.

The *first phase* is ***chemotaxis stage***. Many microbial agents produce chemotactic agents that attract phagocytic cells. Chemotaxis deficiency may account for enhanced susceptibility to certain infections; these defects may be acquired or inherited.

This stage involves phagocyte approaching to the microbe by means of a positive chemotaxis. Under the influence of microbial products activation of phagocytes occurs. It leads to a change in the cellular actin contractility, thus conferring amoeboid motility to phagocytes.

There is a tremendous number of different chemoattractive agents known as ***chemokines*** (more than 100 substances). One of most important chemokines is the above-mentioned ***IL-8***, ***C5a***-complement component and others, which attract macrophages and neutrophils toward the focus of inflammation. Also some bacteria produce chemical substances such as LPS, which can attract leukocytes.

At the *second stage* adsorption of the microorganisms to the surface of the phagocyte takes place (***adhesion stage***). This event is mediated by several recognition mechanisms. Tight binding is maintained by specific interaction of phagocyte receptors (e.g., from ***Toll-like receptor*** family) with ***pathogen-associated molecular patterns***.

Target recognition often involves carbohydrate elements and ***lectins*** both of phagocyte and bacterial origin.

The above mentioned variant of microbial inactivation is known as ***non-immune phagocytosis***. It may develop in the absence of serum antibodies or complement. Such "surface phagocytosis" occurs early in the infectious process before antibodies would become available.

Immune phagocytosis evolves with the help of immune recognition.

Phagocytosis is much more efficient in presence of specific antibodies or complement components like C3b (so-called ***opsonins***) that cover the surface of bacteria, thus facilitating their ingestion by phagocytes. ***Opsonization*** can occur by three basic mechanisms: by antibodies alone, by immune complex or by complement fragments (mainly via C3b and C5a components). Macrophages have membrane receptors to Fc portion of antibody and to C3b and C5a components of complement system. These receptors stimulate the phagocytosis of antibody-coated particles.

Third stage of phagocytosis is named ***ingestion (or engulfment) stage***. The attached bacteria activate the ingestion phase by stimulating intracellular actin contraction with formation of pseudopodia enwrapping the object of phagocytosis. Bacteria become completely encased within a ***vacuole*** (*endosome* or ***phagosome***). In 1 minute the lysosomal granules fuse with the phagosome resulting in ***phagolysosome*** formation, and the lysosomal contents expel nearby the engulfed microorganism. This is followed by the activation of a great number of microbicidal mechanisms.

In the *fourth phase* intracellular digestion of the engulfed microbes is activated (***digestion stage***).

Ingestion of foreign particles (e.g., microorganisms) triggers several effects of phagocytic cells.

There is a tremendous increase in activity of the hexose monophosphate shunt that generates NADPH. Primary key reaction in phagocytosis is catalyzed by phagocyte ***NADPH oxidase***, which initiates the formation of multiple ***reactive oxygen species*** (***ROS***). This process is known as ***respiratory burst*** – the major microbicidal mechanism in phagocytes.

The main ROS agents are ***superoxide anion*** (O_2^-), ***hydroxyl radicals*** (•OH) and ***singlet oxygen***.

Superoxide anion undergoes conversion into hydrogen peroxide under the influence of superoxide dismutase, and subsequently to ***hydroxyl radicals*** (•OH). All of these products have the outstanding chemical reactivity making them powerful microbicidal agents. Hydroxyl radical is one of the most reactive chemicals known. Furthermore, the combination

of ***peroxide*** and ***halide ions*** forms a potent halogenating system capable of killing both bacteria and viruses. Hydrogen peroxide and the halogenated compounds are more stable than free radicals. They diffuse further and subvert the microbials outside the cell.

Reactive nitrogen species (nitric oxide ***NO*** and ***peroxynitrite***) act through the inactivation of certain microbial electron transport enzymes and also by production of (•OH) radicals. Activation of reactive nitrogen species is termed as ***nitrosative burst*** or ***nitrosative stress***. It plays significant role in elimination of certain pathogens, e.g., *Mycobacterium tuberculosis*.

Also the family of cationic proteins and peptides, known as ***defensins***, attacks the bacteria inside the phagolysosome. They are about of 3.5-4 kDa of molecular weight, being rich in arginine. These antibiotic peptides act as biocides against a broad spectrum of gram-positive and gram-negative bacteria, fungi and a number of enveloped viruses.

Further damage of bacterial structures is caused by neutral proteinases (such as ***cathepsin G***) and by other endosomal hydrolytic enzymes like hyaluronidase or nucleases. ***Lysozyme*** and ***lactoferrin*** are also potent microbicidal factors that are oxygen independent and can function under anaerobic conditions.

Low pH within phagosome facilitates microbial degradation.

Finally, the killed microbial bodies are digested by hydrolytic enzymes, and the phagocytized microbes become completely disintegrated (***complete phagocytosis***).

Besides complete phagocytosis, ***incomplete phagocytosis*** occurs in certain bacterial infections (gonorrhoea, legionellosis, leishmaniasis, tuberculosis, leprosy, etc.) In those cases microorganisms are engulfed by phagocytes, but don't lose their viability, and even may reproduce. The mechanisms of microbial survival within phagocytes are supported by capsule production (like *Klebsiella pneumonia*), or by the block of phagosome-lysosomal fusion (e.g., *Legionella pneumophila*) or by chemically resistant structure of the microbial body (e.g., the presence of highly stable lipids and waxes in *M. tuberculosis*).

The last *fifth phase* of phagocytosis is ***the release of degradation products***, where the non-digested microbial remnants are discharged outside from the cell.

Antigen Presentation

Macrophages demonstrate moderate activity in antigen processing and presentation for antigen-specific T cells.

Under phagocytosis and antigen digestion a great amount of low-weight antigenic peptides is formed. They are processed inside the ***endosome*** (exo-antigens) or within special supramolecular cytoplasmic protease complex known as ***proteasome*** (endo-antigens). During ***processing*** antigenic peptides are coupled with the *major hystocompatibility complex* molecules (MHC, in humans – ***HLA***).

The complex "Ag peptide-HLA molecule" is next transferred and expressed upon the cell membrane (***antigen presentation*)**.

If viral or modified endo-antigen has been processed, its fragments of 8-12 amino acids residues are coupled predominantly with HLA I class molecules (HLA-A, HLA-B, HLA-C) to be presented to T-cytotoxic lymphocytes.

Exo-antigens (peptides of 12-25 amino acids residues) are coupled mainly with HLA II class molecules (HLA-DR, HLA-DP, HLA-DQ) and presented to T helper cells.

These interactions provide maximal stimulation for cell-mediated immunity.

However, macrophages are not the most powerful ***antigen-presenting cells*** (***APC***). ***Dendritic cells*** of different origin perform this function with greatest effectiveness. They are localized in skin, mucosal tissues, in thymus, various zones of lymph nodes.

Most active ***Langerhans cells*** transport the antigen from skin to T cells in lymph nodes, where presentation occurs. Membrane of dendritic cells is highly enriched with MHC II class molecules. Similar ***follicular dendritic cells*** present the native antigens to B lymphocytes of B-dependent zones in peripheral lymphoid organs.

Also B-lymphocytes themselves can serve as efficient APCs. They present various antigens to T helper cells mostly of Th2 type.

Cytokine Secretion by Macrophages

Macrophages can be activated by various stimuli, including microbes and their products, immune complexes, cytokines, injured tissue components (alarmins), sensitized T cells, etc. Activated macrophages possess an increased number of lysosomes and produce a broad set of

cytokines. The main macrophage cytokines (*monokines*) are IL-1, IL-6, IL-8, IL-12, IL-18, α-TNF, prostaglandins, leukotriens, and some others.

Pro-inflammatory cytokines (presumably IL-1, IL-6, IL-12, IL-18, α-TNF) have a wide range of biological activity. They cause febrile reactions with fever and chills. Also they participate in activation of lymphoid cells (predominantly of Th1), resulting in the release of other pro-inflammatory cytokines (IL-2, γ-interferon, etc.) with progressive tissue inflammation. Redundant cytokine production especially after bacterial endotoxin exposure can lead to septic shock with fever, collapse, and inner organ failure.

The macrophages, stimulating inflammatory reactions, are termed as *classically activated* macrophages. They were designated as ***M1 macrophage*** subpopulation. Together with potent microbicidal activity, in some situations they may trigger autoimmune inflammatory response (e.g., in rheumathoid arthritis or multiple sclerosis).

Alternatively activated macrophages (or ***M2 macrophages***) are stimulated by anti-inflammatory cytokines IL-4, IL-10, and TGF-β, as well as by products of fungi and helminth infections. Therefore, they take part in anti-parasite immunity. Also they are engaged in allergic reactions.

M2 cells produce low amounts of IL-12, but high levels of IL-10, thus maintaining tissue anti-inflammatory state.

Granulocytes: Neutrophils, Eosinophils and Basophils

It was previously mentioned that microphages comprise ***granular leukocytes***: ***neutrophils***, ***eosinophils*** and ***basophils***. These cells originate from hematopoietic stem cells under the signaling of different cytokines and ***colony stimulating factors*** (granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, etc).

Neutrophils make 47-72% of total leukocyte count, basophils – 0-1%, eosinophils – 1-5%.

Neutrophil Leukocytes

Neutrophils are the most active phagocytic cells maintaining the reactions of innate immunity.

They are short-living cells – average life span of neutrophils is near 7 h.

Neutrophils harbor the granules of various types in their cytoplasm, e.g. ***primary*** azurophilic, ***secondary*** "specific" and ***tertiary***.

Primary granules contain the vast number of enzymes. A pivotal enzyme of primary granules is ***myeloperoxidase*** – the specific enzyme of neutrophils. Also they accumulate ***neutrophil elastase***, catepsins, neutral and acid proteases, β-glucuronidase, and other enzymes as well as microbicidal proteins ***defensins***.

Secondary granules contain lactoferrin, lysozyme, collagenase (*gelatinase*), metalloproteinases and the components of NADPH-oxidase – the enzyme generating respiratory burst.

Tertiary granules predominantly harbor gelatinase.

Neutrophils show instant response to different exo- and endogenous stimuli, moving directly to inflammation focus. Of the most efficient chemoattractants for neutrophils are ***IL-8*** and ***anaphylotoxins*** C3a and C5a.

The basic function of neutrophil cells is ***phagocytosis***. After microbial engulfment NADPH-oxidase triggers the respiratory burst pathway. It is is activated via granular enzyme release and *reactive oxygen species formation* (superoxide anion O_2^-, hydroxyl radicals (•OH), singlet oxygen and many others). These substances render strong bactericidal effects.

Neutrophil myeloperoxidase actively participate in microbial destruction generating highly reactive hypochlorite (HOCl) from hydrogen peroxide.

The process of phagocytosis is followed by intensive production of inflammatory cytokines.

After successful elimination of pathogens by phagocytosis activated netrophils usually undergo apoptosis in order to limit inflammatory reactions. On the contrary, in case of highly aggressive pathogens neutrophil cell can be destroyed resulting in necrosis.

In the situations, where the neutrophils can't cope with the objects of phagocytosis, they may trigger additional microbicidal mechanism known as the production of ***neutrophil extracellular traps*** (or ***NETs***). The process of NETs formation (*NETosis*) includes the controlled release of neutrophil *nuclear DNA* together with *bactericidal enzymes* and proteins from *granules* through the membrane of the leukocyte.

NETosis creates the spatial net-like structures full of microbicidal molecules that cover the large areas within the tissues. As the result, pathogenic agents become captured and confined within the NETs and undergo gradual degradation. Thus, NETs prevent microbial invasion and spread. Complete NETosis results in neutrophil death.

As the particular antimicrobial action, NETosis is especially efficient, if the neutrophil encounters the pathogens of larger sizes like fungal and protozoan cells, or parasites. Overall, in the reactions of innate immunity NETosis is regarded now as the process demonstrating the comparable efficacy with conventional phagocytosis.

Neutrophil cells bear membrane CD16 (FcγRIII), CD32 (FcγRII), and CD64 (FcγRI) markers; receptors to C3b, C5a and C1q complement components, great number of adhesion molecules from various protein families (selectins, integrins and many others).

Basophil Leukocytes

Basophils circulate in the blood carrying large granules that stain in purple-blue by Romanowsky-Giemsa method. ***Mast cells*** are the tissue counterparts of basophils. All of these cells are essential participants in *immediate allergic reactions*.

Basophil granules contain ***histamine***, ***serotonin***, ***leukotrienes***, ***platelet-activating factor*** (***PAF***), ***heparin***, different chemoattractants, and many other potent mediators. They are released from activated basophil after its degranulation that is triggered by specific binding of basophil Fcε-receptor to allergen-specific IgE.

Basophil mediators affect blood vessels; thus they increase blood flow and accelerate inflammatory response.

Eosinophil Leukocytes

Eosinophils take part in allergic reactions and in anti-parasitic immunity. Their maturation and life cycle are controlled by IL-3 and IL-5. IL-5 activates eosinophil movement towards the places of mast cell concentration within target tissues and organs, thereby promoting eosinophil tissue infiltration.

Granules of eosinophils contain various allergic mediators and cytotoxic proteins, predominantly ***eosinophil major basic protein***. It causes the damage of invaded parasites, but can provoke bronchial hypersensitivity, affecting respiratory epithelial cells. Eosinophils are shown to develop the cytotoxic activity to different host cells with subsequent tissue damage.

Eosinophils express the great variety of surface receptors, e.g. for C3b and C4 complement components, Fc receptors to IgG and IgE, and many others.

Chapter 13
ANTIGENS: STRUCTURE AND PROPERTIES. MAJOR INFECTIOUS AND NON-INFECTIOUS ANTIGENS. HLA SYSTEM

Antigens: Structure and Main Properties

Some substances, coming into the body, are able to cause host specific immune response. They were termed "***antigens***".

Antigen *is any simple or complex substance that elicits specific immune response and interacts with the specific products of immune reactions – antibodies or antigen-specific receptors*.

The term *antigen* is a combination of two primary words "***anti****body*" and "***gen****erator*". The term ***immunogen*** has the same meaning.

Antigens (Ags) are characterized by the following basic properties: the capacity to trigger the production of immune antibodies or receptors (***antigenicity*** or ***immunogenicity***), and the ability to bind only to the specific antibodies and receptors (antigenic ***specificity***).

Antigenicity depends on various essential properties of an antigen.

Foreignness (or ***difference from "self"***) is the major function of an antigen. In general, molecular structures of the host are recognized as "self and not immunogenic"; for the immune response the molecules must be recognized as "non-self."

Antigenicity rests on the ***molecular size, nature*** and ***chemical structure*** of antigens. The potent immunogens are usually the large molecular substances. It is commonly assumed that the molecules with a molecular weight less than 10,000 are weakly immunogenic, and very small ones (amino acids, etc.) are non-immunogenic.

Most of antigens are macromolecules such as proteins, polysaccharides, and occasionally lipids or nucleic acids. Molecules differ in their abilty in stimulating antibody production. Proteins and polysaccharides are generally the efficient antigens, whereas lipids and nucleic acids are rarely antigenic.

The breakdown of proteins to peptones, amino acids as well as deep denaturation of proteins bring about a loss of antigenic activity, while the introduction of various radicals and side residues into the protein molecule causes the change of antigen specificity.

A certain amount of ***chemical complexity*** is required – for instance, amino acid homopolymers are less immunogenic than heteropolymers containing two or three different amino acids.

Genetic constitution of the host substantially affects antigenicity. Two lines of the same animal species respond differently to the same antigen because of another composition of genes, encoding immune response proteins.

Dosage, routs and ***schedule of antigen administration*** demonstrate the essential significance for the immune response. Since the degree of immune reactions depends on the amount of antigen given, the immune defense can be changed by different dosage, route of administration, and timing of administration (including the intervals between doses).

The immune response is directed against the certain sites of antigenic molecules. Despite the antigens are usually the large substances, the immune response is not directed towards the entire antigen molecule but mainly to the specific chemical groups of its molecule known as ***antigenic determinants***, or ***epitopes***.

Epitope is the smallest unit of a complex antigen that is capable of binding to an antibody. Corresponding site in antibody molecule able to interact with the epitope is named as ***paratope***. Upon the large protein molecules, sequences of ten to twenty amino acids act as antigenic determinants. Complex structures such as bacterial cell walls have 100 or more different antigenic determinants.

It is possible to enhance the immunogenicity of an antigen by mixing it with an ***adjuvant. Adjuvants*** *are the auxiliary substances that boost the immune response* – e.g., stimulating the antigen uptake by antigen-presenting cells.

Different antigens (proteins, lipids, polysaccharides and the great variety of small molecules) are characterized by unequal immunogenicity. Thus, there are ***complete*** and ***partial antigens*** (***haptens***).

Complete antigens are the substances that elicit full-grade immune response with antibody production solely by themselves (foreign proteins, bacteria and their toxins, viruses, fungal cellular compounds, etc.)

Partial antigens or ***haptens***, if taken solely, do not cause the production of antibodies. They become immunogenic only after binding to a ***carrier protein***. Haptens include large quantity of small molecules (drugs, chemicals, etc), lipids, some carbohydrates, pure nucleic acids and other substances. The addition of proteins to haptens endows them with the properties of complete antigens.

Antigenic Substances of Different Origin

There are ***exogenous*** and ***endogenous*** antigens.

Exogenous antigens are divided into ***infectious*** and ***non-infectious***.

Infectious antigens comprise *bacterial, viral, fungal, parasitic* antigens, *endo-* and *exotoxins*.

Antigenic Structure of Microbial Cell

Bacterium is a complex of antigens that comprises high molecular weight compounds of a protein nature and biologically active specific polysaccharides.

Most of bacteria contain ***O-antigen*** (somatic lipopolysaccharide or ***LPS***) that is thermostable and withstand heating to 80-100^{o}C. O-antigen renders endotoxic activity due to lipid A presence in its structure.

Motile bacteria possess ***H-antigens*** (composed of flagellar protein flagellin), which are thermolabile and readily destroyed at the temperature of 56-80^{o}C.

The antigenic specificity of certain bacteria, e.g., *Enterobacteria* representatives like *Klebsiella pneumoniae,* is associated with the capsular substances (***K-antigens***). The capsular antigens are composed of complex polysaccharides being responsible for the type specificity of microorganisms. *S. pneumoniae*, for instance, has over 80 serological variations (***serovars***).

Capsular K-antigens are distributed widely among the bacteria. They are located upon the O-antigens on the surface of microbial cells. K-antigens in *E. coli* contain thermolabile L- and B-fractions and thermoresistant A- or M-fractions.

In fine, bacterial envelope demonstrates the highly developed antigenic properties, particularly evident in gram-negative bacteria.

A certain K-antigen variation, relatively thermolabile antigen, is called ***Vi-antigen***. For instance, it was determined in strains of enteric typhoid bacteria isolated from the carriers.

Also there are ***group***, ***species***, and ***type-specific*** microbial antigens.

The common ***group*** antigens are determined among the related microbial species. The presence of group antigens in bacteria is based on common genetic and phenotypic links between some microbial communities.

Species-specific antigens characterize particular species.

And finally, there is a large number of ***type-specific*** antigens essential for different bacterial strains and variations of the same species (***serovars***). This kind of specificity is usually associated with the presence of unique polysaccharide or protein residues in various microbial cells. They determine the individual ***serovar*** of bacteria.

Also it has been established that human cells sometimes have the antigens common with staphylococci, streptococci, enterobacteria, some viruses and other causative agents of infectious diseases. Such a condition is called ***antigenic mimicry.***

When the antigenic structures of the host are similar to those of the causative agent, the immune system of the host is incapable of producing the efficient immunity. It is presumed that in individual cases the carrier state and unresponsiveness to the vaccination are related to the antigenic mimicry of shared antigens in bacteria and human body.

On the other hand, microbial antigen, resembling host cell structures, can induce autoimmune reactions. In particular, streptococcal M-protein is able to trigger autoimmune response to endocardial membrane and myocardium due to some structural and charge similarity. In that case host autoimmune attack provokes cardiac valve damage that is typical in rheumatic fever patients.

In addition, ***heterogenic*** or ***heterologic*** antigens (haptens) are also found among the various species of animals (guinea pigs, dogs, cats, etc.) For instance, in case of rabbit immunization with an extract of the organs of guinea pigs, antibodies arising in rabbit recognize not only the primary antigens but also bind to sheep red blood cells (so-called *Forssman's heterologic antigen*). Thus, there are some heterologic antigens within guinea pig tissues and sheep erythrocytes that demonstrate a certain structural similarity.

It has been proven that the non-specific properties of Forssman's heterologic antigen are largely associated with the presence of lipid or polysaccharide antigenic fractions that bear some common properties in different species of animals, plants or microbes. This similarity resembles the antigen mimicry phenomenon.

Protective antigen is the antigenic complex, which develops strong immunogenic activity. The immune response against protective antigens may prevent the host from the infection. For instance, these antigens are found in anthrax bacilli.

Protective antigens have considerable immunogenic properties and can be used in vaccine design for prophylaxis of many infectious diseases.

Microbial toxins also possess remarkable antigenic properties. Having been made harmless by formaldehyde and heat treatment, exotoxins lose their toxic activity but almost completely retain their antigenic functions. They are known as ***toxoids*** or ***anatoxins***. These biological products are used in medicine for vaccination, e.g. against diphtheria and tetanus. Immunization of blood donors or animals by toxoids allows to obtain antitoxic therapeutic antibodies and sera against diphtheria, tetanus, botulism, anaerobic infections, etc.

Superantigens

There is a particular group of antigens, known as ***superantigens***. They display the outstanding biological features being active in lowest concentrations. In very small dose superantigens lead to the activation of a great set of T cells in more than 20% of their total quantity (in comparison, conventional antigens activate not more than 0.01%-0.1% of all T lymphocytes). Similar to ordinary antigens, they are recognized by T cells via TCR. However, superantigens have strong binding capacity to some common variable domains of T cell receptors (their Vb-variants). Consequently, they activate a great number of T-lymphocytes that is followed by redundant pro-inflammatory cytokine production by T cells and macrophages (IL-1, IL-2 IL-6, IL-12, TNF-alpha, etc.) Such activation provokes inflammation with severe tissue damage. A large number of bacterial toxins (staphylococcal, streptococcal toxins, enterotoxins, etc.) pertain to superantigens.

Antigenic structure of viruses

All known viruses contain a certain number of antigens with species and type specificity. According to their antigenic properties various antigenic types (***serotypes***) were determined in many groups of viruses (enteroviruses, adenoviruses, reoviruses, etc.)

For instance, type division of influenza viruses is based on antigenic variation of nucleocapsid (NP) and matrix (M) viral structural proteins. They are distinct in all three viral types – A, B, and C. Further viral subtyping is performed according to antigenic differences of viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA); 18 subtypes of HA (H1-H18) and 11 of NA (N1-N11) are known to date.

All these viral proteins are of the strong immunogenicity, resulting in generation of neutralizing antibodies.

Non-infectious antigens

These antigens are classified according to their origin.

There are ***plant*** antigens, ***animal***-derived and ***human*** antigens, ***chemical*** antigens (e.g., various drugs).

Some of them can interact with immune system as ***allergens***, causing hypersensitivity and ***allergy*** that is followed by tissue damage.

Among the variety of tissue and cell antigens, there are ***nuclear, cytoplasmic***, ***microsomal***, ***mitochondrial***, ***membrane*** antigens.

Xenogenic antigens are the substances, which are immunogenic for the members of another species.

Alloantigens

Alloantigens are the antigenic substances, carried by various individuals of a given species.

For instance, a great set of alloantigens was identified in red blood cells of mammalian species.

At first it was found that human erythrocytes contain at least two main blood antigens (agglutinogens ***A and B***), whereas the sera of individuals carry beta- and alpha-agglutinins (antibodies).

On the basis of AB0 antigenic structure, the erythrocytes of all people can be subdivided into 4 main groups. More than 15 systems of blood alloantigens (e.g., M and N systems, Kelly, Duffy, etc.) including about 100 antigens are known to date. Besides AB0 system, 85% of humans possess erythrocytes expressing ***rhesus factor*** (***Rh***) antigen (so-called *rhesus-positive* persons), while other 15% of nidividuals are *rhesus-negative*.

Alloantigens of Leukocytes and Other Cell Types. Major Histocompatibility Complex of Humans – HLA Molecules

The genes, encoding the antigens of human ***major histocompatibility complex*** (or ***MHC***), were first discovered as the genetic cluster coding for the glycoprotein molecules (*transplantation antigens*) responsible for the rapid rejection of tissue allografts transplanted between genetically non-identical donor and recipient.

Nonetheless, it has become evident later that the main function of MHC molecules is to ***bind peptide antigens*** and ***present them to T cells***.

Thus, MHC molecules were shown to account for antigen recognition by T cell receptor. T cell receptor performs specific binding to the antigen only in case if it presented in complex with MHC molecule. It is possible to conclude that TCR simultaneously recognizes antigenic peptide and self MHC molecule that matches this peptide (***double recognition*** phenomenon).

If the same antigen is presented by another allelic form of the MHC molecule (that can be realized only in experimental conditions), there is no recognition by the T cell receptor. The phenomenon is known as ***MHC restriction***.

The function of antigen presentation is realized by antigen-presenting cells (APCs) such as dendritic cells, B lymphocytes, or macrophages.

In humans MHC is organized as the large genetic cluster located on chromosome 6. It was entitled like ***HLA complex*** (***human leukocyte antigens***) as it was primarily studied in human leukocytes.

The genes of HLA complex encode the ***class I***, ***class II***, and ***class III*** MHC proteins. All of them are located on short *p arm* of 6 chromosome – class I genes occupy more distal position; class II genes are present closer to centromere.

HLA class I molecules comprise three major (***HLA-A, -B***, and ***-C*** antigens) and three minor protein clusters encoded by corresponsing genes.

After gene expression the resulting molecule of HLA class I consists of two chains. ***Heavy chain*** with molecular weight of 43 kDa is highly polymorfic – it has extremely large amount of allelic variants for all three HLA-A, -B, and -C antigens.

Heavy chain is non-covalently linked to a small 11 kDa ***β_2-microglobulin*** peptide thus making membrane heterodimer. By contrast, the molecule of β_2-microglobulin is the same for all variations of HLA class I molecules.

The part of the heavy chain protruded outside the cellular membrane is composed of three globular domains (α_1, α_2 and α_3) that are bound to β_2-microglobulin.

The molecules of HLA class I are expressed in various densities upon ***all nucleated somatic cells*** of humans.

Class II HLA antigens comprises three main protein subsets – HLA II ***DP***, ***DQ***, and ***DR*** molecules encoded by respective genes.

The molecules of HLA II class are the transmembrane glycoproteins composed of two non-covalently linked polypeptide sequences (***α-*** and ***β-***

chains of molecular weight about 33 kDa and 29 kDa). Each chain has 2 external globular domains (α_1 and α_2 or β_1 and β_2, respectively). Both chains are highly polymorphic.

Unlike class I proteins, they have a restricted tissue distribution. HLA class II antigens are predominantly expressed on the membranes of professional ***antigen-presenting cells*** (e.g., dendritic cells) as well as on B cells, macrophages, activated T cells.

Their expression on other cells types (like endothelial cells) can be induced by gamma-interferon and some other cytokines.

The class II MHC region also includes the genes encoding proteins involved into antigen processing (***TAP-antigens***).

The genetic region of ***class III MHC*** encodes several complement proteins and certain cytokines (tumor necrosis factor).

All the genes encoding HLA molecules exhibit a remarkable genetic diversity.

Genetic HLA complex is ***polygenic*** – there are at least 3 kinds of major genes coding for I class (A, B, and C) and II class (DP, DQ, and DR) of HLA molecules.

The HLA complex is also highly ***polymorphic*.** The genes encoding all the chains of HLA I and II class except β_2-microglobulin have enormously great number of allelic variants determined by the methods of genetic typing – more than 2 or 3 thousands for HLA-A, B or C and tens of thousands combinations of allelic variations for 2 chains of HLA II class DP, DQ, and DR.

Futhermore, HLA genes of I and II classes have ***codominant expression*** – the sets of HLA genes inherited from both parents are expressed simultaneously thereby expanding the variability of individual HLA complex.

In addition to the mentioned above, the set of HLA genes present in one chromosome is inherited as a single linkage group or ***haplotype***. Every individual inherits one HLA haplotype from mother and father, thus resulting in final HLA phenotype.

The ***main function of HLA*** molecules *to bind foreign peptide antigens and deliver them for recognition by specific TCRs* of T cells is realized in various ways.

In all of cases the antigen should be degraded by proteolysis into a number of short peptides in the cytoplasm of the host cell (*antigenic processing*). The processed peptides are checked for matching with host HLA molecules. HLA of both classes have the deep cleft between their two chains, where the specific antigenic peptide is inserted after

recognition. From the whole spectrum of antigenic peptides only part of them can find and bind specifically to HLA molecules present in the individual host. Therefore, peptides uncapable of matching with host HLA will escape T cell immune response.

There are striking differences in recognition of foreign exo- and endo-antigens by host HLA molecules.

Exo-antigens (e.g., bacterial or fungal) are processed inside the *endosomes* of APCs (e.g., dendritic cells). Their peptides of 12-25 amino acids residues are coupled mainly with HLA II class molecules (HLA-DR, HLA-DP, HLA-DQ) and presented to ***T helper*** cells.

On the contrary, ***endo-antigens*** arisen inside the host cell after its viral infection or tumor transformation are processed within the *proteasome* – cytoplasmic protease complex. The fragments of viral or other endo-antigen of 9-10 amino acids residues are coupled predominantly with HLA I class molecules (HLA-A, HLA-B, HLA-C) to be presented to ***T-cytotoxic lymphocytes***. Activation of cytotoxic T cells results in killing of virus-infected or transformed host cell.

All these interactions provide the maximal stimulation for cell-mediated immunity.

Determination of individual HLA-antigens (***HLA typing***) is necessary in certain cases. The main reason is the donor selection for organ transplantation.

In addition, strong correlations between particular HLA-haplotype and some diseases are estimated. For instance, the presence of HLA-B27 antigen is observed in more than 95% of patients with spondyloarthritis (autoimmune disease of spine and joints).

Likewise, the combination of HLA-DR3 and HLA-DR4 is found with the high incidence in patients with diabetes mellitus type I.

Autoantigens

Autoantigens are the self substances that elicit immune response against host's cells or tissues.

Usually they become immunogenic after some structural modifications. For instance, one group of autoantigens comprises the *structures* ***isolated behind the blood-tissue barriers***. These substances include the eye lens (behind the blood-ocular barrier), spermatozoids and seminal gland, thyroid gland, CNS cells, and some other structures.

Under the ordinary conditions they do not come into contact with host immune system. Therefore, antibodies dont't give rise against "hidden" cells, tissues and organs. However, if these structures are injured, the autoantigens become exposed to the immune cells. This enables the production of antibodies and autoreactive T cells that might be deleterious against the primary isolated tissues that become autoantigenic.

Also the origination of autoantigens is possible under the influence of various external factors – radiation, tissue crush, extreme cooling, drug treatment (e.g., nonsteroidal anti-inflammatory drugs, sulphonamides, colloidal gold products and others) as well as by impact from bacterial proteins and toxins or under the action of viruses (e.g, in case of infectious mononucleosis, viral hepatitis and many other viral infections).

Pathological autoantigens appear due mainly to the structural changes after the damage of macromolecules, cells and tissues (in burn disease, in cancer patients, after exposure to radiation, severe tissue crush and other similar cases).

Chapter 14

IMMUNOGLOBULINS AND ANTIBODIES, THEIR STRUCTURE AND FUNCTION. COMPLEMENT SYSTEM. PATHWAYS OF COMPLEMENT ACTIVATION

Immunoglobulins

Immunoglobulins *are serum proteins, synthesized by B lymphocytes and plasma cells*. During electrophoresis they migrate in γ-globulin zone.

All immunoglobulin molecules are composed of ***light*** and ***heavy*** polypeptide chains. The terms "light" and "heavy" originate from their molecular weight – light chains are of molecular mass about 25 kDa, heavy chains – approximately 50 kDa.

Light (***L***) ***chain*** is of two distinct types, kappa or lambda; the division is based on amino acid differences in their constant regions. Both types occur in all classes of immunoglobulins (IgG, IgM, IgA, IgE, and IgD), but one immunoglobulin molecule contains only one type of L chain.

Heavy (***H***) ***chains*** are distinct for each of the five immunoglobulin classes. Among them there are gamma-, mu-, alpha-, delta-, and epsilon types of heavy chains.

An individual antibody molecule always consists of identical H chains and identical L chains. The simplest antibody molecule of IgG is composed of four polypeptide chains: two H chains and two L chains. These four chains are covalently linked by disulfide bonds.

After treating of immunoglobulin molecule with a proteolytic enzyme (e.g., papain), peptide bonds in the flexible central part of the molecule (its ***hinge region***) are broken. Two identical ***Fab fragments*** are formed, which carry the ***antigen-binding sites***, and one ***Fc fragment***, which is involved in placental transfer, complement fixation, attachment to various cells, and other biologic activities.

L and H chains are composed of ***variable regions*** and ***constant regions***. All the regions contain globular ***domains***.

An L chain consists of one ***variable domain*** (VL) and one ***constant domain*** (CL). Most H chains consist of one variable domain (VH) and three or more constant domains (CH). Variable regions are responsible for antigen binding; constant regions are responsible for other functions of Ig.

In variable regions of both L and H chains there are three utmost variable (***hypervariable***) amino acid sequences that form the ***antigen-***

binding site. They are known also as ***complementarity-determining regions*** (***CDRs***). Antigen binding is non-covalent, involving van der Waals and electrostatic forces, hydrogen bonds, etc.

Binding power of the single active site (***paratope***) of Ab molecule to corresponding antigenic *epitope* is termed ***affinity***. Binding strength of the whole multivalent antibody molecule to the antigen is known as ***avidity***. It is multiplied depending on antibody valency.

Immunoglobulin Classes

Immunoglobulin G

Each IgG molecule consists of two L chains and two H chains linked by disulfide bonds. Molecular weight of IgG is about 150 kDa. Its serum concentration is between 8-12 g/l (the mid level is 10 g/l).

Because of two identical antigen-binding sites, IgG is divalent. There are four subclasses (IgG1 to IgG4), based on antigenic differences in the H chains and on the number and location of disulfide bonds. The subclass of IgG1 poses about 65% of the total IgG demonstrating the highest defensive potential. IgG2 is directed against polysaccharide antigens and may be an important part of host defense against encapsulated bacteria.

IgG is the predominant antibody of ***secondary immune response*** that ensures the protection against the bacteria and viruses. It possesses the *highest affinity*. IgG activates complement system via classical pathway. It also plays a role as efficient *opsonin*, activating phagocytosis.

IgG is the only immunoglobulin, which passes the placenta, thus IgG antibodies prevail in self-protection of newborns and infants.

Immunoglobulin M

IgM is the main immunoglobulin produced early in the ***primary immune response***. IgM is present on the surface of majority of uncommitted B cells. It is composed of five H, L units (each similar to one IgG unit) and one molecule of J (***joining***) chain. The final pentameric molecule (MW 900 kDa) has ten identical antigen-binding sites and thus a valency of 10. Hence, it has the highest avidity of all classes of immunoglobulins. Serum concentration of IgM is from 0.8 to 1.5 g/l.

It is the most active immunoglobulin in agglutination, complement fixation, and other antigen-antibody reactions. It creates the primary line of the defense against bacteria and viruses. IgM stimulates phagocytosis (opsonin action) and activates complement system via classical pathway.

Immunoglobulin A

IgA is the major immunoglobulin of the body secretions such as milk, saliva, and tears, secretions of the respiratory, intestinal, and genital tracts. It protects mucous membranes from attack by bacteria and viruses.

Each secretory IgA molecule (MW ~ 350 kDa) consists of two H, L units and one molecule each of ***J chain*** and ***secretory component***. Secretory component binds to IgA dimers and supports their transport across the mucosal epithelial cells. Some IgAs exist in serum as monomeric H-L molecules (with MW of 170 kDa). Serum concentration of IgA is about 1.0-4.0 g/l

There are at least two subclasses, IgAl and IgA2. Some bacteria (eg, neisseriae) can destroy IgAl by producing a specific IgA-protease. This way they may overcome antibody-mediated resistance of mucosal barriers.

Immunoglobulin E

Molecular weight of IgE is near 190 kDa. Its serum concentration is extremely low; thus it is expressed in international units (IU). One IU is about of 1.5 ng. Normal serum range for IgE is between 0-100 IU per 1 ml of serum.

IgE are the ***main antibodies in allergy*** (***reagins***); they do not penetrate the placenta. Fc portion of IgE binds to a receptor on the surface of mast cells and basophils. This bound IgE acts as a receptor for the antigen. The resulting antigen-antibody complex triggers allergic response of the immediate (anaphylactic) type with the release of allergy mediators. Serum IgE is also typically increased during helminth infections.

Immunoglobulin D

IgD acts as an antigenic receptor, when present on the surface of mature B lymphocytes. It also occurs on the cells of some lymphatic leukemias. Its molecular weight is about 160 kDa. In serum it is present only in trace amounts (0.04 g/l). IgD do not fixate complement; and don’t penetrate the placenta. Maybe it is responsible at least in part for anti-viral immunity.

Antibodies

Antibodies *are immunoglobulin molecules capable of specific binding to the antigen that induced their synthesis*. They make up about 20% of plasma proteins.

There are ***natural*** and ***immune*** antibodies.

Natural antibodies are the agents of the innate immunity that react with many bacterial and viral antigens without previous immunization. They show polyspecifity but low affinity. Another example of natural Abs is the presence of serum α- and β-agglutinins, which can interact with agglutinogens A and B of human red blood cells.

Immune Abs arise after the immunization being capable of acting against the immunogen.

There are anti-bacterial, antiviral, anti-toxic, anti-fungal, anti-parasitic antibodies.

The mechanisms of antigen-neutralizing effect of Ab are the following:

- blocking of active sites of toxins, inactivation of venoms;
- complex of antibodies with antigens activate complement classical pathway resulting in cell lysis;
- opsonization of antigens with phagocytosis enhancement;
- stimulation of killing effect of NK cells and cytotoxic lymphocytes (***antibody-dependent cell cytotoxicity***);
- antibodies can develop inner enzymatic activity (***abzyme antibodies***) and may break down some antigenic substances (proteins, nucleic acids, etc.)

Monoclonal antibodies

Antibodies that develop in response to a single antigen are heterogeneous, because they are synthesized by many different clones of plasma cells. These antibodies are named ***polyclonal***.

Antibodies produced by a single clone of plasma cells or by tumor plasma cells (***myeloma*** cells) are homogeneous, being ***monoclonal***.

Monoclonal antibodies (***mAb***) can be produced by fusion of a myeloma cell with an antibody-producing lymphocyte. Such ***hybridoma*** synthesizes monoclonal antibodies *in vitro*. Important information about the structure and function of antibodies was gained from the investigation of monoclonal antibodies.

There are several common steps in standard ***hybridoma technology***.

First, inbred line mice are immunized with necessary antigen. After the end of immunization course mouse spleen is taken out and immune splenocytes, containing antigen-specific B cells, are derived. These cells are fused with non-Ig-secreting mouse myeloma cells. As other cancer

cells, mouse myeloma cells possess immortality, but cannot produce antibodies. Fusion is made by polyethylene glycol (PEG) or by electric field. The created hybrid cells are placed into selective media permissive only for hybridoma cells.

Hybrid cells are diluted with cultural media to the limit of one hybridoma cell per one well of culture plate. These cells are cloned. After propagation hybridoma culture is tested for specific antibody production. In case of positive test specific hybridomas are sub-cloned. Mass hybrid culture is able to produce large amounts of monoclonal antibodies with the same specifity and affinity.

Due to their homogeneity, monoclonal antibodies have become an extremely powerful tool in biology and medicine, especially for immunochemical testing. All cell markers (CD antigens, enzymes, signal proteins, etc.) were investigated with monoclonal Ab. They are used in any kind of immune assay (ELISA, RIA, immune hystochemical testing, etc.)

Numerous problems were primarily arisen in use of monoclonal Abs for therapy of human diseases. First generations of therapeutic mAbs were totally of mouse origin; thus, they induced immune response in humans after several injections. To avoid this obstacle, *humanization* of mouse monoclonal Ab is performed now. In that case variable antibody fragments of mouse origin are coupled with human constant immunoglobulin parts by methods of genetic engineering or the total sequence of primary monoclonal antibody is substituted with human one. These mAbs are of reduced immunogenicity, being available for therapy in humans. As they demonstrated extremely high specificity, the medical applications for therapeutic monoclonal antibodies were termed as "***targeted therapy***".

Targeted therapy is actively used now for cancer and autoimmune disease treatment, for prevention of allograft rejection, etc. The last barrier still limiting the common employment of curative mAbs is a high cost of technology.

Genetic Control of Specificity and Diversity of Antibodies and T Cell Receptors (TCRs)

A total number of specificities of Ab active sites (known as ***antibody repertoire***) is extremely high – in humans it is generally estimated as about 10^{11} of variations and perhaps even more.

Antibody of a certain specificity is produced by single clone of B lymphocytes and plasma cells. Hence, it is clear that virtually any

foreign substance (antigen), when exposed to the immune system, "finds" enough clones of B lymphocytes bearing specific Ab receptors to antigenic epitopes. This way the antigen selects Ag-specific lymphocyte clones (***clonal selection***) and activates their prolipheration (***clonal expansion*** of Ag-specific lymphocytes). It leads to accumulation of Ag-specific immune cells that eventually eliminate the invaded foreign substance.

The tremendous diversity of human immune repertoire is based on complex and highly versatile genetic mechanisms.

Genes (gene segments), encoding human immunoglobulin heavy chain are located in 14 chromosome, light chain of kappa isotype – within 2 chromosome, light chains of lambda isotype – in 22 chromosome.

Variable parts of immunoglobulin ***heavy chains*** are encoded by 3 kinds of gene segments present in chromosome 14 – ***V*** (*variable*), ***D*** (*diversity*) and ***J*** (*joining*). Also this chromosomal region carries 9 gene segments encoding all the versions of ***constant parts*** of Ig heavy chains (from μ to ε) that determine the class and subtype of Ig molecule.

The ***variable parts*** of immunoglobulin ***light chains*** are encoded by 2 gene segments – ***V*** and ***J***. The constant part of kappa light chain is controlled by a single gene segment, whereas the constant part of lambda light chain – by 4 functional genetic segments (from λ_1 to λ_4).

Initial (or ***germline***) configuration of Ig-encoding genes is observed in lymphocyte precursors in fetal period before the beginning of differentiation and maturation of human B cells.

Germline genetic organization includes the *multiple variants of gene segments* that encode the ***variable part*** of Ig molecule.

For heavy chain there are about 50-100 different V gene segments, 20-30 versions of D segments and 6 copies of J segments. All of them are placed linearly along the chromosome.

For light chains there are about 30-40 of V gene segments, and several copies of J segments.

However, the genes in germline configuration cannot undergo transcription and translation to produce functional Ab receptor. It is possible only in developing B cells. Therefore, B cell development and maturation is followed by ***rearrangement*** of gene segments encoding Ab binding site. ***Multiple recombination events*** result in random selection and joining of single V, D, and J segment for heavy Ig chain as well as in single V and J segment joining for light chain.

Thus, any clone of developing B cell has a unique randomly chosen combination of single V, (D), and J gene segments that encode the variable part of Ab receptor of B cells.

The next recombination event brings together the newly formed V (D) J exon and the gene segment encoding the constant part of Ig molecule (μ- and, later, δ-chain). The process of joining of various genetic segments is followed by multiple local mutations that expands the spectrum of Ab diversity.

Furthermore, primary RNA transcript of Ig chain undergoes splicing with intron removal resulting in final mRNA that codes for the functional light or heavy chain of B cell Ab receptor. As the result of alternative RNA splicing in mature B cells, Ab receptors of IgM and IgD classes appear together upon B cell membranes.

The process of Ab rearrangement is governed by recombinase protein complex ***Rag-1/Rag-2*** encoded by corresponding *recombination-activating genes 1* and *2*. These recombinases are lymphocyte-specific, being active only in developing B cells.

Further enrichment of human antibody repertoire occurs after the stimulation of human B cells with specific antigen under the influence of Ag-specific Th2 and T_{FH} cells.

T cell help leads to ***isotype switching*** of Ab molecules produced by B cells – this process results in change of Ig class from IgM to IgG, IgA or IgE under the influence of various cytokines secreted by T helper cells (IL-4, TGF-β and others).

Genetic mechanism ensuring isotype switching is known as ***switch recombination*** that joins existing V D J gene of Ig variable part with new gene segment encoding a new constant part of Ig molecule (α-, γ-, or ε- heavy chain instead of primary μ- or δ-chains). This results in multiple joining mutations that expand the diversity of Ab molecules.

Finally, antigen-stimulated differentiation and proliferation of B cells is followed by activation of ***somatic hypermutagenesis*** in genes encoding variable parts of antibodies. Here the total rate of somatic mutations within Ab active sites elevates more than 1000 times in comparison with the background rate. This greatly broadens the final Ab repertoire of B lymphocytes and plasma cells.

Genetic mechanism that accounts for somatic hypermutations is not totally clarified. For instance, it includes the action of ***deaminase enzyme*** that converts cytosine (C) into uracil (U) in antibody V genes. Subsequent process of DNA repair removes uracil residues and substitutes them with other nucleotides.

Somatic hypermutagenesis creates the opportunities for ***affinity maturation*** of Ag-specific B cells that enhances the binding power of Ab active sites. As the result, only B cell clones that express antibodies with

the highest affinity will survive; some part of them undergoes transformation into long-living memory cells. The rest of Ag-specific B lymphocytes with dimmed affinity is eliminated by apoptosis.

The genetic mechanisms involved into generation of ***immune repertoire of T cells*** bearing Ag-specific TCRs are principally the same as for Ab receptors of B cells.

Genes (gene segments), encoding alpha and delta chains of human TCR are located in 14 chromosome; beta and gamma chains – within 7 chromosome.

Similarly to Ab molecules, ***variable parts*** of ***beta chain*** of TCRs are encoded by 3 kinds of gene segments – ***V***, ***D***, and ***J***.

The ***variable parts*** of TCR ***alpha chain*** are encoded by 2 gene segments – ***V*** and ***J***.

Both 7 and 14 chromosomes carry gene segments encoding the limited number of the ***constant parts*** of human TCRs.

In ***germline*** genetic configuration TCR beta chain has about 50 different V gene segments, 2 distinct D segments, and 12 J segments.

Likewise, TCR alpha chain locus comprises about 45 of V gene segments and 55 versions of J segments.

The progenitors of T cells (thymocytes) undergo maturation in thymus that is followed by ***rearrangement*** of gene segments encoding variable and constant parts of T cell reseptor. It result in random selection and joining of V, (D), and J segments with genes encoding the constant parts of TCR.

Thus, each clone of T cells carries a randomly created TCR molecule with unique specificity. This generates the broad immune repertoire of Ag-specific T cells.

Unlike B cells, T lymphocytes are devoid of affinity maturation and isotype switching.

Complement System

Complement *is a system of enzymes and proteins contributing to the lysis of target cells*.

Complement system includes serum and membrane-bound proteins that function in both specific and non-specific immune reactions. They are activated via a chain of proteolytic cascades. The term "***complement***" indicates the ability of these proteins to complement (augment) the effects of other components of the immune response (antibodies, phagocytes, etc.).

Complement system consists of about 30 proteins designated by the numbers C1 through C9 (***components*** of ***classical pathway***), in addition to various ***factors*** of ***alternative pathway*** (e.g. B, D, H, and I factors, properdin and others). These proteins act in a series of steps, where the activated component of complement splits the next one in the cascade of activation.

Complement system has the following main effects:

– the ***lysis*** of target cells (for instance, bacterial and tumor cells);

– ***production of chemoattractants*** and ***mediators*** that actively participate in inflammation and allergy;

– ***opsonization*** of microbial cells and immune complexes for clearance by phagocytosis.

Complement proteins are synthesized mainly in the liver and by different phagocytic cells. The complement is quite sensitive to heating. It is inactivated after exposure at 56°C for 30 minutes and also during long-term storage, under the influence of UV-radiation and by various chemical substances, whereas immunoglobulin molecules are more resistant and can withstand heating at 56°C.

Complement Activation

Most of complement components are *proenzymes*, which must be cleaved to form the active molecules. The components of the classical pathway are numbered from C1 to C9; and the reaction sequence is C1→C4→C2→C3→C5→C6→C7→C8→C9.

Up to C5, activation involves proteolytic cleavage, releasing smaller fragments from C2 through C5. The smaller fragments are marked with the letter ***a*** (e.g., C4a) and the larger fragments with ***b*** (e.g., C5b).

The complement reaction can take one of three pathways. The first one is the ***classical pathway*** that is initiated by certain specific immune reactions, predominantly by *antigen-antibody complex* formation.

The second is the ***alternative pathway***, which is triggered by versatile non-specific stimuli (e.g., polysaccharides of microbial cell wall and capsule, aggregated immunoglobulins, drugs and various chemical substances).

The third ***lectin pathway*** is strongly related with the mechanism of classical pathway. It is activated by host ***lectin*** proteins that bind to carbohydrate residues of microbial cells.

Classical Pathway

Only IgM and IgG can fix and activate the complement via the classical pathway. Antibodies of these Ig classes were named ***lysins***, which initiate the lysis of bacteria, fungi, parasites and animal cells.

Activation starts from C1 component. It binds to a CH_2-domain of Fc portion of immunoglobulin molecule.

The component C1 is composed of three proteins: Clq, C1r, and C1s. The completed $C1qr^2s^2$, attached to the antibody-antigen complex, cleaves C4 and C2 to form C4b2b. The latter complex is an active ***C3 convertase*** or ***convertase of classical pathway***, which transforms C3 molecules into two fragments: C3a and C3b.

C3 molecule is the mainstay of all pathways of complement system. Its ***C3b fragment*** promotes further complement activation that eventually results in cell lysis and death.

Also C3b plays a role of potent ***opsonin***, when it binds to the foreign cells and other particles. Consequently, this event is followed by C3b interaction with complement receptors on phagocytic cells (e.g., CD35), thereby promoting opsonization.

One more reaction cascade is activated through the release of C3a. C3a is a highly reactive ***anaphylatoxin*** with pleotropic biological effects. In particular, it intensively stimulates macrophages resulting in acute inflammation.

In classical pathway C3b comes into contact with a complex C4b2b, producing a new enzyme, ***C5 convertase***, which cleaves C5 to form C5a and C5b.

C5a is the most potent ***anaphylatoxin*** and a chemotactic factor (***chemoattractant***). C5b binds to C6 and C7 to form a complex that plunges into the membrane of target cell. Then C8 binds to the C5bC6C7 template and joins up to 16-20 C9 molecules to produce the ***membrane attack complex (MAC)*** that directly causes cytolysis. MAC resembles in its action cytotoxic protein ***perforin*** of killer cells.

The Alternative and Lectin Pathways

Great variety of substances with multiple biological activities (various chemicals, bacterial endotoxins, numerous infectious agents – fungi, parasites, and others) activates an alternative pathway.

Traces of ***C3b*** are normally present in sera. They may bind to ***factor B*** (non-active serine protease); this reaction is stimulated by Mg^{+2} ions. Factor B is cleaved here by factor D (active serine protease) into Ba and Bb proteins.

As the result, alternative pathway ***C3 convertase*** (***C3bBb***) is formed that acts on C3 and generates more C3b molecules. The additional C3b binds to the C3 convertase to produce ***C3bBbC3b***, which is the alternative pathway ***C5 convertase***. This complex is stabilized with serum protein ***properdin***. At next step it cleaves C5 into C5a and C5b, making C5b active. The rest of activation sequence is the same as in classical pathway leading to the production of ***membrane attack complex***.

One more additional pathway of complement activation is termed as ***lectin pathway***. Its main activators are the proteins ***lectins*** that specifically bind to the carbohydrate moiety of microbial cells.

For instance, ***mannose-binding lectin*** (***MBL***) of human plasma actively binds to sugar residues like mannose found in polysaccharides of microbial outer membrane (e.g., LPS). Binding of MBL to microbial cell activates MBL-associated serine protease that acts on C4 of classical pathway. Thus, except the initial phase of activation by lectin molecule, the steps of lectin pathway become literally the same as in classical pathway of activation of complement system.

Vast number of serum and tissue proteins regulates the complement system at different stages. For example, ***C1 inhibitor*** inactivates the serine protease activity of C1r and C1s. Other proteins have the ability to accelerate the decay of the complement molecules. Among them is ***decay-accelerating factor*** (***DAF***), a membrane-bound protein found on most blood cell membranes. It can accelerate the dissociation of C3 convertases of both pathways. Similarly, factor S or ***vitronectin*** prevents the assemblage of membrane-attack complex.

The Role of Complement Activation in Immune Response

Cytolysis

Insertion of the C5bC6C7C8C9 MAC complex into the cell membrane promotes lysis and killing of many types of target cells, including erythrocytes, bacteria, and tumor cells.

Opsonization

If coated with C3b, antigen-antibody complexes, microbial cells, and other particles become much more efficiently ingested by phagocytes

(macrophages, neutrophils, etc.) It results from phagocyte expression of C3b receptors on their surface. Successful clearance of immune complexes out of blood stream prevents the emergence of autoimmune reactions.

Chemotaxis

Several fragments of complement proteins, e.g. C5a or C3a, stimulate directed movement of neutrophils and other cells towards the inflammation area.

Allergy development

C3a, C4a, and C5a can provoke degranulation of mast cells and basophils with massive release of mediators of allergy, leading to increased vascular permeability and smooth muscle contraction.

Chapter 15
PRIMARY AND SECONDARY IMMUNE RESPONSE. DYNAMICS OF IMMUNE RESPONSE

Primary and Secondary Immune Response

Immune response evolves in several stages. Most of antigens require *antigen presentation*. *Antigen-presenting cells* (or APC) ingest Ag, process it and present to T helper cells in complex with HLA antigens. During *inductive phase* APC activate T helpers by means of direct contact and cytokine production. T helpers differentiate into Th1 or Th2. These subpopulations re-route immune response towards cell-mediated reactions (Th1) or to humoral immunity activation (Th2). *Effector phase* is characterized by T cells cytotoxic reactions, transformation of B cells into plasma cells, which produce specific antibodies. They inactivate and eliminate antigen. At the same time suppressor mechanisms evolve, which confine immune reactions. Resting long-living specific T- and B-memory cells remain capable to future extensive reactivity in case of secondary contact with the same or similar antigen.

From that point there is ***primary*** and ***secondary*** immune response.

Primary immune response is developed after short latent period. IgM are shown to be major antibodies, produced at primary immune reactions (in 2-3 days). IgG start to rise in 5-7 days after stimulation. Amplitude of primary response is not very high. In 2-3 weeks it declines but trace amounts of specific antibodies and immune cells are maintained in the body. Finally immune memory cells are formed.

Secondary immune response is characterized by intensive proliferation of specific T- and B cells, followed by the high rate of antibody production predominantly of IgG class. The elevated levels of IgG tend to persist much longer than in the primary response. The affinity of T cell receptors and antibodies is increased. All these events promote rapid antigen elimination.

Dynamics of Immune Response – General Characteristics

Once penetrated the host, a complex antigenic substance such as bacterial cell or virus immediately triggers the broad set of non-specific and specific immune reactions. In vast majority of cases (90% and even

more events) non-specific cellular and humoral mechanisms of ***innate immune response*** are able to eliminate the limited number of the pathogen invaded. The most efficient reactions of innate immunity include the phagocytosis of corpuscular antigens by neitrophils and macrophages, cytolytic activity of NK cells, binding of bacterial and viral pathogens with Toll-like receptors, activation of complement system via alternative or lectin pathways, neutralization of microbial agents by natural antibodies.

But in other situations (massive microbial load, rapid pathogen penetration and dissemination, intensive toxin release, etc.) powerful specific reactions of ***acquired*** (or ***adaptive***) ***immunity*** are activated.

These reactions are much more complex and intricate affecting all the parts of immune system.

There is a substantial difference of immune response against thymus-dependent and thymus-independent Ags.

T-independent Immune Response

Activation of immune system towards thymus-independent pathway is provoked by relatively limited number of antigenic substances restricted by their structural organization and molecular charge.

As the result, a polymeric complex Ag binds to specific Ab receptors of IgM class anchored within cytoplasmic membrane of B cells. This leads to cross-linking of two or more molecules of superficial Ab receptor resulting in next signal transmission and B cell activation. Activated B lymphocytes start transformation into plasma cells that intensively produce specific Ab but of IgM class only. Without T cell help (particularly of IL-4 absence) B cells are unable to switch Ab synthesis from IgM to other Ig classes thereby averting further Ab maturation. Thus, T cell-independent response has essential limitations demonstrating rather low grade and specificity.

By contrast, specific response against thymus-dependent Ags initiates multiple immune reactions that profoundly affect the host immune system.

T-dependent immune response demonstrates three principle phases:

1. Antigen processing and presentation.
2. Inductive phase with activation and differentiation of T helper cells.
3. Effector phase.

Antigen Processing and Presentation

Processing and presentation of Ags is the main function of specialized ***antigen-presenting cells*** (APC). Among them are dendritic cells (DC) of myeloid and plasmacytoid origin, Langerhans` cells, follicular dendritic cells, and some others. B lymphocytes are also capable of presenting Ags for T cells.

Once captured by APCs (e.g., by different types of DCs) exogenous Ag degrades within APC *endosome* up to small antigenic peptides (***antigen processing***). Resulting fragments of Ag are next coupled with HLA II class molecules inside APC cytoplasm. This interaction requires specific binding of intracellular HLA chains predominantly to antigenic peptide that demonstrates high affinity to them. Therefore, processed antigenic substances that don't match well any HLA molecule will evade T cell response.

After transportation the specific complex (antigenic peptide - HLA II class molecule) is exposed upon APC membrane for recognition by Th0 cells (***antigen presentation***).

The protein antigens of intracellular microbial pathogens (viruses, chlamydiae, rickettsiae and others) are processed directly inside the cytoplasm of infected cells within the special cytoplasmic protease complex known as *proteasome*. Resulting antigenic peptides associate with HLA I class molecules for further presentation to cytotoxic T cells.

It is generally ascertained that antigen-presenting dendritic cells of various origin play a key role in activation of T cell-dependent reactions. These reactions hinge strongly on the type of initial Ag recognition by APC.

Ag-presenting cells bear multiple *pathogen-binding receptors* (PBR) of different types. For instance, high pathogen-binding activity is essential for molecules of ***Toll-like receptor*** (***TLR***) family. These receptors possess strong binding capacity to *pathogen-associated molecular patterns* (PAMP) – structural units that are similar or common for vast groups of microbial pathogens. Other families of receptors (e.g., NOD) are also implicated to primary binding of some groups of Ags.

Hence, antigenic uptake by dendritic cells via TLR-4 activates production of pro-inflammatory cytokines (IL-1, IL-6, IL-12, IL-18, TNF-α). This leads to next transformation of Th0 to Th1, promoting cell-mediated reactions and inflammation.

By contrast, immune activation resulted from Ag binding to TLR-2 upon APC membrane triggers secretion of another set of regulatory

cytokines (IL-4, IL-10, IL-13, etc.) The latter ensures conversion of Th0 into Th2 lymphocytes thereby promoting B cell transformation and antibody secretion by plasma cells (see below). This pathway is also stimulated by antigen-presenting B lymphocytes.

Inductive Phase:
Activation and Differentiation of T Helper cells

Principal event that biases immune reactivity towards the ***cellular inflammatory response*** is the transformation of naive Th0 cells into ***Th1*** cell type, whereas conversion of Th0 lymphocytes into ***Th2*** cells activates ***humoral response*** and antibody production (***functional polarization*** of T helper cells).

This needs a number of specific stimulatory signals from APC to T helper cells:

a) processing of Ag and presentation of membrane Ag-HLA complex for recognition by T helper cells;

b) expression of costimulatory molecules upon APC membranes for additional activation of T helpers;

c) release of specific set of cytokines that stimulate T helper differentiation.

T helpers recognize complex "Ag-HLA II" by antigen-specific membrane receptor TCR. CD4 and CD3 molecules of Th cells also take part in the reaction making the recognition more stable. This delivers ***first signal*** for T helper activation.

At the same time the expression of costimulatory molecules upon the membranes of helper cells is elevated. T cells bear specific costimulatory molecule CD28 that binds to its counterpart molecules CD80 or CD86 expressed on the membrane of APC. This interaction provides ***second signal*** for activation of T helpers. Without costimulation T cell binding to processed Ags leads not to activation but to suppression and apoptosis of reactive cell clone. It results in *anergy* (cell unresponsiveness) in concern to recognized Ag.

If antigen-specific B-lymphocyte serves as antigen-presenting cell it also expresses costimulatory molecules for activation. The most essential is CD40 receptor that reacts with specific CD40 ligand (CD40L or CD154 molecule) upon Th0 membrane. This re-directs transformation of Th0 into Th2 cells stimulating humoral response with antibody production.

Third signal for differentiation of Th0 into Th1 or Th2 cells ensues from alternative cytokine activation of T helper cells. Type of cytokine secretion substantially depends on primary Ag recognition by APC via specialized pattern-recognizing receptors. As mentioned above, stimulation of APC by TLR-4 activates production of pro-inflammatory cytokines (IL-1, IL-12, IL-18, α-TNF, etc.) thereby causing Th1 differentiation. In contrast, Ag binding to TLR-2 in most of cases triggers the release of IL-4, IL-10, IL-13 that promotes Th0 maturation into Th2 cell type.

Th1 and Th2 are characterized by opposite type of cytokine secretion. In particular, major cytokines, produced by Th1 cells are γ-interferon, IL-2 and β-TNF, whereas cytokine array of Th2 encompasses IL-4, IL-5, IL-10, IL-13, and some others. Th1/Th2 activity re-routes next immune reactions into ***cell-mediated inflammatory response*** or towards B cell stimulation and ***antibody synthesis*** (***humoral response***).

Besides Th1/Th2 differentiation, some other types of specialized T helper cells become activated.

For instance, ***Th17 helper*** cells originate from Th0 under the activation by IL-23 from dendritic cells and by IL-21.

Follicular T helper cells T_{FH} give rise from Th0 by the contact with follicular antigen-presenting B cells supported by stimulation with IL-21.

Activation of Effector Cells

Multiple effector mechanisms ensuring the elimination of invaded pathogen include cell recruitment to inflammatory focus, stimulation of phagocytosis and antigenic presentation, cytotoxic action of T killers and NK cells, conversion of B cells into plasma cells with secretion of antibodies of various Ig classes.

The reactions of specific adaptive immunity are also followed by the ***clonal expansion*** of activated immune cells that leads to the increase of number of antigen-specific cells reacting against the pathogen.

When differentiated, ***Th1*** produce ***γ-interferon*** that activates macrophages and dendritic cells. They greatly enhance production of pro-inflammatory cytokines IL-1, IL-12, IL-18, alpha-TNF thus augmenting further Th0 differentiation into Th1. Furthermore, these cytokines arrest Th2 maturation. Progression of ***cell-mediated reactions*** results in delayed type of hypersensitivity with granulomatous inflammation.

And vice versa, ***Th2*** cells produce a broad spectrum of interleukins (***IL-4***, IL-5, IL-10, IL-13). Activation by IL-4 and costimulation of B cells

via CD40L of Th2 triggers blast transformation of B lymphocytes into plasma cells. This results in antibody affinity maturation and secretion (***humoral immune response***). Newly generated highly specific Abs neutralize Ags or opsonize Ags for phagocytes, activate the complement pathways thereby eliminating invaded pathogens.

Additional T helper populations also intensively stimulate effector immune reactions.

For instance, activated ***Th17*** start to produce ***IL-17***, IL-21, and IL-22 cytokines. This leads to the activation of multiple lines of immune and non-immune cells (T cells, neutrophils, macrophages, NK cells, B cells, epithelial and endothelial cells), resulting in progressive chronic infllammation, enhancement of phagocytosis and antibody synthesis, maturation of myeloid cells, and in some cases, generation of autoimmune response.

Activated ***follicular T helper cells*** stimulate the transformation of follicular B lymphocytes into *long-living antibody-secreting plasma cells* and *memory B cells*.

In case of immune response against viruses, intracellular bacteria (chlamydia, rickettsiae, etc.) or tumor cells another type of Ag elimination occurs. In these situations antigen-specific ***cytotoxic T cells*** (or ***Tc***) become activated.

Mechanisms of immune cytolysis by Tc cells include:

(1) target cell recognition and activation of Tc lymphocytes via HLA I-Ag complex expressed by infected or malignant cell;

(2) secretion of killing molecules by Tc cells with subsequent target cell elimination.

Literally all nucleated cells are capable of presenting Ag for ***T-cytotoxic cells*** (Tc or T killers). When infected, the cell expresses antigenic peptides of invaded pathogens in complex with HLA I class molecules (HLA-A, HLA-B, or HLA-C) upon its cytoplasmic membrane. Complex "Ag-HLA I" is recognized by cytotoxic Tc bearing specific TCRs. This binding is supported by CD8 and CD3 molecules. Activation of Tc needs also additional costimulatory molecules and cytokine stimulation (mainly, γ-interferon and IL-2 from Th1). Once activated, Tcs render Ag-specific cytotoxic activity against infected or tumor cells.

Activated (CD8+) T cells (*T killers*) release the vast number of cytotoxic factors that eventually cause target cell death. Among them are effector proteins perforin, granzymes, and granulysin.

For instance, ***perforin*** is highly active cytotoxic protein demonstrating lytic activity similar with membrane attacking complex of complement.

Perforin acts as pore-forming toxin allowing ***granzymes*** to enter the infected cell and stimulate apoptosis via activation of caspases. In addition, Tcs stimulate apoptosis by elevated expression of FasL that binds to apoptosis receptor CD95 Fas/Apo upon the membranes of target cells.

Finally, Tcs activate secretion of γ-interferon that arrests viral replication and stimulates antiviral and anti-tumor activity of NK cells.

Likewise, a great variety of cytotoxic substances is produced by ***natural killers*** or ***NK*** cells. When stimulated by signals from their inhibitory or activation receptors, they destroy the target cells by secretion of perforin, granzymes or lymphotoxins.

Natural Inhibition of Immune Response

After coming to the peak of highest intensity, immune reactions gradually fade. Clonal expansion of immunocytes results in formation of ***long-living memory T- and B cells*** that come into dormant state until next antigenic challenge. This dampens overexuberant or detrimental immune reactivity thus preventing autoimmune disorders.

There are numerous versatile mechanisms of natural inhibition and restriction of immune response.

Normally all secreted cytokines are short-living substances. They render discernible activity only within the limited space affecting the cell itself and/or neighboring cells. This prevents undesirable immune expansion.

Furthermore, multiplex interrelationships within cytokine network are characterized by reciprocal suppression of their actions. For instance, IL-4 precludes Th1 differentiation and next cell-mediated response, whereas IL-12 inhibits Th2 thereby down-regulating humoral immunity; IL-10 blocks literally all pro-inflammatory cytokines; potent inhibitory activity is proven for TGF-beta (transforming growth factor beta) cytokine.

In addition, cell activation terminates after the change of superficial costimulatory molecules. While CD80/86 promotes Th differentiation via CD28, later substitution of CD80/86 with CD152 inhibits further proliferation of T helpers.

And finally, many of activated cell subsets (e.g., plasma cells) raise the expression of apoptosis receptor CD95. It causes their programmed death, for example, after interaction with regulatory T lymphocytes, bearing specific CD95L.

It has been established quite recently that the group of ***regulatory T cells*** includes diverse T cell populations endowed with powerful ***suppressive function***.

Number of adaptive regulatory T cells is activated directly after antigenic challenge. Among them are CD4(+) Tr1 lymphocytes, producing IL-10, Th3 cells, secreting TGF-beta, and some others.

Another substantial part of T lymphocytes (more than 3% of total T cell count) is primarily differentiated as ***regulatory suppressor cells***. They demonstrate striking inhibitory capacity contributing to immune reactions as natural regulatory T cells (***T regs***). Molecular markers of this cell type are CD4 and CD25 that are co-expressed together upon the cell membranes.

Unlike other lymphocytes, natural regulatory T cells contain active form of specific transcriptional factor Foxp3. It is encoded by X chromosome foxp3 gene H – a special molecular label for this cell subset.

During recognition of Ags presented by DCs natural regulatory T cells expose inhibitory molecule CTLA-4 (CD-152). This leads to silencing of cell activation and prevents effective co-stimulation of other T cells by APCs. Furthermore, natural regulatory lymphocytes produce large amounts of suppressive cytokines TGF-beta and IL-10 that averts proliferation of reactive cell populations.

Another way of immune control is realized by ***idiotypic network regulation***. Any antigen that encounters with the immune system triggers polyclonal immune response, i.e., stimulates production of specific Abs and TCRs by various cell clones. They display similar but not identical specificity and affinity. It means that generated Abs and receptors have minor structural differences in their antigen-binding sites. Therefore, active sites of newly appeared Abs and receptors would bear unique antigenic determinants (named *idiotopes*) that specify only one certain clone of immune cells. Total number of idiotopes carried by single molecule of antibody was termed idiotype.

First generation of Abs raised against specific Ag during immune response was named idiotypic (i.e. bearing idiotype). Once appeared, idiotypic Abs start to trigger second generation of Abs against their own active sites (so-called *anti-idiotypic Abs*). The latter inhibit overexpression of primary idiotypic Abs, receptors and cell clones.

Chapter 16
NON-IMMUNE AND IMMUNE DEFENSIVE MECHANISMS IN ORAL CAVITY
(***For studens of Dentistry Faculty***)

Normal or pathogenic microflora of oral cavity is continuously affected by wide network of defensive factors operating in this area. And vice versa, every microbial agent harbors countless antigenic ensembles that stress all the sides of the host defensive forces.

Once penetrated, a complex antigenic substance such as bacterial cell immediately triggers the vast set of *non-immune* and *immune reactions*. Notably, *immune reactions* can be both *non-specific* and *specific*. In most of cases non-specific response from innate immunity is able to eliminate the pathogen. Nevertheless, in more critical situations the specific immune reactions of adaptive immunity are activated.

Non-Specific Defensive Factors of Oral Cavity

The decisive role in protection of oral tissues from harmful microbial activities is played by ***saliva*** and epithelial cells of ***mucous membranes*** with their ***barrier function***. Continuous salivary flow washes out carbohydrates from dental surfaces. Salivary glands produce 0.5 to 3 liters of saliva per day. Saliva efficiently controls supragingival environment and the same way prevents microbial entry into subgingival space. The most powerful salivary bicarbonate buffer maintains pH of oral cavity within the range of 6.7-7.3. However, the diffusion of salivary components through dental plaque is slow; that's why pH in central part of dental plaque falls down to 5.0 or even less.

Oxidation-reduction or ***redox potential*** substantially influences the growth and reproduction of microbial populations and the rates of enzyme reactions in oral cavity. The levels of redox potential depend strongly on local concentrations of molecular oxygen. Positive values of redox potential indicate aerobic conditions, negative – anaerobic ones. Saliva, tongue body, mucous epithelium of cheeks and palate have positive redox potential (+158-540 mB), dental crevices and approximal dental surfaces display negative potential with its value about (–300 mB).

The process of dental plaque maturation reduces local redox potential from about (+290 мВ) to (–140 мВ). This leads to successful propagation of anaerobic bacteria.

Powerful ***innate immunity*** of oral cavity is maintained by multiple humoral and cellular non-specific immune reactions.

The ***system of humoral non-specific defense factors*** encompasses *mucins*, *glycoproteins*, *lactoferrin*, *lysozyme*, *peroxidase*, short-chain basic defensive proteins *histatins* and *cystatins*. They are present in saliva and crevicular fluid in relatively large amounts.

Mucins are highly polymeric viscous glycoproteins secreted predominantly by submandibular and sublingual salivary glands. There are 2 major mucin glycoproteins in saliva – *MG1* and *MG2*.

MG1 has the molecular weight more than 1 mln Da. It tightly covers mucous membranes of oral cavity. ***MG2*** of molecular weight for about 125 kDa hinders aggregation and adhesion of oral streptococci. Viscous mucin layer captures dental microflora thereby preventing bacterial penetration. Mucins also reduce acidic demineralization of hard dental tissues.

Likewise, other *salivary glycoproteins* block microbial adhesion to underlying oral mucous membranes.

Small cationic enzyme ***lysozyme*** (or ***muramidase***) hydrolyses glycosidic bonds between N-acetyl-glucosamine and N-acetyl-muramic acid within bacterial cell wall *peptidoglycan* (or murein). It exerts easy lysis of bacterial cells.

Furthermore, lysozyme binds to monovalent anions, e.g., perchlorates, iodides, bromides, fluorides, thiocyanates, and some others, and at the same time it binds to salivary proteases. This complex destabilizes the envelope of bacterial cells thus activating bacterial autolysins with subsequent cell lysis.

Lactoferrin is the iron-binding salivary glycoprotein that is synthesized by intercalated duct cells and granulocytes. Antimicrobial action of lactoferrin depends on its *specific iron-binding capacity*. Under binding it makes ferric ions unavailable for bacterial cells. Iron-deprived bacteria stop their growth and reproduction.

Iron-free lactoferrin or *apolactoferrin* demonstrates direct antibacterial effect agglutinating microorganisms that stimulate caries progression (*S. mutans, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*).

Salivary peroxidase is secreted by acinar cells. It is thermostable enzyme active in broad pH range (3.0 to 7.0) and resistant to proteolysis. It

inactivates hydrogen peroxide generated by oral microflora and diminishes acid accumulation within dental plaque. Peroxidase retains stability being absorbed on hard dental tissues, e.g., enamel. As the result, this enzyme slows down the progression of dental plaque, caries and periodontal diseases.

Histatins are low molecular weight (3 to 5 kDa) antimicrobial salivary peptides. They comprise the group of small basic peptides secreted by acinar cells, which are enriched with histidine.

Histatins block the growth of common oral pathogens (*S. mutans*, *C. albicans*), aggregation of porphyromonads and streptococci.

Cystatins pertain to one more family of salivary antimicrobial peptides. They diffuse to saliva from gingival crevicular fluid. *Cystatins* work as the inhibitors of bacterial thiol proteases thereby impairing normal microbial metabolism.

Substantial part of salivary antimicrobial defense is related with ***complement system***. Complement proteins leak from gingival capillaries into crevicular epithelial cells and reach gingival crevice. Then in smaller amounts they may spread to saliva. This gradual overflow strongly accelerates in case of oral inflammation.

Complement *proteins* render the variety of defensive reactions against the invaded pathogens: *lysis* of target cells (for instance, bacterial or viral-infected); *production of chemoattractants and mediators* that participate in inflammation and allergy, *opsonization* of bacteria and immune complexes for clearance by phagocytosis.

As in any body compartment, complement activation can take one of three pathways. The first is the *classical pathway*, which is initiated by specific *antigen-antibody complexes*. *Lectin pathway* closely resembles classical one except the primary step: lectin pathway is stimulated by reaction of host carbohydrate-binding proteins or *lectins* (e.g., human mannose-binding lectin) with bacterial polysaccharides.

The third route of complement activation is the *alternative pathway* that can be triggered by the components of bacterial cells, their endotoxins, host IgA molecules, some chemical substances, etc. Complement activation via this pathway is the most common in oral cavity.

In all three pathways of complement activation the resulting membrane-attack complex or MAC exerts the lysis of microbial cells. Nevertheless, general conditions for complement action in oral cavity are not so beneficial as in bloodstream.

Biological Activity of Secretory IgA

Despite the evident protective power of all above-mentioned defensive mechanisms, it remains uncontested that ***secretory immunoglobulin A*** is clearly the most significant factor of humoral response in oral cavity. Secretory IgAs display their protective features by action on multiple targets: both specific and non-specific, humoral or cellular.

It must be pointed out that in large amounts IgA is present not only in saliva, but in milk, tears, colostrum, etc., thus being the dominant human immunoglobulin in secretions of the respiratory, intestinal, and genital tracts. It rids mucous membranes of attacks made by bacteria and viruses.

IgAs together with other immunoglobulin classes are produced by plasma cells. Plasma cells of secretory glands are located in their stromal part being adjusted to acini and ducts.

Every ***secretory IgA molecule*** (with molecular weight for about 350 kDa) consists of two *heavy* (H) and *light* (L) protein chains and one molecule each of ***J chain*** (15.6 kDa) and ***secretory component*** (70 kDa). J chain and secretory component have excessive amounts of carbohydrates in their structure.

Secretory component binds to IgA dimers and supports transportation of IgA molecules across mucosal epithelial cells towards the lumen of salivary ducts.

Some IgAs exist in serum as a monomeric $(H\text{-}L)_2$ molecule (MW 170,000 Da). Serum concentration of IgA is about 1.0-4.0 g/l.

There are at least two IgA subclasses, namely IgAl and IgA2. Salivary IgA1 subclass antibodies bind preferably to bacterial proteins and carbohydrates, whereas IgA2-subclass molecules react with lipoteichoic acids of gram-positive bacteria and lipopolysaccharide (or LPS) of gram-negative ones.

Some oral bacteria (e.g., streptococci and neisseriae) can destroy IgAl by producing a specific protease thereby overcoming antibody-mediated resistance of mucosal surfaces.

Internal and external secretions of oral cavity are different in IgA content. Internal secretions are present in gingival crevices and pockets (e.g., crevicular fluid); and IgA/IgG ratio there is similar to serum proportions (nearby 1 to 6). On the contrary, IgA dominates within external secretions (saliva), where IgA/IgG ratio exceeds 100/1.

The induction of secretory IgA synthesis by plasma cells can be triggered locally in oral cavity or after the migration of stimulated B cells

from gut-associated lymphoid tissue (GALT) towards salivary glands and lymphoid tissue of oral cavity.

Secretory IgAs are endowed with multiple protective functions. They slow down microbial adherence, block the action of bacterial toxins, and neutralize viruses. They successfully perform so-called *immune exclusion*, preventing antigenic transposition from oral cavity through the epithelium of mucous membranes to regional lymph nodes and blood flow. For instance, sIgA antibodies inhibit the attachment of *S. mutans* to dental enamel thus breaking down the starting point for caries. Furthermore, sIgAs inactivate streptococcal glycosyltransferase enzyme that catalyzes exo-polysaccharide synthesis required for efficient bacterial adhesion. Similar action they demonstrate against fungal pathogen *C. albicans.*

Secretory IgAs foster antimicrobial activities of oral mucins, lactoferrin and salivary peroxidase mainly owing to extensive non-immune interactions of these substances with oral IgA molecules. In fact, it exerts tight cross-agglutination of adherent microbial cells thus limiting bacterial spread and propagation.

Finally, IgAs intensively stimulate several lines of immune cells via binding to Fc-receptors for IgA on the membranes of macrophages, dendritic cells, granulocytes and T cells thereby triggering *antibody-dependent cell cytotoxicity* (or *ADCC*).

Sufficient levels of secretory IgA antibodies may prevent the flare-up of certain oral infections of viral etiology, e.g., herpetic exacerbations. In patients with selective IgA deficiency the viruses can easily reproduce in oral mucosa resulting in specific viral lesions.

Cellular Innate Immune Responses in Protection of Oral Cavity

Primary cellular reactions of *innate anti-bacterial immunity* are generally based on ***phagocytosis*** and activity of phagocytic cells within oral cavity. The latter comprise the members of ***mononuclear phagocyte system*** (blood monocytes and resident tissue macrophages) and ***granulocyte system*** (*polymorphonuclear leukocytes* – neutrophils, basophils, eosinophils).

Saliva of healthy individuals steadily contains cellular elements usually moved from gingival crevices and pockets. Approximately 90% of the cells within gingival crevice pertain to polymorphonuclear leukocytes,

10% are mononuclear cells. Mononuclear leukocytes encompass 60% of B cells, 20-30% of T cells and for about 10-15% of macrophages.

Granulocytes and macrophages of oral cavity engulf and then destroy the invaded bacterial pathogens. Phagocytes migrate and accumulate within inflammatory focus under the action of versatile groups of *chemokines* both of host or microbial origin (*IL 8* and other cytokines, C3a and C5a complement fragments or *anaphylotoxins*, bacterial peptidoglycan, teichoic acids, and many others).

Microbial killing depends on the vast number of bactericidal reactions generated by phagocytes. The most potent microbicidal mechanism is known as ***respiratory burst*** resulting in large amounts of *reactive oxygen species* (*ROS*). The main ROS agents are *superoxide anion* (O_2^-), *hydroxyl radical* (•OH) and *hypochlorite* (OCl^-) that break down biopolymers of bacterial cells within phagosomes. Digestion of bacteria is also supported by numerous antimicrobial peptides (defensins) and hydrolytic enzymes (cathepsins, elastase, lysozyme, and many others) produced by phagocytes.

In parallel to microbial digestion, macrophages and dendritic cells activate antigenic presentation and enhance secretion of pro-inflammatory cytokines (IL-1, IL-12, IL-18, α-TNF, etc.). This stimulates the differentiation of naive Th0 cells into Th1 generating local inflammatory response in oral cavity.

Inflammatory state can be profoundly aggravated in case of infection spread from primary oral site to regional lymph nodes and further to bloodstream (*systemic inflammatory response*). It is followed by macrophage stimulation with bacterial LPS (or bacterial endotoxin) via its binding to membrane CD14 receptor molecule. This leads to massive overproduction of pro-inflammatory cytokines resulting in fever, hypotension, and if not treated – to multiple organ dysfunction and toxic shock.

Viral infections of oral cavity are controlled by another powerful mechanism of innate immunity – activation of ***natural killers*** or ***NK cells***.

It is a rather small cell population containing great number of granules in cytoplasm. NK cells exert the lysis of virus-infected target cells regardless of their antigenic specificity (so-called ***non-immune cytolysis***). In addition, natural killers can destroy some bacteria.

NK cells release a great variety of cytotoxic substances – *perforin*, β-tumor necrosis factor or *lymphotoxin*, some special cytotoxic enzymes, or *granzymes* that activate apoptosis. Also they induce cell death activating apoptosis of target cells via CD95-Fas ligand interaction.

Overall, non-specific reactions of innate immune response can efficiently eliminate most of microbial invaders. But in situations of progressive oral infections *antigen-specific acquired immunity* should be stimulated.

The mechanisms of acquired immune response in oral cavity

In general, the reactions of acquired (adaptive) immunity are much more complex and intricate affecting the all lineages of immune cells. In oral cavity antigen-specific immune response originates from two main sources.

First, oral pathogens trigger *direct activation of resident immune cells* of salivary glands. Among them are ***antigen-presenting cells*** (APC), consisting of dendritic cells (DC), macrophages, and B lymphocytes. Microbial antigens undergo natural retrograde flow along the salivary ducts. Next they are delivered to immune cells via endocytosis by ductal epithelium.

Second, specific immune response in oral cavity is maintained by continuous *migration of Ag-specific immune cells* to salivary glands from primary sites of antigenic exposure, mainly from *gut-associated lymphoid tissues* or GALT. These cells comprise activated T lymphocytes and specific B cells capable of IgA synthesis. The lymphoid follicules of GALT carry follicule-associated epithelial cells (FAE cells) or *microfold cells* (*M cells*) that capture and transport exogenous antigens from the lumen of small intestine to neighboring dendritic cells for antigen presentation. Afterwards, antigen-stimulated immune cells begin to spread to various peripheral sites of immune system including oral cavity.

Similar with GALT, expansion of primed immunocompetent cells to oral cavity occurs from NALT, or *nasal mucosa-associated lymphoid tissues* (tonsils and other accumulations of lymphoid follicles within Waldeyer's pharyngeal lymphatic ring).

Taken together, it can be concluded that IgA-producing plasma cells and effector T lymphocytes of oral cavity originate from ***common mucosal immune system***. The latter is the sum of local mucosal lymphoid tissues present in peripheral body compartments and organs.

Specific acquired response against thymus-dependent Ags initiates multiple immune reactions that profoundly affect the host immunity.

Principal event that biases the immune reactivity towards cellular inflammatory response is the transformation of naive Th0 cells into Th1

cell type, whereas conversion of Th0 lymphocytes into Th2 cells activates humoral response and antibody production.

Th1 and Th2 are characterized by the opposite type of cytokine secretion. The basic cytokines, produced by Th1 cells are γ-interferon, IL-2 and β-TNF, whereas cytokines of Th2 encompasses IL-4, IL-5, IL-6, IL-10, IL-13, and some others. Th1/Th2 activity re-directs next immune reactions into ***cell-mediated inflammatory response*** or towards B cell stimulation and antibody synthesis (***humoral response***).

Multiple effector mechanisms ensuring the elimination of invaded pathogen include cell recruitment to inflammatory focus, stimulation of phagocytosis and antigenic presentation, cytotoxic action of T killers and NK cells, conversion of B cells into plasma cells with secretion of antibodies of various Ig classes.

Chapter 17

IMMUNODIAGNOSTICS. EVALUATION OF IMMUNE STATUS. SEROLOGICAL REACTIONS AND THEIR PRACTICAL APPLICATIONS

Immunodiagnostics and Immune Status

Immunodiagnostics *is a complex of laboratory tests based on immunological reactions and methods that are used for evaluation of immune status, laboratory diagnosis of diseases or antigen identification.*

Immune system state (or ***immune status***) is characterized by methods of immunodiagnostics.

Immune status *is the state of immune system at the certain moment of life.* It is the result of continuous balance between the host immune system and specific and non-specific environmental challenges.

For the assessment of immune status the vast number of tests and methods is elaborated. Immune status is described by versatile ***quantitative*** and ***functional indices***. Both can be ***specific*** and ***non-specific***.

Specific factors of immunity (*antigens* or *antibodies*) are evaluated by ***serological reactions***. Immune T- and B-cells, bearing antigen-specific receptors are determined with blast transformation test and some other specific cellular reactions (see below).

Non-Specific Parameters of Immune Status

Lymphoid system characteristics

Total quantity of blood lymphocytes, granulocytes and monocytes are determined by *total leukocyte count* and *leukocyte differential count*.

Lymphocytes make 18-37% of total leukocyte count, monocytes – 3-11%, neutrophils – 47-72%, basophils – 0-1%, eosinophils – 1-5%.

Total quantity of leukocytes is $4\text{-}9*10^9$/l, lymphocytes – about $2*10^9$/l.

Evaluation of different lymphocyte subsets is performed by determination of their specific cellular markers – membrane CD molecules.

The major method for identification of subpopulations of immune cells is the ***indirect fluorescent assay*** with anti-CD mouse monoclonal antibodies.

It is carried out as follows: patient's white blood cell suspension is fixed on slide. Mouse monoclonal antibodies to appropriate CD-Ag are

added to the cells. They should interact directly with CD antigens upon the membranes of particular cell subset. After incubation and subsequent wash the slide is treated with a fluorescent-labeled anti-mouse immunoglobulin, which binds to the immune complex Ab-CD Ag.

Finally, the slide is examined by luminescent microscopy. Cells, bearing specific CD-Ags show bright luminous halo. The part of specific cell subset among the all leukocytes is evaluated.

Likewise, immune fluorescent technique is applied to automatic cell subpopulation count that also allows automatic cell sorting.

This technology is named ***flow cytometry***. ***Fluorescence-activated cell sorter*** (FACS) is used for automatic cell sorting.

Flow cytometry analyzes a single-cell suspension flowing along the capillary unit of sorter crossing several laser beams. It measures the light scattering of cells, thereby estimating total cell count, and the relative fluorescence of cell subpopulations, tagged with monoclonal fluorescent antibodies. Cells, bearing specific CD antigens, are detected by luminescence sensors.

By means of electrostatic field the cells passing through the capillary unit and bearing fluorescent label can be separated from the total cell population. This technology is widely used in clinical medicine and biomedical research.

Using the above-mentioned methods different lymphoid subpopulation can be readily estimated.

Basic markers of total T cells are CD2 and CD3, T helpers – CD4 (35-50% of total lymphocytes), cytotoxic T cells – CD8 (18-25%).

Th/Tc ratio – immunoregulatory index – is in the range 1.4-2.0. It declines in patients with immunodeficiency (e.g., in AIDS patients it may be less than 0.04) and arises in autoimmune diseases.

B cells markers are CD19-22, CD40, CD72.

The quantity of interleukins and other ***cytokines*** produced by different cell types is evaluated by ***ELISA*** or radioimmunoassay.

Evaluation of functions of T- and B cell

Different methods can be used to determine T- or B cells functional activities.

One of the basic methods is the ***test of lymphocyte blast transformation***. It estimates proliferative response of T- and B cells to ***mitogens*** (mitogen-activated blast transformation) and ***antigens*** (specific blast transformation).

For these purposes lymphocyte culture taken from examined patient is cultivated in presence of ***mitogens*** (cell mitotic activity stimulators) or antigens. After incubation the responding cells differentiate into their blast forms, which can be identified by microscopy. Blast cells have the large basophilic nucleus surrounded with narrow ring of cytoplasm.

Also radiometric count is used, where DNA synthesis in proliferating cells is determined by detection of ^{3}H-thymidine incorporation into replicating cell DNA.

The example of T cell mitogen is ***phytohemagglutinin*** (***PHA***). Almost 40-70% of lymphocytes respond to PHA. Bacterial ***lipopolysaccharide*** (***LPS***) is the strong mitogen for B cells; 15-25% of B cells respond to it.

Specific antigens, involved into blast transformation reaction, activate populations of antigen-specific T- and B cells that can be detected similar ways.

Investigation of complement system

Serum ***concentration*** of complement proteins is determined by ***ELISA***. Serum content of major complement fractions varies significantly (C1s – 0.12 g/l; C4 – 0.43 g/l; C3 – 1.30 g/l; C9 – 0.16 g/l).

Functional activity of complement system is estimated by hemolysis reaction. It is expressed in 50% hemolysis units (CH50).

During infection after the initial increase complement serum concentration is diminished substantially due to immune complex formation that results in complement consumption..

Determination of immunoglobulin concentration

Quantitation of major serum and secretory immunoglobulins of G, M and A classes is estimated by ELISA or Mancini single radial immune diffusion test (see below).

Serum IgE concentration is evaluated by ELISA or RIA taking into account negligible serum IgE content.

Macrophage and granulocyte system assessment

Total quantity of blood granulocytes and monocytes is determined by leukocyte differential count.

Different aspects of phagocyte activity could be examined. Bacterial engulfment and phagocyte digestive capacity are evaluated by incubation of leukocyte culture with model microorganisms with subsequent Giemsa stain.

Phagocytic index and phagocytic number are determined here.

Phagocytic number means an average quantity of ingested bacteria within the single phagocyte (normally 3-8). ***Phagocytic index*** is the percentage of phagocytes taking part in phagocytosis (about 70-80% in immune competent persons).

Digestion is estimated by inoculation of phagocyte lysates into nutrient media after the processing of native bacteria by leukocytes. If the abundant growth of bacteria has been appeared, the phagocytosis is regarded as incomplete and the digestion is impaired.

Respiratory burst in phagocytes is measured by ***nitroblue tetrazolium reduction test*** (***NBT-test***).

Metabolic activity of phagocytes (e.g., neutrophils) that reflects respiratory burst activation is determined by cytoplasmic oxidative conversion of *nitroblue tetrazolium* (NBT) dye into insoluble *formazan*. In normalcy about 15-18% of neutrophils are positive. In case of serious infections this index arises above 40%.

Indirect immune fluorescent test with monoclonal antibodies is used for phagocyte cell typing. Phagocytes express the vast number of membrane markers; among them are CD14, CD11/CD18, CD16, CD32, CD64, CD35, etc.

Cytokines, produced by phagocytic cells, are determined by ELISA test.

Serological Reactions and Their Practical Applications: General Characteristics of Serological Reactions

In most cases interactions of antigens (Ag) with antibodies (Ab) are ***highly specific***. Antibodies can distinguish between very similar antigenic molecules – they can perform even stereospecific recognition of several isomeric forms of the same Ag molecule. Also these interactions are extremely ***sensitive*** – Ab may reveal complementary antigens in nano- or pikomolar concentrations. Under these circumstances specific Ag-Ab reactions are very powerful tool in immunochemistry for antigen or, in advert case, antibody determination.

Taking into account that almost any molecule may play a role of an antigen, a tremendous variety of antigenic substances can be revealed and investigated with the help of ***serological reactions*** owing to another component of these reactions – ***immune serum*** that contains ***specific antibodies***.

Antigen-antibody binding is a particular type of non-covalent interaction. Antigen and antibody molecules react by means of their active sites (***epitopes*** vs ***paratopes***) and form ***immune complexes*** of various structures.

Serological reactions possess some common properties.

They demonstrate ***two phases*** in their development. The first phase is ***specific***. It is characterized by recognition and binding of the complementary groups of antigens and antibodies. The ***second non-specific phase*** is an expanding progressive reaction that is followed by visible manifestations – at this stage the visible complexes and aggregates are formed.

All of these reactions occur within the electrolyte solution. Optimal pH is neutral or weakly alkaline, optimal temperature is about 37^{o}C.

In some conditions at acidic (below 4.5) or alkaline pH (>9-10), or at high salt concentrations the serological reactions become reversible.

The reversible interaction between Ag and Ab is defined by the ***mass action equation*** at equilibrium point:

[free Ag]*[free Ab] ↔ *Kd* [Ag-Ab complex],

where *Kd* is the dissociation constant of complex.

As the result of the reaction (formation of immune complex) depends on Ag and Ab concentrations, it is possible to estimate Ag or Ab quantities in serological reactions by means of known reagent (Ab or Ag). Hence, in any serological reaction one of the reagents (unknown) should be determined with the help of known second one.

For semi-quantitative assessments of Ag or Ab amounts in serological reactions, the consecutive dilutions of reagents are used that determine the ***titer*** of an ***antibody or antigen***.

*The limiting dilution of unknown Ab (or Ag) still able to produce the distinct positive result of serological test is termed as the **titer of antibody or antigen** for this reaction*.

Immune Reagents for Serological Reactions

Immune reagents are the antibodies and antigens taking part in serological tests.

For unknown antigen determination ***specific immune serum*** is used. It contains ***specific antibodies***, which were generated after the *immunization of laboratory animals* (rabbit, etc.) with necessary antigens.

For instance, to obtain *agglutinating serum*, animals are immunized with a suspension of freshly isolated bacteria of a certain species or type. Immunization is accomplished according to special protocols, taking into account the dose and the intervals between injections. At the end of immunization blood is taken from the animals, and the serum obtained is inactivated, conserved and titrated. For improvement of reaction specifity *pure specific antibodies* can be isolated from sera.

The titer of agglutinating serum or antibodies is known as *the smallest amount or the greatest dilution of antibodies, which causes a clearly marked agglutination reaction.*

The vials, containing the manufactured sera, carry the labels, where the titers of Abs are present, indicating the maximum dilution of serum (1/500, 1/4000, 1/16000, etc.) that may cause the agglutination of the specific antigen (*agglutinogen*).

In turn, for test of unknown antibodies, antigen-containing immune reagents are used. They are named as ***immune diagnosticums***. It is a highly broad group of reagents. Their origin depends on the immune reaction they take part. It can be suspension of known inactivated viruses, toxin or toxoid products, various soluble antigenic substances. More complex antigenic diagnosticums are the particles with naturally anchored or artificially bound to their surface antigenic substances (***microbial*** and ***erythrocyte diagnosticums***).

Classification of Serological Reactions

According to their types, serological tests are classified as follows.

1. ***Agglutination reactions***.
2. ***Precipitation reactions***.
3. ***Neutralization reactions***.
4. ***Lysis reactions*** (bacteriolysis, hemolysis, *complement fixation test* and some others)
5. ***Reactions with labeled reagents*** (immune fluorescence test, enzyme-linked assay, radioimmune assay, western-blotting technique, etc.)

Agglutination Reactions

Agglutinins *are the antibodies that enable to clump the antigen-containing particles* (*microbial cells, erythrocytes, etc.*) *with formation of visible agglomerates*.

The addition of specific immune serum to a suspension of microbial cells leads to their agglomeration into the large visible complex, looking like flakes or granules. This phenomenon is determined as microbial ***agglutination***.

Overall, the agglutination reaction results from the interaction of erythrocytes, microbial and other cells with the specific immune serum. Antigen in agglutination reaction must be presented in ***corpuscular*** (particle) form.

In 1896 F. Widal indicated that the serum of patients with enteric typhoid fever was able to cause the specific agglutination of salmonellas – the causative agents of this disease.

Further it was found that in great sets of infectious diseases the specific antibodies (or ***agglutinins***) begin to raise, and their growth becomes the specific hallmark of the disease.

Specific antibody (***agglutinin***) and the corpuscular antigen (***agglutinogen***) are the immunochemical reagents for the agglutination reaction. Their specific binding occurs in saline-contaning medium and requires the definite quantitative ratio of the reagents.

The mechanism and external manifestations of the agglutination test depend, from one side, on the corpuscular nature of an antigen with the large number of epitopes (***multivalent structure***).

From the other side, agglutinating antibodies must be bivalent or better polyvalent with two or more active sites. In that way agglutination of at least two microbial cells is performed via the bridge of antibody molecule, where two active sites of the same antibody are bound to different bacterial cells. Spatial spread of agglutinate lattice results in visible cell agglomeration.

Meanwhile, the mechanism of antigenic precipitation is also very similar. Both reactions are accompanied by the production of visible aggregates.

The agglutination reaction is characterized by high specificity. Nevertheless, the antigenic structure of bacteria for agglutination is extremely variable. The same bacterial cell can exhibit ***group***, ***species***, and ***type specific antigens***. Type-specific antigens are also known as ***serovar-specific***. Group or species-specific antigens show cross-reactivity between

many related bacteria. The variation of antigens in the microbial cells is a regular process; it reflects intraspecies and interspecies variability or similarity of bacteria.

Thus, upon the immunization of an animal with the cells of one microbial species, agglutinins can appear not only to the species of immunization, but also to some other related bacterial species that possess the common group-specific antigens.

That is why ***group agglutination*** is observed sometimes, resulting in non-specific clumping of closely related microbial cells bearing the same group antigens.

For isolation of specific agglutinins in sera of animals immunized by a complex of bacterial cell antigens the method of adsorption of cross-reactive agglutinins on the related bacterial cells is employed. Agglutinating sera obtained by this technique (proposed by A. Castellani in 1902) is called ***adsorbed monospecific serum***.

Monospecific antibody reagents make possible to determine more precisely the species and serovar specificity of the causative agents of various diseases (e.g., salmonellosis, shigellosis, etc.)

Thus, agglutinating sera are used as ***non-adsorbed*** or ***adsorbed products*** that are ***group***, ***species*** or ***serovar specific***.

Motile bacterial cells carry somatic O- and flagellar H-antigens. Upon animal immunization, both O-agglutinins and H-agglutinins are produced. The bacteria, covered with capsular K- or Vi-antigen, poorly react with O-antisera, but easily agglutinate with anti-Vi-sera.

The manifestations of agglutination reaction depend on the ratio of antigen/antibody concentrations, density of microbial suspension, pH and ionic strength of the medium, the temperature of incubation, the quality of reagent mixing, etc.

Microbial agglutination test has many practical applications being used for ***serological diagnosis*** of various infectious disorders.

In most of clinical cases ***serological diagnosis*** *means the laboratory diagnosis of the infectious diseases made by determination of* ***specific antibodies*** *directed against their causative agents*.

In some other cases the serological diagnosis of the disease is made by the detection of specific antigens in patient's serum.

Determination of specific antibodies is essential for the diagnosis of enteric typhoid fever and paratyphoidal diseases (*Widal's agglutination test*), brucellosis (*Wright's reaction*), as well as for leptospirosis, tularemia, and other diseases.

In all these cases the specific antibodies or agglutinins are determined in patients' sera by means of *known microbial cells* containing specific microbial antigens called as ***diagnosticums***.

In turn, the agglutination reaction is also used for the *identification of unknown microbial cultures* isolated from patients and sick animals. It is performed by means of agglutinating sera that contain antibodies of known specificity. This test determines ***microbial serological properties***, resulting in ***serological identification*** of microbial species.

To get the fast preliminary results of serological reactions, rapid agglutination tests are commonly used. They are performed as tentative ***slide agglutination tests*** with concentrated specific immune serum. These reactions can be applied either for microbial identification (for instance, to identify cholera vibrios in patients with cholera) or for the determination of antimicrobial antibodies (*Huddleson's test* in brucellosis, reactions for tularemia, etc.)

In case of positive results of slide agglutination test the extended ***tube agglutination test*** with serial dilutions of immune serum is elaborated.

Hemagglutination and Other Indirect Agglutination Techniques

Besides direct agglutination, ***indirect agglutination*** is employed for laboratory diagnosis of infectious diseases. For instance, indirect agglutination is used for detection of antibodies produced against the antigens devoid of corpuscular structure (e.g., *soluble proteins*).

The most effective is the ***indirect hemagglutination test***. For this reaction the antigen is usually adsorbed on the surface of chemically fixed xenogenic (e.g., cattle) erythrocytes (***erythrocyte antigenic diagnosticum***).

Hemagglutination is performed with erythrocyte diagnosticum and patient’s sera. As an example, Vi-antibodies present in sera of carriers of enteric fever salmonellae are determined by hemagglutination with Vi-Ag erythrocyte diagnosticum.

Hemagglutination reaction is quite sensitive and allows the detection of antibodies in greater dilutions.

These tests are usually carried out in the wells of plastic agglutination plates, where the agglutination patterns of the cells on the bottom of the well are readily observed. This technique provides a more sensitive detection than macroscopical clumping.

In medical practice the *reaction of isohemagglutination* is used for determination of human blood groups in case of blood transfusion.

Modern variations of indirect agglutination tests include latex-agglutination, acrylic beads agglutination, gold nanoparticles agglutination followed by microscopical count of the reactions and some other tests. Quantification of initial levels of agglutination can be achieved by laser nephelometry.

These advanced reactions develop highest sensitivity and specifity than conventional ones.

Reactions with Incomplete Antibodies. Coombs` Test

Incomplete antibodies *unlike the complete ones are monovalent with one active antigen-binding site*. The rest of antibody active sites can be destroyed by proteolytic enzymes or due to irreversible block by various agents.

During the interaction of incomplete antibodies with complete antigen no visible immunological reaction proceeds.

Incomplete (*monovalent*) or *blocking antibodies* bind to the antigens, but don't lead to their visible precipitation or agglutination. By fact, these antibodies are regarded to be more stable to heating and chemical treatment.

Incomplete Abs encompass the variety of Ab molecules that give rise in certain pathological conditions such as rhesus-agglutinins (Abs directed to human rhesus-positive red blood cells), reagins of allergic patients, autoantibodies of patients with systemic lupus erythematosus (SLE) and rheumathoid polyarthritis, antibodies against tumor antigens in cancer patients, anti-infectious antibodies in patients with brucellosis, syphilis or other diseases. Incomplete autoantibodies may cause drug-induced leukopenia, hemolytic anemia or thrombocytopenia.

In pregnant rhesus-negative women the incomplete antibodies can arise against the rhesus (Rh)-antigens of Rh-positive fetus.

In general, rhesus Ags elicit the production of two types of antibodies: complete bivalent anti-Rh-agglutinins and incomplete monovalent anti-Rh-Abs unable to cause the agglutination of Rh+ red blood cells.

The peak of accumulation of incomplete anti-Rh Abs usually occurs in case of the repeated pregnancy with (Rh+) fetus. These antibodies cross the placental barrier and enter the fetal bloodstream. They cause the damage of fetal erythrocytes, thereby developing hemolytic anemia and resulting in hemolytic disease of newborns.

For the ***detection of incomplete antibodies*** the special immunological reaction known as the ***Coombs' test*** was designed being based on the principle of hemagglutination.

The ***direct Coombs`test*** (also called as *direct antiglobulin test*) reveals the presence of *incomplete antibodies*, which are *fixed upon patient's erythrocytes*. To determine the fixation of agglutinins on patient's red blood cells, the animal antiglobulin serum containing ***antibodies against human immunoglobulins*** is added. This serum is produced by immunization of laboratory animals with human immunoglobulins.

Being divalent (or complete), the animal antibodies bind to incomplete human antibodies fixed upon erythrocytes. This interaction causes the agglutination of erythrocytes covered with incomplete agglutinins. The mechanism of agglutination is based on fact that one molecule of antiglobulin simultaneously interacts with two molecules of incomplete agglutinins independently bound to the surfaces of two distinct erythrocytes, thus resulting in hemagglutination.

The direct version of Coombs' test is used for detection of anti-erythrocyte autoantibodies that may cause hemolytic anemia in humans.

Indirect Coombs' test (or *indirect antiglobulin test*) detects *free incomplete antibodies* in patient serum (e.g., in pregnant women). To aim this, patient's serum containing free incomplete antibodies is taken; next it is adsorbed upon human erythrocytes of I (0) group like carriers. After successful adsorption of monovalent antibodies on the membranes of carrier erythrocytes, animal antibodies against human immunoglobulins are added, and the reaction of hemagglutination occurs.

Indirect Coombs' test is successfuly used for testing of pregnant women and for testing of blood samples from donor and recipient before blood transfusion for the presence of anti-Rh antibodies.

Precipitins and Precipitation Tests

Precipitins *are the antibodies, which produce the formation of minute insoluble deposits* (***precipitates***) *owing to their specific interaction with a* ***soluble*** *antigen.*

Precipitation ensues from binding of a soluble antigen (***precipitinogen***) to the specific antibody (***precipitin***) in presence of electrolyte (e.g., saline) that results in formation of molecular-based insoluble complex or ***precipitate***.

The mechanism and external manifestations of precipitation test depend on the nature of *soluble* molecular antigen that should contain multuple epitopes (*multivalent structure*). From the other hand, precipitating antibodies must be bivalent (or polyvalent) with two or more active sites. In this case the cross-linkage of at least two molecules of an antigen occurs via the bridge of single antibody molecule, where two active sites of the same antibody become bound to the different molecules of antigens. Spatial expanding of precipitate results in visible manifestations of precipitation (formation of insoluble immune complex).

The precipitation is most clearly observed, when the transparent filtrate (colloid antigenic solution) is layered upon the transparent precipitating serum. A turbid white ring appears rapidly in the test tube at the borderline of the reacting components.

The precipitin reaction is quite specific and sensitive. It allows the detection of an antigen (precipitinogen) in the dilution up to 1:1,000,000.

The molecular extractions of antigens (i.e., precipitinogens) of anthrax, plague, and tularemia causative agents are thermostable. Some precipitinogens are resistant to heat up to 120-180°C.

Therefore, precipitation is used in the diagnosis of anthrax, tularemia, and other diseases by the detection of antigens of their causative agents.

Precipitation reaction is actively performed in the diagnosis of anthrax to identify the antigen of anthrax bacilli in the extractions from the animal organs and tissues and in various raw materials (animal skin, wool, or hair). This reaction is known as the ***thermoprecipitin Ascoli's test***. Antigenic extraction of interest is preliminarily boiled, then filtered to obtain a transparent solution and next layered upon the precipitating anti-anthrax serum with antibodies. As the result, the ***ring of precipitation*** is to be formed.

Similar termoprecipitin reactions (***ring precipitation tests***) can be used for the diagnosis of plague and tularemia. In these cases the antigen-containing extractions prepared from the inner organs of died rodents are investigated.

In forensic medicine precipitation tests are elaborated to determine the origin of blood spots, sperm or other biological fluids, in sanitary microbiological testing – to detect the falsification of various foodstuffs (e.g., the spoilage of natural honey with artificial one, the falsification of fish, meat, and flour goods, etc.)

A great set of precipitating species-specific sera is manufactered, which are obtained by the multiple and long-term immunizations of animals by the corresponding antigens. At the end of the immunization

course the blood is taken from the immunized animal (rabbit, sheep, goats, etc.), a serum is isolated and its strength is determined.

The ***titer*** of the precipitating serum is established as *the maximum dilulion of antibody* (***precipitin***), *where a clearly visible precipitation is detected*.

There is a great variety of precipitation techniques.

Immunodiffusion tests are carried out in the agar or another gel medium. One of such techniques, ***Ouchterlony`s test*** is based on ***double agar diffusion***. Here the antigen and antibody are placed into separate wells cut in the agar. After the start of incubation, the well contents diffuse within the agar towards each other.

When the specific antigen and its antibody meet between the wells in equivalent concentrations, one or more lines of immune precipitate occur. This method is useful in identifying of unknown antigenic substances. Also it can be used to test the serum sample that contains the various types of antibodies against the certain antigens.

Immunoelectrophoresis is a variation of gel precipitation technique. It combines precipitation test with electrophoresis. Electrophoresis is used for rapid separation of the complex mixture of proteins under the influence of electrical field.

After electrophoresis the final distribution of antigenic fractions within the gel is developed by specific complex antibodies placed into a groove that is made in gel along the line of antigen movement. Each antigen gives an individual band of precipitation with the specific antibody. The method allows to detect various pathological antigenic fractions in their complex mixtures (tissue extractions, patient's sera, etc.)

Single radial immunodiffusion (***SRID***) or ***Mancini test*** is used predominantly to evaluate the concentrations of serum immunoglobulins.

Sensitivity of method is enough to determine the concentrations of 3 major immunoglobulin classes – IgG, IgA, IgM.

This gel precipitation technique is based on agar incorporation of the specific anti-immunoglobulin sera that further react with immunoglobulin samples placed into agar wells.

To make the reaction, three separate gels with incorporated anti-sera to IgG, IgA and IgM are melted and then solidified upon three glass plates. The wells in agar are prepared, and specimens of patient's sera with unknown Ig concentrations are placed into wells. Immunoglobulins of sera diffuse into agar and interact with corresponding anti-serum that results in appearance of radial zones of precipitation. The higher the concentration of Ig, the greater the diameter of precipitation zone is. By parallel testing of

serum with different known Ig concentration, a calibration curve can be plotted. The method is used for quantitation of IgG, IgA, and IgM classes in patient's serum samples.

Reactions of Toxin Neutralization by Antitoxin

Many bacteria produce various exotoxins (*C. botulinum, C. tetani, B. anthracis,* etc.)

Antitoxins *are specific antibodies, which bind to microbial toxins and neutralize them.*

In this reaction both toxin and antitoxic serum (antibodies) can be determined.

Exotoxins are obtained *after cultivation of toxigenic bacteria in liquid nutrient media with subsequent filtration*.

Antitoxic sera are derived from blood of animals (e.g. horses) hyperimmunized with toxins and/or toxoids. Also antitoxic antibodies can be obtained from the blood of human donors immunized with toxoids.

Overall, neutralization tests comprise the versatile number of biological and serological reactions that result in common final effect – the blockade of toxin activity by specific antibodies. Various models for the development of neutralization activity are used (laboratory animals, cell cultures, serological tests, etc.)

In vitro manifestation of toxin-antitoxin binding in liquid phase is ***flocculation reaction***. Flocculation results in specific clumping of toxin by antitoxin within the test tube. The mechanism of flocculation test is similar to precipitation.

Flocculation reaction is used for determination of the ***strength of antitoxic sera***.

One International unit of antitoxic serum or antibodies (1 IU) is the dose of antitoxic serum that neutralizes the equivalent amount of toxin.

For the titration of antitoxin by flocculation method a known amount of toxin is used. It is added to different dilutions of antitoxic serum. After the incubation, the most rapid "*initial*" flocculation (formation of turbid aggregates) appears in the test tube, where the quantity of antitoxic serum and toxin is equivalent.

For the ***assessment of toxigenicity*** of diphtheria bacterial cultures the method of neutralization on solid nutrient medium is used. It is based on the interaction of antitoxic serum and the toxin produced by the strains of *Corynebacterium diphtheriae* during their growth.

For this purpose, a sterile strip of filter paper soaked with antiserum against the diphtheria toxin is placed upon the center of a Petri dish. Testing cultures are planted perpendicularly to the strip with anti-toxic sera. If the culture produces exotoxin, it diffuses into the nutrient medium, and the reaction of precipitation with antitoxin occurs. The resulting precipitate is detected as fine whity lines on both sides of the streaked culture.

Special variant of ***neutralization test on animal model*** is broadly used in microbiology. In that case mixture of toxin and anti-toxic serum is injected into experimental group of animals (e.g., mice). Control group is treated with toxin alone. In case of toxin neutralization by antibodies experimental animals survive but the control group members die due to the toxin action.

Antitoxic sera of known strength are applied for treatment of infections caused by toxigenic bacteria – botulism, tetanus, diphtheria, clostridial anaerobic infections, as well as for treatment of snake bites.

Complement-Dependent Serological Reactions (or Lysis Reactions)

Complement system can be used in certain serological reactions for Ab or Ag determination. These tests are known as ***lysis reactions***. However, many of them demonstrate mostly the historical interest due to their moderate sensitivity and hard test performance (they are laborious and time-consuming).

Lysis reactions comprise ***bacteriolysis***, ***hemolysis*** and ***complement fixation tests***.

Cell lysis occurs under the action of three main components of these reactions: *specific antibodies* from the immune serum, corresponding *antigen* and a *lytic substance* present in any serum – *complement system*.

Bacteriolysis reaction is only of historical interest. It was discovered in 1890s, when V. Isaiev and R. Pfeiffer revealed the antibodies (***bacteriolysins***) capable of dissolving bacteria in the blood of the immune animals. This reaction was primarily used for bacterial identification and for antibody determination. Being of limited sensitivity, it is not in practical use now.

Hemolysis Reaction

After immunization of rabbits with a suspension of sheep red blood cells (SRBC), the specific antibodies (***hemolysins***) arise in the rabbit's blood that are capable of interacting with sheep erythrocytes. This ***hemolytic serum*** is used in ***hemolysis reaction*** and ***complement fixation test***. It has to be heated at 56° C for 30 minutes for inactivation of its own complement. The addition of fresh animal serum, even a non-immune one, restores the hemolytic properties of the immune serum. For that purpose *guinea pig serum as complement source* is used.

If hemolytic serum (antibody), sheep erythrocytes (antigen) and complement are placed into a test tube in definite quantitative proportions, then in a few minutes hemoglobin starts to pass out of the erythrocytes into the surrounding fluid, and the medium is colored reddish. It results from the complement damage of erythrocyte membranes. The amount of hemolysis can be estimated visually or by colorimetric methods.

The reaction of hemolysis has a strictly marked specificity. It is used as a detection system for complement-fixation test and for ***determination of serum complement activity***. When quantitatively measured, serum complement activity is expressed in units of 50% hemolysis (CH50). The levels of serum complement increase at the beginning of infectious process, but may fall significantly in autoimmune diseases (e.g., in systemic lupus erythematosus) due to the complement consumption by immune complexes.

Complement Fixation Test

The specific interaction of antibody and antigen leads to the adsorption of complement. This action cannot be directly visible. In 1901 J. Bordet and O. Gengou introduced an indicator second system ***(hemolytic)*** into this reaction. It is composed of a suspension of sheep red blood cells and the corresponding hemolytic serum containing hemolysins.

In complement fixation test ***two systems*** of reagents are included: antigen with antibody and complement (***first specific system***), and mixture of sheep erythrocytes with hemolytic serum (***second indicator hemolytic system***). This reaction can determine either unknown antibodies or antigens depending on test modification.

Both systems are placed into thermostat for incubation. Then they are mixed together in the separate test tube, and the mixture is incubated again

in a thermostat for 30-60 minutes at 37°C or at 4°C overnight. Complement fixation test (***CFT***) is ***regarded as positive*** in case of ***hemolysis absence***. This result is obtained because of the complement consumption by antigen-antibody complex has been formed in the specific system. Therefore, the second system is not hemolyzed as no complement is left for it, and the medium remains opaque. In case of positive result the erythrocytes finally settle to the bottom of the tube, and the supernatant fluid becomes transparent and colorless.

In case of a ***negative reaction*** of the ***CFT*** the complement doesn't bind to Ag-Ab complex but reacts with the complex "sheep erythrocytes-hemolysin", causing ***hemolysis*** – the medium becomes transparent and red after the lysis of sheep red blood cells.

Complement fixation reaction has rather high specificity and retains its significance for some medical applications. For instance, this test is used in *serological diagnosis of syphilis* (***Wassermann's reaction***), Q fever, epidemic typhoid fever and other rickettsioses, glanders, and several viral diseases (e.g., influenza). It may be used either for determination of the titer of specific antibodies or for the antigen identification.

Reactions with Labeled Antibodies and Antigens

Reactions, involving labeled antigens or antibodies as tagging reagents, are the most sensitive among the all of serological reactions. They comprise ***immune fluorescence assay***, different variants of the ***enzyme immunoassay*** including ***ELISA***, ***radioimmunoassay***, ***western blotting*** and some other tests (*immune electron microscopy*, the tests of *immunohistochemistry*, etc.) Besides of extreme sensitivity, these reactions are not cumbersome, being available for specimen mass screening by the versatile automatized registering facilities.

Immunofluorescence Assay

In this method a great number of fluorescent dyes (e.g., *fluorescein*, *rhodamine*) are used as specific reporter labels. As a typical example, ***fluorescein isothiocyanate*** (***FITC***) can be attached to known specific antibodies. This labeled antibody is used to identify epitopes on the surfaces of complex corpuscular antigens (microbial and eukaryotic cells,

erythrocytes, etc.) Dye, covalently attached to antibody molecules, becomes visible by ultraviolet irradiation in the luminescent microscope.

In another case, dye-labeled antiglobulin antibody may be employed to reveal the presence of a specific antibody in patient or animal serum.

These tests are quick, accurate and readily performed. They are divided into two distinct types: ***direct*** and ***indirect immunofluorescence tests***.

In first case of ***direct reaction*** the microorganism to be tested is fixed to a slide. The known antibodies labeled with ***fluorescein isothiocyanate*** (***FITC***) are added to the slide. They should interact directly with unknown microbial antigen. After short-term incubation the slide is washed thoroughly. Antibodies fixed upon the microbial antigens remain hold on the slide. Then the specimen is examined by UV-light luminescent microscopy. If the microorganisms correspond to the antibody, they will be surrounded with bright yellow-green halo.

The indirect fluorescent antibody test is used in both directions: for identification of unknown bacterial cells or for estimation of specific antibodies in patient's serum. In latter case the known microorganism is fixed on the slide, and a sample of the unknown serum is added to it. After incubation the slide is washed. If the unknown serum antibodies match the antigen, they remain fixed to it. The attached unknown antibody is to be detected by next treatment with a *fluorescent-labeled antiglobulin antibody* (anti-human gamma globulin). Finally, the slide is examined by ultraviolet microscopy. When viewed with UV light, the complex appears yellow-green. The indirect test is often more sensitive than the direct one because the larger amounts of labeled antibodies absorb by antigen molecules. Moreover, the labeled antiglobulin is a "universal reagent" that binds to all antibodies that pertain to the same species.

Both types of immune fluorescence reactions are employed widely in immunology and microbiology. They are used in microbial identification, cell typing, for detection of virus-infected cells, etc.

Indirect assay is available for serological diagnosis of Lyme disease, Q fever, syphilis and many other pathological processes, where specific antibodies to infectious agents arise.

Enzyme Immunoassay

In ***enzyme immunoassay*** (or EIA) antibody or antigen conjugated with the enzyme (enzyme-labeled) take part. This test has many technical

variations. The resulting enzymatic activity is revealed by reaction with appropriate substrate. Horseradish peroxidase or alkaline phosphatase are used frequently for enzyme labeling.

In immunological testing the basic are ***homogenous*** and ***heterogeneous*** (or ***solid-phase***) modifications of enzyme immunoassay. The latter is widespread especially for specific antibodies determination. It is usually named as ***enzyme-linked immunosorbent assay*** (or ***ELISA***).

To detect antibodies here, the known antigens are attached to a solid phase (typically, to the bottom of plastic microdilution plate wells). Test serum dilutions are put into the wells. If Abs match the antigen, immune complex is formed. After incubation serum excess is removed, and the wells are washed. Then the specimens are re-incubated with an ***anti-immunoglobulin antibodies*** labeled with an enzyme (***horseradish peroxidase***). Anti-immunoglobulin-enzyme conjugate binds to antibodies present in the immune complex. After next wash enzyme activity is evaluated by adding the specific ***substrate*** (***hydrogen peroxide***) and ***chromogen*** (e.g., o-phenylene-diamine). Chromogen is a colorless substrate substance that produces a color end-product, when acted upon by an enzyme. Enzyme reaction is stopped by adding of sulphuric acid. Brown color of reaction medium appears, and the reaction is assessed with multichannel colorimetric analyzer (microplate reader). Optical density of samples is proportional to the amount of antibody bound.

For the determination of an antigen by ELISA, the antigen-specific antibodies are first absorbed on the bottom of plastic wells of solid phase. Then the antigen-contaning clinical specimens are added and incubated with antibodies. If the specific antigen is present in the sample, it binds to specific antibodies creating an immune complex. After thorough wash the bound antigen is repeatedly treated with a new generation of specific antibodies usually from another species origin. This is known as the "***sandwich***" version of ELISA test. The remaining steps of this test (like addition of species-specific antiglobulin conjugate and application of other reagents) are virtually the same as for conventional ELISA technique.

ELISA test is very sensitive (as RIA, see below) but unlike RIA it doesn't require special expensive equipment or safety measures for technical personnel.

Radioimmunoassay (RIA)

Radioimmunoassay was introduced into medical practice still before the enzyme immunoassay. At first it had many practical applications. By

means of RIA it became possible to test a large number of samples in a very short period of time using very small amounts of reagents.

Nevertheless, being more difficult in handling, RIA required high cost equipment, radioactive isotopes and special safety measures. Thus, it is being ousted by EIA. Now RIA is used predominantly to determine the quantity of antigens or haptens that can be radioactively labeled. These antigens are usually the substances of low molecular weight (drugs, chemicals, toxins, etc.) This variation of RIA is based on the competition for specific antibody between the labeled and non-labeled antigen of unknown concentration (***competitive RIA***).

In this case the specific antibodies are adsorbed on the solid phase (e.g. plastic globular particles). The known quantity of labeled antigen is added to Ab-coated particles. Usually ^{125}I or ^{131}I isotopes are used here as a radioactive tag. After antigen-antibody binding the immune complexes are formed.

Then the clinical samples containing the unknown concentrations of an Ag are introduced into the system. The unlabeled antigens start to replace the labeled antigens in Ab-Ag-complex. This replacement is proportional to unknown antigenic concentration in the sample. As the result, the labeled antigens are released from the solid phase to the surrounding liquid medium. After separation of solid and liquid phases of the reaction the amount of radioactivity is measured using a counter for radioactivity. The concentration of the unknown (non-labeled) antigen or hapten is determined by comparing the results with those obtained with several concentrations of a pre-determined standard antigen.

This method can detect less than 1 ng/ml of an antigen.

RIA is extremely sensitive method applied to the assay of hormones or drugs in biological fluids. A specialized RIA, the ***radioallergosorbent test*** (***RAST***), is used to estimate the quantity of serum IgE antibodies that react with a known allergen (antigen).

Immunoblotting

Immunoblotting or ***western blotting*** is a method for identification of necessary antigen or antibody in a complex mixture of various protein substances.

In this method the mixture of proteins (antigens) undergoes sodium dodecyle sulfate (SDS)-***polyacrylamide gel electrophoresis*** (***PAGE***). SDS can decompose any non-covalent complexes and aggregates in the

antigenic mixture. Polyacrylamide gel electrophoresis separates the proteins according to their molecular size and charge. The gel is washed and covered with a membrane (e.g., a sheet of nitrocellulose).

At next step separated proteins are transferred from gel to the membrane by electrophoresis in perpendicular direction. The nitrocellulose membrane (***blot***) acquires a replica of the proteins separated by SDS-PAGE. During the transfer, the SDS is largely removed from the proteins, so the antibodies can react with the proteins on the membrane.

The nitrocellulose membrane is then treated with enzyme-labeled antibody. Both direct and indirect registrations are possible. In latter case antibodies are revealed by enzyme-labeled antiglobulin antibodies. This looks very similar to ELISA tests.

After substrate conversion the protein antigen becomes visible. It looks like a color band on the membrane. The band appears after the enzyme action that leads to the production of insoluble color product of the reaction. None of the other proteins in the mixture would be visualized.

In the same way the different kinds of specific antibodies directed aganist the number of specific antigens can be discovered by western blotting technique. The latter analysis is commonly used as the confirmatory test for the detection of anti-HIV antibodies.

Chapter 18

IMMUNOPATHOLOGY: TYPES OF HYPERSENSITIVITY. ALLERGY AND AUTOIMMUNE DISEASES. PRIMARY AND SECONDARY IMUNODEFICIENCIES

Immunopathology

Immunopathology encompasses the disorders with substantial role of immune mechanisms in their emergence and progression.

They are divided into three main groups:

1) *diseases with immune system hyperactivity* (***allergy*** and ***autoimmune diseases***);

2) *diseases with suppressed state of immune system* (***primary*** and ***secondary immunodeficiences***);

3) *leuko- and lymphoproliferative disorders* (acute and chronic leukemias; lymphomas, e.g., Hodgkin`s disease; multiple myeloma, Waldenström's macroglobulinemia, and others)

Types of Hypersensitivity

All allergic and autoimmune diseases develop on the ground of various types of hypersensitivity of the immune system.

Hypersensitivity means *the excessive immune reaction against some antigens or allergens*.

There are two basic types of hypersensitivity: ***immediate*** and ***delayed***.

Immediate type of hypersensitivity depends largely on humoral immune response that is followed by synthesis of antibodies of different classes. This type of reactions evolves rapidly and even instantly after the contact with an antigen – from several seconds (in case of anaphylactic shock) to 12-24 hours (urticaria), in typical cases – in about 30 minutes.

Various kinds of immunopathological reactions (anaphylactic, cytotoxic, immune complex-mediated, anti-receptor stimulatory and blocking reactions) are based on the mechanisms of immediate hypersensitivity.

Manifestations that develop in 4-12 hours or even later after the onset of antigen exposure are regarded as "***late***" reactions of immediate type.

Delayed type of hypersensitivity evolves in 24-72 h after antigenic challenge. Delayed hypersensitivity is predominantly maintained by

cooperation of antigen-specific T cells, dendritic cells and phagocytes. It is ***cell-mediated type*** of hypersensitivity.

Well-known immunologists P.G.H. Gell and R. Coombs proposed the classification of different types of hypersensitivity reactions. This division was grounded on the distinct immune mechanisms essential for certain kinds of immunopathology.

According to Gell and Coombs, five predominant types of immunopathological reactions were defined:

– ***anaphylactic*** hypersensitivity (***type I***);
– ***cytotoxic*** hypersensitivity (***type II***);
– ***immune complex-mediated*** hypersensitivity (***type III***);
– ***cell-mediated*** or *delayed* hypersensitivity (***type IV***);
– *stimulatory* and *blocking* (***receptor mediated***) hypersensitivity (***type V***)

First Type of Hypersensitivity – Anaphylaxis or IgE-dependent Reactions

The majority of allergic diseases is developed according to the first type of hypersensitivity reactions (anaphylactic shock, atopic bronchial asthma, urticaria, angioneurotic edema, pollinoses, allergic rhynitis, insect allergy, food allergy, etc.)

By contrast, these reactions are almost negligible in autoimmune disease pathogenesis.

Allergy. Mechanisms and Stages of Development

Allergy *is the specific excessive secondary immune reaction to allergen (antigen) followed by tissue damage and organ dysfunction.*

The specifity of I^{st} type of allergic reactions is maintained by production of anti-allergen antibodies named as "***reagins***" or "***cytophilic antibodies***" due to their ability to interact with different cell lines.

These antibodies arise after primary antigen challenge, and their concentration increases during subsequent contacts. Clinical manifestations of allergy emerge only after the ***secondary antigenic challenge***.

Time period between the primary interaction of allergen with immune system and their secondary contact with allergy development is termed as

sensitization period. It lasts from several days to several months, years and even decades from primary antigenic stimulation.

At this latent period allergen-specific T- and B cells populations proliferate and differentiate with memory cell formation. Th2 cell subpopulation activates B cells by direct contact and by secretion of IL-4, 5, 9, 13, and 15. Activated B lymphocytes switch the production of allergen-specific antibodies to IgE class capable of stimulating mast cells and basophils. This is next followed by basophil/mast cell degranulation resulting in allergy manifestations.

Secretion of gamma interferon, IL-1, or IL-12 by Th1 and macrophages inhibits allergic reactions.

There are several consecutive stages in progression of allergic reactions:

– ***sensitization*** stage (period);
– ***immunological*** stage;
– ***pathochemical*** stage;
– ***pathophysiological*** stage;
– stage of ***clinical manifestations***

In ***sensitization period*** allergen-specific antibodies of IgE class and some subclasses of IgG (e.g., IgG4) arise after antigen priming. These antibodies eventually fix to specific membrane Fcε-receptors on basophils and mast cells.

In ***immunological stage*** as the result of *secondary* contact the allergen binds to IgE-antibodies on basophils and mast cells. This interaction promotes basophil/mast cell activation. It occurs due to the cross-linkage of two membrane IgE-receptors that is followed by membrane signal transmission into the cells. Cellular *src-thyrosine kinases* phosphorylate the number of regulatory proteins, resulting in basophil degranulation. It is noteworthy that after the degranulation basophils retain their viability.

Pathochemical stage begins from the moment of granules release. Pre-existing ***primary*** allergy ***mediators*** are liberated immediately. There are ***histamine***, ***serotonin***, ***platelet-activating factor (PAF)***, specific proteases of mast cells – ***tryptase*** and ***chymase***.

At the same time the other mediators of allergy begin to synthesize *de novo*. The major ones are ***arachidonic acid metabolites***.

They are formed by the ***cyclooxygenase*** and ***lipoxygenase pathways*** due to ***phospholipase*** $\boldsymbol{A_2}$ action.

First pathway results in production of ***prostaglandins*** and ***thromboxanes***. Most active are prostaglandin $F_{2\alpha}$, D_2 and thromboxane A_2. By contrast, prostaglandin E_2 inhibits allergic reactions.

Second lipoxygenase pathway is slower. It provides the synthesis and liberation of the most powerful allergy mediators – ***leukotriens B4***, ***C4***, ***D4***.

Usually allergy mediators decrease intracellular concentration of cyclic AMP.

At the same time different cytokines and chemoattractants are produced (IL-4, 5, 6, 8, eosynophil and neutrophil chemotactic factors). Eosynophils, neutrophils, macrophages and other cells become accumulated in allergy area (late phase of reaction). They produce other ***secondary messengers*** (***bradykinin***, ***heparin***, ***complement factors*** and many others). Interaction of mediators with vessel endotheliocytes accelerates cells extravasation.

Mediator action provokes significant local or systemic hypersensitivity response resulting in ***pathophysiological stage*** of allergic reaction. Tissue inflammation, skin rashes, glandular hypersecretion, bronchial obstruction, arterial hypotension with collapse are developed.

The last phase is the ***stage of clinical manifestations***. The symptoms of various allergic diseases (anaphylactic shock, bronchial asthma attack, urticaria, pollinose, angioneurotic edema or Quincke's disease, food and drug allergy, allergic rhynitis and others) develop in this stage.

Type II – Cytotoxic Hypersensitivity. Autoimmune Diseases Developed by Type II Hypersensitivity

The reactions of second type ensue from antibody recognition of ***cell bound antigens***. Antibodies engaged in these reactions are presumably of ***IgM*** and ***IgG*** class. As immune complex on the cell membrane has been formed, it initiates complement classical pathway activation. Membrane attack complex destroys target cells causing tissue damage.

Different autoantigens, bacterial antigens, chemical substances and drugs, adsorbed on the cell surface, can trigger these cytotoxic reactions.

Another pathway of cytotoxic hypersensitivity is provided by killer cells and phagocytes – ***antibody-dependent cell-mediated cytotoxity – ADCC***. It is exhibited by phagocytic myeloid cells (polymorphs and monocytes) and by natural killer (NK) cells bearing Fc receptors to Ig molecules. Contact between the effector and target cells via immune

complex triggers the liberation of cytotoxic molecules from leukocytes thereby promoting target lysis.

The large number of autoimmune processes is evoked by cytotoxic mechanism. It dominates in various diseases affecting blood cells (autoimmune hemolytic ***anemia***, aplastic anemia, agranulocytosis, ***thrombocytopenic purpura*** or Werlhof's disease, etc.), where blood cells are destroyed by antibody-mediated lysis.

Type III – Immune Complex-Mediated Hypersensitivity. Autoimmune Diseases Mediated by Type III Hypersensivity

Soluble immune complexes are responsible for these immunopathological reactions. Immune complex formation is an ordinary event within immune response, but in some cases redundant quantity of immune complexes is neither seized no degraded by phagocytes. These immune complexes appear in blood stream. After a period of circulation they attach to endothelium of capillary vessels. Ag-Ab aggregates form deposits under vascular basal membrane.

Settled immune complexes are able to activate both classical and alternative pathway of complement system. Complement activation promotes endovascular inflammation (***autoimmune vasculitis***). Complement activation products inhibit cell migration with their involvement into vasculitis intensification. Tissues and organs with rich capillary net are greatly susceptible to immune complex damage (lungs, kidneys, skin, sinovial and connective tissue).

The examples of autoimmune diseases, caused by immune complex-mediated hypersensitivity are ***systemic lupus erythematosus*** (***SLE***), ***rheumathoid arthritis*** (***RA***), ***autoimmune glomerulonephritis***, ***serum sickness*** and many others.

Primary antigen, triggering immune inflammation in ***SLE***, is a protein-DNA complex. Immune complexes settle in different organs (kidneys, lungs etc.) Complement fixation and further activation of immune cells results in tissue inflammation that leads to deep organ dysfunction. The most dangerous in SLE is severe glomerulonephritis ("*lupus-nephritis*").

Joint inflammation in ***rheumathoid arthritis*** is induced by immune complex attachment to sinovial tissue and cartilages. Primary autoantigen epitopes in RA are localized in Fc portion of patient's IgG, which becomes autoantigenic due to its abnormal glycosylation. Autoantibody (so-called

"***rheumatoid factor***") usually of IgM class binds to own IgG molecules thereby provoking complement activation and immune cell involvement.

Serum sickness is developed following repeat injections of large quantities of foreign proteins or drugs. The antigen is slowly cleared from the circulation, and therefore, elicits the generation of specific antibodies. It may happen during intensive treatment of life-threatening diseases (diphtheria, botulism, tetanus, etc.) with xenogenic horse antitoxic sera. Reaction is followed by fever, vasculitis, urticaria, arthralgia, and kidney damage.

In current situations serum sickness appears to be more often in case of beta-lactam antibiotic treatment.

Type IV– Cell-Mediated (or Delayed) Hypersensitivity

Delayed hypersensitivity is observed in many diseases of different etiology (contact dermatitis, multple sclerosis, allergy to metal ions, sarcoidosis and great number of infections – tuberculosis, leprosy, brucellosis, tularemia, Lyme disease, etc.)

Cell-mediated reactions are predominantly stimulated by Th1 cells. The reactions are developed in 1-3 days after antigen exposure. Th1 secrete IL-2 and γ-interferon, involving macrophages and dendritic cells into the reaction. They produce pro-inflammatory cytokines (presumably IL-1, IL-6, IL-12, IL-18, α-TNF) supporting chronic productive inflammation. Connective tissue proliferation restricts the pathogen spread forming ***chronic granuloma*** in the site of inflammation.

Assessment of delayed type hypersensitivity is useful in various infections, where the slow cell allergic reactions evolve. It is essential for mentioned above tuberculosis, leprosy, brucellosis, as well as for syphilis, tularemia, glanders, actinomycosis and some other disorders.

The development of delayed hypersensitivity depends on bacterial antigenic structure. Some bacterial antigens (e.g., cell wall components) activate predominantly Th1 and macrophages promoting cell-mediated infectious allergy. These diseases are characterized by long incubation period with unclear manifestations. At that time the diagnosis of such disorders is performed with different laboratory methods including ***skin tests***.

Skin tests for laboratory diagnosis of infectious pathology *evaluate* ***delayed hypersensitivity*** *to infectious antigens* (*allergens*). The latter are usually injected intracutaneously.

Well-known example of disease diagnosis made by skin test is tuberculin *Mantoux* test in tuberculosis (***tuberculin skin test*** or ***TST***).

Tuberculin is an infectious allergen for estimation of patient hypersensitivity to *M. tuberculosis*. R.Koch obtained it by filtration of the old culture of *Mycobacterium tuberculosis* after continuous cultivation of bacteria in liquid nutrient medium. At first it was applied for tuberculosis treatment but without success. Nevertheless, it was proven to be quite suitable for tuberculosis diagnostics. For this purpose it was purified further to derive protein fractions (***tuberculin PPD*** or ***purified protein derivative***).

When a small amount of tuberculin is injected into the skin of a patient previously exposed to *Mycobacterium tuberculosis*, skin induration and redness develop, which reach a peak in 24-72 hours. Mononuclear cells settle the skin and subcutaneous tissues. A positive skin test indicates that the person has been infected by *M. tuberculosis*. Test explanation is not very easy because the reaction is influenced by different conditions (previous BCG vaccination and chemotherapy, patient anamnesis, immune status conditions, etc.) However, a change of skin test from negative to positive implies recent infection and possible current activity of tuberculosis. A positive skin test response assists in diagnosis being helpful for control of tuberculosis treatment.

In leprosy, a positive ***lepromin*** skin test indicates tuberculoid form of disease with non-suppressed cell-mediated immunity, whereas a negative test corresponds to lepromatous leprosy with severe inhibition of cell-mediated immunity.

Skin tests are applied successfully in brucellosis (***Burnet test*** with ***brucellin***) and tularemia laboratory diagnosis (skin test with ***tularin***).

In systemic mycotic and some protozoan infections (histoplasmosis, blastomycosis, toxoplasmosis, etc.) a positive delayed-type skin test with the specific microbial antigen supports to diagnose the corresponding infection. Also cell-mediated hypersensitivity develops in many viral infections (herpes simplex, mumps, etc.)

Type V – Stimulatory and Blocking (or Receptor Mediated) Hypersensitivity. Autoimmune Diseases, Developed by These Reactions

In certain pathological cases autoantibodies against membrane receptors of the host cells are produced. These antibodies are able to modulate receptor function activating or repressing cellular activity.

Sometimes receptor-mediated hypersensitivity is referred to as a special kind of II type or cytotoxic hypersensitivity. Nevertheless, taking into account the specific mechanisms and manifestations of stimulatory and blocking reactions it became relevant to separate them into the distinct type of hypersensitivity (*type V*).

Bright example of autoantibody stimulating function is ***Graves` disease*** development. Antibodies known as ***long acting thyroid stimulator*** (***LATS-factor***) play the main role in its pathogenesis.

LATS-factor was proven to be the autoantibody of IgG class that binds to receptors of thyroid-stimulating hormone (thyrotropin) on thyroid gland cells. Autoantibody stimulates the receptors and initiates uncontrolled thyroid hormone production by thyrocytes. Hyperfunction of thyroid gland leads to patient`s hyperthyroidism.

The opposite action of autoantibodies – cell receptor block – is essential for the neuronal disorder ***Myasthenia gravis***. In that case autoantibodies hinder the impulse transmission within neuromuscular synapse. These antibodies bind to acetylcholine receptor on post-synaptic muscle membrane thus impairing its activity. Receptor inactivation promotes the progressing muscular weakness with poor disease prognosis.

Imunodeficiencies

According to their origin all ***immunodeficiences*** are divided into ***primary*** and ***secondary***.

Primary immunodeficiences are the hereditary diseases originated from various genetic abnormalities.

Secondary immunodeficiences arise as the result of deleterious influences of various external or internal factors (starvation, severe acute and chronic diseases, unfavorable environmental conditions – chemical and radiation pollution, low-quality foodstaffs, etc.)

Immunodeficiencies can affect any part of immune system. Most important are immunodeficiences of lymphoid system. Likewise, they can affect mononuclear phagocytes and granulocytes, complement system and many other subsets of immunity. In large number of cases the combined immune disturbances occur.

However, in many cases immunodeficiences remain latent owing to the great reserves and cross-reactivity between the links within the immune network.

Primary Immunodeficiences

Severe combined immunodeficiency syndrome (SCID)

This state is characterized by profound impairment of differentiation of various immune cell precursors, including lymphoid stem cells. There are several SCID variations.

The main variant of SCID is based on gene abnormality localized in X-chromosome. It impairs the synthesis of *IL-2 receptor gamma-chain*. This common chain is also the obligate constituent of IL-7 receptor molecule. As IL-7 is one of the major differentiation factors for blood cells, the maturation of T- and B cells is ceased.

SCID emerges also in case of several enzyme deficiencies, where the cells are lack of production *adenosine deaminase* or *purine nucleoside phosphorilase*. This leads to accumulation of toxic metabolites of nucleic acids degradation within immune cells.

The prognosis of SCID is very poor, and patient survival is possible only owing to advanced treatment methods, including gene therapy or bone marrow transplantation.

Ataxia-telangiectasia or Louis-Bar's syndrome

It is a human autosomal recessive disorder associated with chromosome impairment in genes of the Ig heavy chains and T cell receptors. It is characterized by progressive cerebellar ataxia with Purkinje cells degeneration, high incidence of cancer, greater susceptibility to respiratory infections, microcirculation disturbances. The process is followed by profound combined T- and B cell deficiency.

Wiskott-Aldrich syndrome

Patients suffering from ***Wiskott-Aldrich syndrome*** possess immunodeficiency with thrombocytopenia and eczema. Immune cells in that case lack a *surface molecule* ***sialophorin*** (CD43), which is a ligand for adhesion molecule ICAM-1. Wiskott-Aldrich syndrome is associated with a low IgM and a poor response to many polysaccharides due to the dysfunctions of T- and B cells cooperation.

T cell immunodeficiences

DiGeorge syndrome

This disease is evolved due to severe disorders in thymus development (*thymus dysgenesis*) from the third and fourth pharyngeal pouches in

embryogenesis. The sick children also lack parathyroid glands and have severe cardiovascular abnormalities.

Thymus dysgenesis (*aplasia* or *hypoplasia*) results in the block of T cell precursor differentiation. Cell-mediated immune response is absent. Antibody response can appear but it is impaired against majority of antigens because of T helper depletion.

Treatment by transplantation of thymus may perform the restoration of immunocompetence, but this method is being only worked out.

B cell immunodeficiences

Bruton's syndrome

Bruton's syndrome, or ***Bruton's congenital agammaglobulinemia*** is developed in males and affects B cells maturation on the level of pre-B cells differentiation. The disease is associated with X-chromosome. The main cause of it is the failure of variable genes rearrangement due to tyrosine kinase gene mutations. Serum immunoglobulin levels are negligible. Cell-mediated immune response is normal. These children are very susceptible to infections caused by pyogenic bacteria. In case of adequate substitutive therapy with intravenous immunoglobulin (IVIG) the patients with Bruton's syndrome can survive.

Common variable immunodeficiency pertains to the most common primary immunological disorders. It comprises the number of related syndromes resulted from B cell dysfunction, T helper insufficiency, dysregulation of cytokines and costimulatory molecules. This ailment is usuallly manifested at the age of 25-30 years by lymphoadenopathy, splenomegaly, recurrent infections of respiratory and gastrointestinal tract. The patients demonstrate the elevated risk of lymphoid-derived tumors. Blood concentrations of all immunoglobulin classes are seriously reduced.

Selective IgA deficiency is the most frequent variant of ***disimmunoglobulinemia***, where only one or few Ig classes are decreased or absent. The incidence of this syndrome is about 1:700. Sometimes it comes without manifestations but usually patients are susceptible to respiratory and enteric infections.

Immunodeficiencies of mononuclear phagocytes and granulocytes

These disturbances can impair any stage of phagocytosis. First group comprises the disorders in phagocyte chemotaxis, second one – in opsonin functions, the third – in surface receptor expression, and one more includes the defects in respiratory burst with impaired microbial killing.

The example is ***chronic granulomatous disease***, where granulocytes are *lack of functional NADPH oxidase* for respiratory burst activation. Phagocytes ingest microbes normally, but subsequent microbial killing is slow or absent. These patients suffer from different bacterial infections.

Chediak–Higashi syndrome is a rare disease inherited by autosomal recessive type. It is characterized by mutations affecting *lysosomal trafficking regulator protein*. This is followed by formation of giant vesicles within phagocytes uncapable of fusion with lyzosomes due to cytoskeleton impairment. The dampening of phagocytosis results in recurrent opportunistic bacterial infections. The disease is manifested by fever, low neutrophil and platelet count; the patients demonstrate partial albinism with photophobia and bleedings.

Leukocyte adhesion deficiency or ***LAD syndrome*** is also rare autosomal recessive disease. Several clinical variants of this disorder include the deficiency of beta-chain (CD18) of leukocyte integrin molecules and the lack of expression of leukocyte selectins.

The disturbances in leukocyte chemotaxis, adhesion, migration to inflammatory focus and exravasation lead to severe bacterial infections; their treatment ultimately requires bone marrow transplantation.

Immunodeficiencies in complement system

Levels of any complement component may be decreased in case of primary complement immunodeficiency. Patients with C1, C2, C4 and C5 deficiency develop lupus-like state that affects microcirculation, whereas C3 deficiency is characterized by recurrent bacterial infections.

Hereditary angioedema is the result of Cl inhibitor deficiency. The disease has the dominantly autosomal inheritance.

Non-inflammatory edema of tongue, pharynx, and neck tissues appears by action of excess of vasoactive C2 fragment. These patients are usually heterozygotic and may synthesize small amounts of the inhibitor. Its concentration can be raised to sub-normal levels by androgen steroidal treatment. For urgent treatment of the disease C1 inhibitor concentrate isolated from donor’s blood is used.

Secondary immunodeficiencies

Secondary immunodeficiencies evolve under the pressure of different environmental conditions. They develop with a far greater rate comparing with primary ones. Secondary immunodeficiencies are followed by

multiple viral and bacterial acute and chronic infections of respiratory, urogenital and digestive tracts.

These disorders are not inherited. They usually have a specific cause, which provokes the immune system dysfunction, but the immune changes retain even after the causative factor disappearance.

Many infectious and non-infectious factors can trigger secondary immunodeficiencies.

Infectious agents can directly suppress immune cells. Many viral, fungal, parasitic and bacterial diseases stimulate secondary immunodeficiency. The most severe secondary immunodeficiency develops in the course of HIV-infection resulting in AIDS progression.

Non-infectious factors include immunosuppressive therapy, starvation, any severe chronic diseases (diabetes, cancer, cardiovascular or pulmonary failure, etc.), stresses, burns, post-operative traumas, intoxications, drug abuse, or unfavorable external factors (chemical and radiation pollution, low-quality food-staffs and others).

Any part of immune system can be affected with secondary immunodeficiency.

Chapter 19
IMMUNOPROPHYLAXIS AND IMMUNOTHERAPY. VACCINES, IMMUNE SERA AND ANTIBODIES

Active Immunoprophylaxis. Vaccines, Their Classification and Characteristics

Vaccinoprophylaxis is the most prominent discovery of medical science that reduced to negligible level the incidence of many life-threatening infections like poliomyelitis, yellow fever, rabies, tetanus, diphtheria, measles and others or even resulted in their complete eradication (e.g., in case of smallpox).

Vaccine (from Lat. *vacca* – cow) was named according to the first anti-smallpox substance prepared from the virus of cowpox by E. Jenner at the end of XVIII century.

Vaccines and ***toxoids*** (***anatoxins***) *are the antigenic biological products derived from corresponding infectious agents but deprived of their pathogenic effects*.

Vaccines are predominantly used for the ***prophylaxis of infectious diseases***. They confer ***artificial active immunity*** stimulating both cellular and humoral immune response.

Commonly used vaccines are classified as follows:

1) vaccines from live microorganisms (***live vaccines***) containing ***attenuated microbes*** devoid of virulence;

2) vaccines from inactivated microbial cultures (***inactivated*** or ***killed*** vaccines);

3) vaccines obtained by chemical treatment of microbial agents (***chemical vaccines***);

4) ***subunit vaccines*** containing individual protective antigens;

5) ***recombinant vaccines*** and ***DNA vaccines*** designed by methods of genetic engineering;

6) ***toxoids*** or ***anatoxins*** – molecular antigenic products derived from bacterial exotoxins usually by their formaldehyde treatment.

Live vaccines comprise biological antigenic products designed to prevent a great number of diseases – smallpox, tuberculosis, anthrax, tularemia, yellow fever, measles, poliomyelitis, mumps and others. The main advantage of live vaccines grounds on their capacity to confer full-

value immunity similar with natural post-infectious one. Nevertheless, a minimal likelihood to provoke disease-like complications by immunization with live vaccines still exists especially in immunocompromised patients.

For vaccination with live vaccines specially modified ***avirulent*** microbial strains are used. Diminishing of microbial virulence under the influence of various physical, chemical or biological factors is known as ***attenuation*** of microbial strain.

Famous example of attenuated vaccine is ***BCG vaccine*** for tuberculosis specific prophylaxis. A virulent strain of *M. bovis* was first attenuated in 1908, when the scientists A. Calmette and Ch. Guerin at Pasteur Institute started to cultivate it in nutrient media supplemented with bile. After 13 years of continious culturing in bile-containing medium the strain almost lost its virulence and became attenuated. The strain ***BCG*** (or *bacille Calmette-Guerin*) remains a single efficient vaccine for human immunization against tuberculosis.

Inactivated vaccines are created on the templates of microbial strains treated by various chemicals (ethanol, formaldehyde and other substances) or inactivated by heat. This group of vaccines comprises enteric fever, cholera, whooping cough, poliomyelitis and many other vaccines. Traditionally they are regarded as someway less effective but generally more safe than the live vaccines.

A special group of inactivated viral vaccines comprise so-called "***split***" ***vaccines***. They include viral particles (virions) decomposed by detergents. Detergent treatment removes lipid envelope from complex viruses, thus exposing inner protein antigens to the immune cells on vaccination. For instance, high efficacy is demonstrated by influenza split vaccines that contain viral coats (capsids) with immunogenic outer proteins.

Chemical vaccines are the vaccine products composed of microbial complexes isolated by chemical and physical methods from initial microbial cells. Well-known example here is meningococcal chemical polysaccharide vaccine.

Subunit vaccines encompass the number of purified ***protective antigens***, which trigger high-grade immune response to corresponding infectious agents. Usually they are ***polyvaccines*** composed of many antigenic substances.

The design of tailor-made subunit vaccines includes the series of common steps. At first the protective epitopes are determined; then they are derived and combined together. Finally the protective antigens should be absorbed on the carrier with adjuvant properties.

Vaccination with isolated protective antigens avoids some post-vaccinal complications characteristic for previous generations of vaccines (hypersensitivity development, incapability to induce protective immunity, local inflammation on injection site, etc.)

Typical representative of this group is influenza subunit vaccine *grippol*. It contains a number of specific viral superficial antigens, hemagglutinin and neuraminidase, in combination with immune stimulator polyoxidonium with adjuvant activity.

Recombinant vaccines are the most advanced vaccine tools with excellent perspectives. After the identification of protective antigen, its DNA is introduced by vector into recipient cell culture, where the antigen is expressed in large amounts.

Antigen-specific DNA may be also fused with DNA of other epitope sequences, enhancer elements, or immunostimulators by methods of genetic engineering. Plasmides, bacteriophages and other viruses (for instance, vaccinia virus) serve as vectors.

There are only few examples of currently used recombinant vaccines, but they are strong effective (hepatitis B vaccine, experimental rabies vaccine).

DNA vaccines are the most promising novel vaccines based on recombinant technologies. They include specific sequences of DNA coding for the most immunogenic epitopes of infectious agent. Then antigen-coding DNA fragment is incorporated into DNA delivery system (e.g., avirulent bacteria capable of intracellular propagation in the host). After successful delivery the host cells start to express the protective antigens on specific DNA template in quantities enough to confer high-grade immunity.

Toxoids (also known as ***anatoxins***) are prepared from exotoxins of bacteria by formaldehyde treatment at a temperature of 38-40°C for several days or weeks.

Usually purified toxoids are further coupled with adjuvants, e.g. aluminium hydroxide. Anatoxins induce the production of antitoxin antibodies, thus reproducing antitoxic immunity.

For instance, ***diphtheria*** and ***tetanus toxoids*** are highly efficient tools for prophylaxis of corresponding severe infections.

These toxoids are also the essential constituents of complex ***ADPT polyvaccine*** (*adsorbed diphtheria, tetanus, pertussis vaccine with aluminium hydroxide* as adjuvant) or combined toxoid product ***ADT***. Under the broad immunization campaigns tetanus and diphtheria became the completely preventable diseases.

Immunotherapy. Immune Sera and Immunoglobulins

Immunotherapy means *the treatment of infectious, autoimmune or cancer diseases with versatile immunobiological products or chemical drugs that influence the state of immune system*.

It is further divided into ***activation*** and ***suppression*** *immunotherapy*. A special field is ***passive immunotherapy*** that can be regarded as *substitution* immunotherapy.

Activation immunotherapy comprises the treatment measures that *stimulate* or *enhance* host immune response.

A great variety of substances is employed for activation immunotherapy.

Among them are the ***vaccines*** used for therapeutic objectives (e.g., intravesical treatment of bladder cancer with BCG vaccine) and numerous products of ***recombinant cytokines*** (IL-1β or betaleukin, IL-2 or ronkoleukin, colony-stimulating factors like G-CSF or filgrastim, recombinant alpha- and beta-interferons, recombinant chemokines and many others) as well as chemical ***synthetic drugs*** for immune stimulation (e.g., *imiquimod* that activates innate immune response via TLR7).

By contrast, ***suppression immunotherapy*** presumes the treatment that *inhibits* host immune reactions.

To aim this, various medications are used. For instance, numerous groups of ***cytostatic drugs*** are applied for *chemotherapy of cancer* (methotrexate, cisplatin and many others); ***immunophilins*** (*ciclosporin*, *tacrolimus* and *rapamycin*) inhibit T cells, thus preventing allograft rejection and autoimmune disease progression; ***glucocorticoids*** and therapeutic humanized ***monoclonal antibodies*** are used in all of these clinical situations.

Passive immunotherapy means *the treatment or prevention of diseases by immunobiological products* (*e.g., immune sera, immunoglobulins and antibodies, cytokines, immune cells and others*) *obtained from the external sources to create* ***artificial passive immunity***.

Passive immunotherapy replenishes the immune factors, which are deficient in patient's body.

Immune serum for passive immunotherapy is produced by host ***immunization*** with corresponding antigen. These sera contain specific antibodies against pathogenic agent.

Immunoglobulins are derived from immune sera after additional purification. They can be isolated from several sources – ***animals*** (e.g., horses) or ***humans*** (blood donors or healthy volunteers).

Immune sera are divided into ***antitoxic*** and ***antimicrobial***.

Antitoxic sera include anti-diphtheritic, antitetanic serum, immune serum against botulotoxin, anaerobic clostridial infections, snake venoms, etc. Antimicrobial serum may be used against anthrax or some other diseases.

Therapy with horse immune serum can provoke serious *complications* during treatment course. It depends on foreign nature of injected proteins. In case of massive antigenic exposure the immune response to horse serum proteins is triggered, and ***serum sickness*** can develop.

In order to prevent these adverse effects the administration of immunoglobulins instead of sera is preferable.

Immunoglobulins **(**or ***gamma globulins***) are usually of human origin. They are obtained from blood donors and used for curative and prophylactic purposes against measles, poliomyelitis, whooping cough, viral hepatitis A and B, etc. Specific gamma globulin is also administered together with vaccine against rabies.

Highly active immunoglobulins with direct protective effect are produced against staphylococcal toxin. They are isolated from donors immunized with staphylococcal toxoid.

Isolated immunoglobulins tend to form a lot of minute aggregates, and this may cause serious anaphylactic reactions. To prevent undesirable effects, the material should be injected intramuscularly.

Nevertheless, modern Ig products free of aggregates are available now. They are generally known as ***immunoglobulins for intravenous injection*** (or ***IVIGs***). Such immunotherapeutic drugs contain higher titers of antibodies and can be used for treatment of severe disorders, including profound immunodeficiency, septicemia and autoimmune thrombocytopenia.

The most recent advances in passive immunotherapy are related with the development of ***therapeutic monoclonal antibodies*** (***mAbs***). They notably expanded the opportunities for successful control of human cancer and autoimmune diseases. Because of their strict specificity and selectivity, the treatment with therapeutic mAbs is called "***targeted therapy***".

However, there were many primary difficulties on this way, as standard murine mAbs rapidly trigger immune response when administered to the human hosts. Therefore, the procedure of "***humanization***" of monoclonal antibodies becomes mandatory that substitutes antibody sequences of murine origin with human ones. It can be performed, for example, by design of phage genetic libraries of active sites of human

antibodies by methods of genetic engineering. Similarly, the “humanized” constant parts of antibodies can be produced.

As the result, a broad panel of therapeutic monoclonal antibodies is introduced into clinical practice nowadays. Among them are powerful anti-cancer tools like ***trastuzumab*** (mAb against specific receptor of breast cancer cells) or ***rituximab*** (anti-CD20 mAb active against human lymphomas) as well as the remedies against autoimmune diseases – ***infliximab*** (mAb against TNF-α), ***tocilizumab*** (mAb directed against IL-6 receptor) and many others.

These perspective investigations are in great progress now.

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для студентов лечебного и стоматологического факультетов высших медицинских учебных заведений

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