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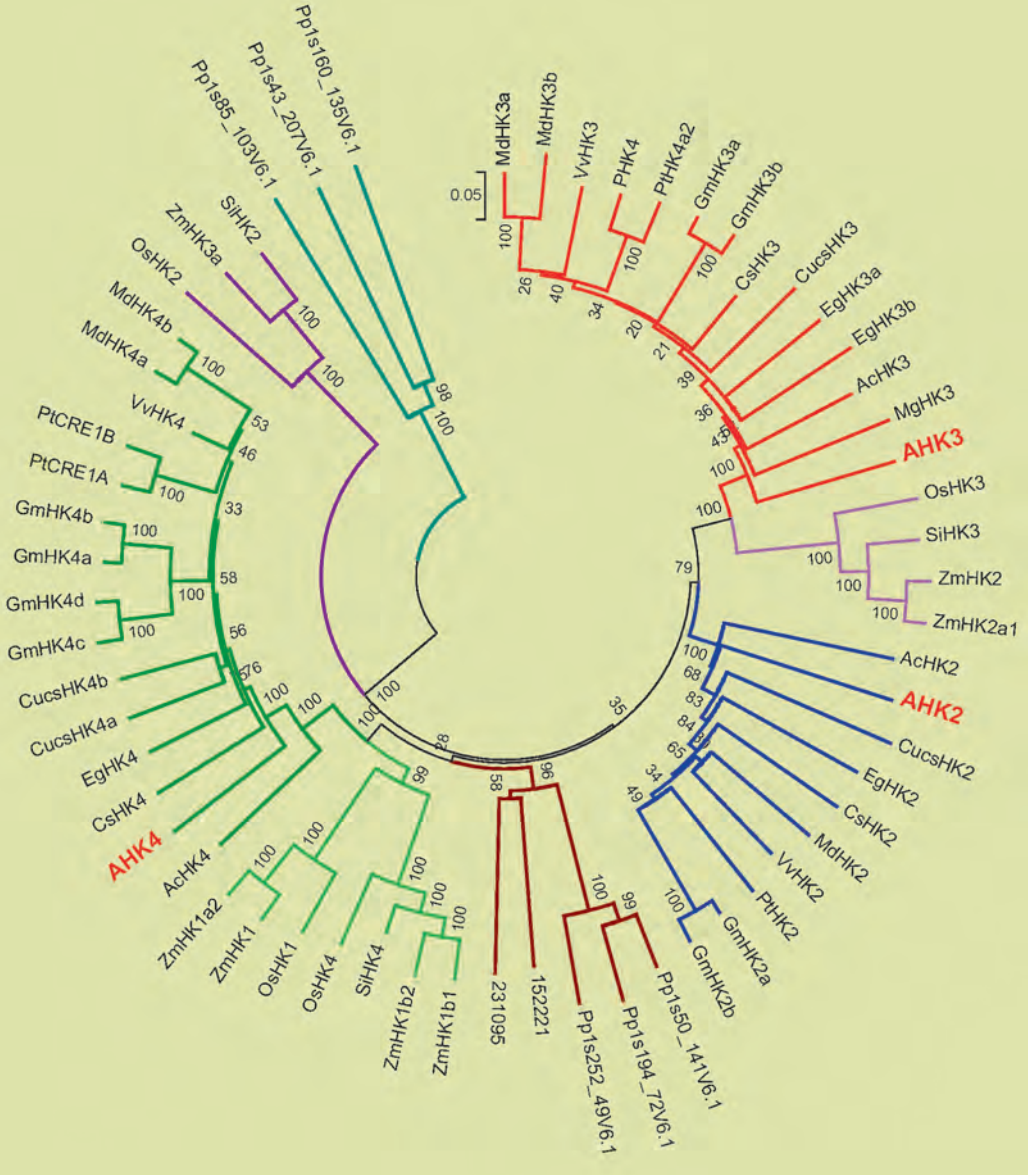
ActaNaturae

Receptor Properties and Features of Cytokinin Signaling



THE USE OF CELLULAR TECHNOLOGIES IN TREATMENT OF LIVER PATHOLOGIES
P. 16

A POLYGENIC APPROACH TO THE STUDY OF POLYGENIC DISEASES
P. 59

Letter from the Editors

Dear colleagues and readers,

We would like to bring you the fourteenth issue of *Acta Naturae*. The issue begins with an interview with R.M. Khaitov, academician of the RAS and RAMS, who won the State Award 2011 "for outstanding achievements in fundamental and practical development of Russian immunology" (sharing with academician R.V. Petrov). Rakhim Musaevich shares his ideas about the development of immunology and Russian science, in general. For our part, we want to seize on this opportunity to once again congratulate the laureates on this well deserved award.

Now to the contents of this issue. It contains five review articles devoted to the topical problems of life sciences. Three of them are related to the field of fundamental medicine: *The Use of Cellular Technologies for the Treatment of Liver Pathologies* (O.S. Petrakova *et al.*), *Molecular-Genetic Approaches to the Investigation of Polygenic Diseases* (D. Lvovs *et al.*), and *Cardiologic Biopharmaceuticals* (A.V. Maksimenko). The remaining two review articles deal with more "academic" aspects, such as the receptor properties and features of cytokinin signaling (S.N. Lomin *et al.*) and the structural and functional studies of lipopolysaccharide of the plague microbe (Y.A. Knirel and A.P. Anisimov). It is obvious from the titles above that the reviews cover a rather wide subject area. We therefore hope that these articles will delight many interested readers.

All three research articles in this issue are devoted to the study of cellular-model systems. We would like to particularly single out the article by A.I. Tukhvatulin *et al.* The study was carried out at the intersection between two apparently unrelated disciplines, plasma physics and cell biology. We believe that this is an absolutely novel approach to biomedical research.

We would like to remind our readers and future authors that our journal has now been included in the PubMed database (with full-text versions of the articles available). This calls on all of us to remain vigilant as to the quality of our publications if we want *Acta Naturae* to score high in the international scientific journals ranking. We are convinced that our joint efforts will allow us to fulfill this ambitious task.

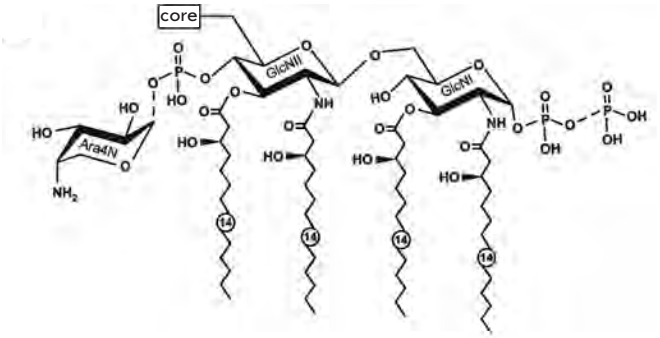
See you again in our next issue! ●

Editorial Board

Lipopolysaccharide of *Yersinia pestis*, the Cause of Plague: Structure, Genetics, Biological Properties

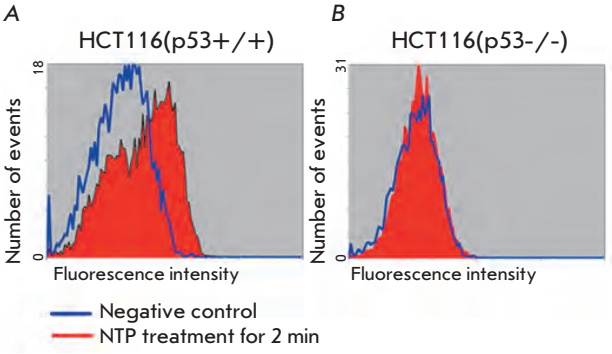
Y. A. Knirel, A. P. Anisimov

The present review summarizes data pertaining to the composition and structure of the carbohydrate moiety (core oligosaccharide) and lipid component (lipid A) of the various forms of lipopolysaccharide (LPS), one of the major pathogenicity factors of *Yersinia pestis*, the cause of plague. The review addresses the functions and the biological significance of genes for the biosynthesis of LPS, as well as the biological properties of LPS in strains from various intraspecies groups of *Y. pestis* and their mutants. The prospects of development of live plague vaccines created on the basis of *Y. pestis* strains with genetically modified LPS are discussed.



Structural variants of lipid A of Y. pestis –the tetraacyl form synthesized by wild-type strains at 37°C.

Non-thermal Plasma Causes p53-Dependent Apoptosis in Human Colon Carcinoma Cells



Intracellular concentration of protein p53 in HCT116(p53+/+) and HCT116(p53-/-) cells.

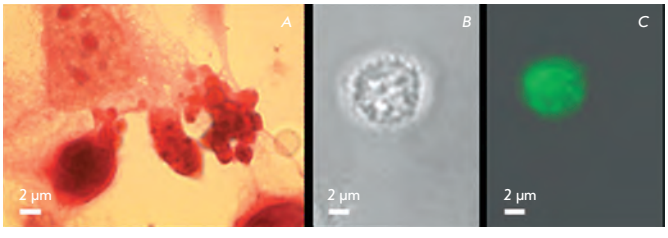
A. I. Tuhvatulin, E. V. Sysolyatina, D. V. Scheblyakov, D. Yu. Logunov, M. M. Vasiliev, M. A. Yurova, M. A. Danilova, O. F. Petrov, B. S. Naroditsky, G. E. Morfill, A. I. Grigoriev, V. E. Fortov, A. L. Gintsburg, S. A. Ermolaeva

Non-thermal plasma (NTP) consists of a huge amount of biologically active particles, whereas its temperature is close to ambient. The study was the first to demonstrate that treatment of human colon carcinoma cells with NTP results in p53-dependent apoptosis. The results obtained contribute to our understanding of the applicability of NTP in antitumor therapy.

Mitochondrial Pathway of α-Tocopheryl Succinate-Induced Apoptosis in Human Epidermoid Carcinoma A431 Cells

M. A. Savitskaya, M. S. Vildanova, O. P. Kisurina-Evgenieva, E.A. Smirnova, G. E. Onischenko

Vitamin E derivatives are known to act as agents exhibiting cytotoxity against tumor cells. Thus mitochondria are shown to be crucial targets in α-tocopheryl succinate-induced caspase-dependent cell death in human carcinoma A431 cells.



Apoptosis in human epidermoid carcinoma A431cells.

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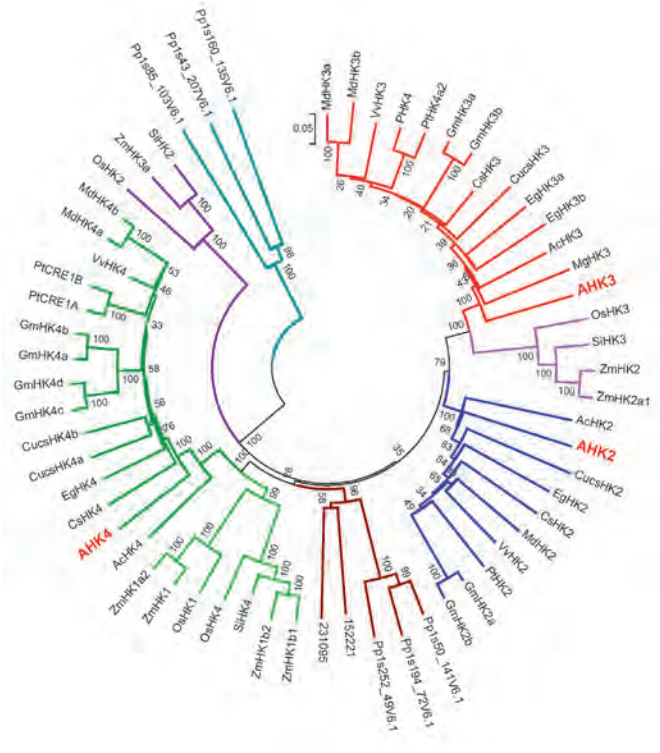


IMAGE ON THE COVER PAGE
Phylogenetic analysis of cytokinin receptors (S. N. Lomin *et al.*)

Immunology in the XXI Century – Progress and Achievements

The awarding ceremony for the laureates of the Russian Federation National Award 2011 in the field of science and engineering was held in Moscow this summer. Two interviews with the laureates are published in the popular science section of this issue of *Acta Naturae*. **Rakhim Musaevich Khaitov**, who has been awarded for outstanding achievements in the fundamental and practical development of Russian immunology, talks about the current state of biomedicine.

– Rakhim Musaevich, you have been working in immunology for 40 years now. How did it begin?

– It all began in the Samarkand State Medical Institute. Back then, student scientific societies were very popular. I was a member of the radiobiology society.

There was a boom in this field of science at that time; many researchers were interested in the effect radiation has on "living systems." I became interested in the effect of radiation on the immune system. The first books devoted to this subject were just starting to be published. One such book, *Immunology of Acute Radiation Injury* by R.V. Petrov, left a deep impression on me. When in the radiobiology student society, I started to simulate various immune reactions. I irradiated animals and made attempts at checking whether any antibodies against toxemia products emerged due to irradiation, similar to what occurs in other diseases. I understood back then that there was no way a vaccine against radiation sickness could be designed and that transplantation of bone marrow stem cells was the most adequate approach (the first studies devoted to stem cells were already being carried out). The major challenge was to devise the right method for selecting a donor and a recipient based on immune response genes. I became so interested in the subject that I abandoned all other areas of interest and entirely focused on immunology. Unfortunately, there was no immunology in Samarkand in those days. It did not exist as an independent field of science in the USSR. It was a part of virology, microbiology, and infectious disease specialties; just a few hours within various courses and departments were devoted to this subject area. Then I decided to find an institute where I could carry out serious immunological research; so I set off for Moscow, the scientific center of the USSR.

I visited a number of institutes; however, the themes of immunological research were either rather weak or did not sound interesting to me. Finally, I came to the Institute of Biophysics, Ministry of Health of the USSR. They housed the Laboratory of Immunology headed by the already mentioned Professor R.V. Petrov. At the time, he had just brought complex methods of chromosome analysis from England. Those methods were unknown in Russia at the time. The professor suggested that I implement those methods for 3 months. He hinted that if I succeed, I would be hired to work at the laboratory. It took me two weeks to develop them, so I was hired. In just a few months, I had the data published not only in leading journals of the Soviet Union, but in foreign journals, as well.

– You have done great research. What can you say about the condition of the immune system in modern humans and that in humans in the beginning of the XX century? Are there any changes?

– I believe that the immune systems in modern humans and humans who lived in the XIX–XX centuries are fundamentally the same. It is our knowledge and concepts relating to the immune system that have changed. For example, the term "immunity" already existed in the XIX century (the term was proposed by Louis Pasteur and originates from the Latin word "immunitas"); it denoted nonsusceptibility to infectious diseases. Later, in the end of the XIX century, people started to realize that it was not just some kind of abstract nonsusceptibility and that it was associated with particular things (specific proteins and antibodies recognizing bacteria and viruses) and with the cells killing these bacteria and viruses. The immune system remains the same; it is its response to the environment that changes. The system responds to new infections and new

Rakhim Musaevich Khaitov, Director of the Institute of Immunology, Federal Medical-Biological Agency of Russia; Chairman of the Russian Association of Allergology and Clinical Immunology; member of the Council of the European Academy of Allergology and Clinical Immunology; leading expert with the Ministry of Health and Social Development of the Russian Federation for allergology and immunology problems, Academician of the RAS and RAMS, Doctor of Medical Sciences, Professor. Twice-Laureate of the National Award of the Russian Federation and the Russian Federation Government Award.

antigens. There are only two systems in the human organism which possess memory and are capable of learning: the nervous and immune systems. Therefore, if an infection did not exist in the XVIII century – for example, the HIV or T-cell leukemia virus – it is natural that the immune system had no cells/proteins that could recognize such foreign elements. These viruses exist today, so the immune system can recognize them.

– An immunological map of Russia has been created on your initiative. Where and how is it used?

– The immune status shows the state of the immune system in the complex. The immune system is very sophisticatedly organized; it includes central and peripheral organs and a large number of circulating cells, namely, T-lymphocyte, B-lymphocyte, monocyte, and macrophage families. It is very important that all cells of the immune system interact with each other and are regulated by T cells (I call them "conductors of the immune system"). There are genes that conduct this immune "orchestra," and it is crucial that all the parameters of the immune system are normal in each person. So we raised the question as to whether the immune system parameters of individuals living in various regions of Russia are identical. For example, a person arrives from the polar region or comes down from the mountains. He can come to our institute and say: "I'm sick. Could you please check my immune system?" And what if the immune system parameters for the region he came from and the new location

are different? In order to assess the immune status, we established about 120 clinical immunology laboratories in different regions of Russia. The parameters turned out to differ; but this is normal rather than a pathology. In some regions, the amount of T-lymphocytes is higher; in other regions, it is lower. Still other regions are characterized by the highest amount of B-lymphocytes. Of course, the differences are small, but they still exist. Thus, it is important to know the parameters of a specific region so that a person knows his/her normal immune status.

– It is a known fact that it is easier to prevent a disease than to cure it. What measures are being taken to prevent immune system disorders?

– There is a national immunization schedule, which includes *N* vaccines. Immunized children develop nonsusceptibility to these diseases (since the immune system possesses memory). This is one of the measures for fighting an infection. However, there can be genetic pathologies in the immune system; i.e., a child born has congenital disorders of the immune system. The immune system can also be suppressed by such factors as radiation, burns, injuries, a high level of stress, starvation, and exposure to harmful environmental factors. A permanently suppressed immune system results in the development of various disorders: autoimmune diseases, cancer, etc. The immune system is connected to all the other systems in the organism; that is why immune system disorders frequently cause pathologies in a number of other systems in the organism. Therefore, the immune status needs to be checked.

– How can we check it?

– Once you feel there is something wrong with your immune system, you need to take certain measures. What are the first signs? First of all, they include frequent respiratory tract diseases and other symptoms. In this case, you need to consult an immunologist to determine your immune status. If the immune status is suppressed, immunomodulatory agents are prescribed to reactivate it. We were able to pinpoint the regions where large population groups suffer from immunodeficiency. Preventive immunotherapy was carried out in these groups. It turned out that restoration of the immune system parameters abruptly reduces morbidity.

– Rakhim Musaevich, can the use of immunomodulatory agents be harmful to the organism?

– If your immune system status is fine, there is no need for immunomodulatory agents. One of my articles is even called *I Have Never Taken Immunomodulators*. Why? I check my immune status once a year. If it is fine, why should I use drugs?

– You mean, there is no need to be overcautious?

– There is no need if your immune status is normal, since it is fraught with autoimmune diseases, can cause allergic reactions, etc. Another situation is if a doctor (I mean not a self-taught doctor, but an immunologist) has diagnosed immune insufficiency. In this case, you need to take immunomodulatory drugs.

– As for allergic diseases, why are so many people susceptible to them nowadays? And a lot of people acquire these diseases in their adulthood…

– You are right. The frequency of allergic diseases has significantly increased recently. In the early XX century, only a negligible percentage of the population suffered from allergies, whereas today this figure is 25–30% in developed countries. There are regions in Russia where 15% (and even 30%) of the population suffer from allergies. These are extremely high numbers. If you remember, there were a large number of allergic diseases this spring. Spring came early and was warm, so the "green clouds" that emerged made people think about an environmental disaster. In fact, it was abundant blossoming of trees – birch, alder, and hazel trees. The pollen of these trees is responsible for the so-called "seasonal allergy." A lot of people with various symptoms of allergic diseases came for consultations to our institute. Some of them had never had any allergies prior to that. It was the "first wave"; the second wave has already started: other plants and grass are blossoming. There will also be a third wave in autumn.

– And the number of people susceptible to allergies is increasing…

– The point is that the surrounding world is changing. The number of infectious diseases used to be larger and the number of allergic ones smaller. The immune system used to be in a permanent fight against infections, but now we protect children against infections from early childhood. The position of the infections became vacant, so the immune system switched to allergens, which in fact are not infections at all. So apparently harmless proteins cause annoying allergic reactions, which sometimes can be very dangerous. In addition, a great number of triggers of allergy reactions have emerged, including pollutants, exhaust gases, and environmental factors. A number of them are not allergens, but they trigger the development of allergies.

New drugs are constantly coming to market. Most of them are also allergens; therefore, drug allergy is one of the main problems nowadays. Food allergies are rather common: people used to eat traditional food, whereas now a lot of the new foodstuff is produced. In fact, there is no substance today

that could be called completely allergy-free.

– But some people develop an allergic reaction, whereas others do not…

– Nobody knows why this is a selective process. Scientists know how an allergy develops, what causes it, and what the allergens are. They know the mechanisms of allergy development and how it can be blocked; however, it remains unclear why a person develops an allergy to ovalbumin, while another does not. I think that it is the genes that are responsible for this phenomenon. For example, one person carries genes encoding resistance to ovalbumin, whereas another carries a gene making him susceptible to this allergen and facilitating the development of an allergic reaction. It is possible that once these genes are identified, the allergy problem will be solved.

– Your research group has designed antiallergy drugs. To what extent are you satisfied with the results? Do the drugs have any side effects?

– Yes, antiallergy drugs, allergotropins, have been designed in our institute. These drugs exhibit much higher efficacy compared to the conventional allergens used to cure allergies. No specific side effects have been observed during clinical trials. We have forwarded these medications to the production stage and are awaiting registration and production.

– When is that going to happen?

– That depends on the enterprise that is responsible for processing the documents and obtaining the license for production and application.

– What is the mechanism of action of these drugs?

– First of all, our drugs inhibit the formation of "allergy antibodies." Second, they cause the secretion of protective antibodies, which block the allergic reactions. In other words, we used special molecules to affect different points within the mechanism of allergy development to finally design drugs that are practically significant.

– How do they differ from existing antiallergic agents?

– The already existing drugs – the first-, second-, and third-generation antihistamine agents – provide conditional treatment of allergies. They inhibit the allergic reaction; so it will return if you stop taking them. Allergen-specific immunotherapy is another type of treatment. What does it consist in? The causal allergen (i.e., the allergen causing a disease) is identified in the patient. The patient then receives the purified form of this allergen for a very prolonged period with the dose increasing gradually. The course lasts 2–3 months and needs to be repeated during several years. Complete recovery is possible in this case; however, this method is very complex, time-consuming, and fraught with complications. The drugs designed reduce therapy duration to two weeks and 15 injections; they also provide a more than twofold decrease in the need for pharmacotherapy.

– To what extent will this therapy be affordable?

– It is not up to us to decide. I do not think these medications will be more expensive than the allergens used for treatment today.

–An anti-AIDS vaccine is being developed in your institute. What stage are the researchers at?

– It actually is a rather complex problem. It is very difficult to design this vaccine, since the virus mutates quickly. So each patient may host a new variant of the virus. Therefore, many researchers believe that mining for a vaccine is a dead-end approach. However, not all scientists support this point of view. The fact is that even the AIDS virus has conserved regions that do not mutate; therefore, certain proteins or protein fragments in these regions remain intact. So there is a chance that these proteins can be used to design the vaccine. Scientists all over the world are working to solve this problem; however, almost no success has been achieved. Only one American–French group has had some results: two of their vaccines have provided a 35% reduction in morbidity.

As for me, I believe that another approach should be used in designing the vaccine. The conserved antigens should be combined with immunostimulating agents; this will ensure robust immunity. Since a large number of people have already been infected, there should not only be preventive AIDS vaccines, but therapeutic ones, as well. HIV affects the immune system. Vaccines are inefficient in this case. Therefore, we hope that combination with immunomodulatory agents will ensure a therapeutic effect in infected people and probably even in people with AIDS. Our research group has designed a vaccine of this type; it is known as "Vichrepol."

– What does it consist of?

– We used genetic engineering techniques to design a chimeric protein. This protein contains fragments of various proteins found in the virus (both surface and inner ones, which do not undergo mutation). The protein alone causes no immunological reactions; i.e., it cannot be used as a vaccine. Therefore, it was bound to an immunostimulating agent. This form of the protein had very strong immunostimulating properties. A vast range of preclinical studies and phase I trials in volunteers have been carried out. We are now preparing for phase II trials, which will be more extensive.

– A question emerges: are there any difficulties with clinical trials and recruiting volunteers?

– The phase I trials need to be carried out only in healthy individuals to make sure that the vaccine is safe and indeed causes two forms of immune response: both antibodies and killers that destroy virus-infected cells are formed. Phase II trials will also be carried out in volunteers. Recruiting volunteers is a very difficult task. Healthy people often do not want to be vaccinated; nevertheless, there are people who are enthusiastic about vaccination (in particular, among the cohorts of sexual minorities).

– What phase of clinical trials is carried out in infected individuals?

– If phase II trials are successful, we will be allowed to proceed to the next phase and include infected people in trials.

– If a vaccine is designed, which population groups will be the first to receive it?

– How was smallpox defeated? Each person was vaccinated with an effective vaccine. There is an influenza vaccine; its effectiveness is lower than that of the smallpox vaccine but still rather high (70–80%). Influenza epidemics still occur from time to time. Virus mutation is not the only reason. The fact is that only part of the population has been vaccinated; this includes the so-called "risk group," people who have higher chances to be infected: namely, schoolchildren, children attending kindergartens, doctors, teachers, and public transport employees. If every single person received the vaccine, influenza would presumably be defeated as well. Therefore, with allowance for the enormous scale of the epidemic, if an AIDS vaccine is eventually designed, it will certainly be optimal to vaccinate everyone. A lot of venereal diseases (syphilis and gonorrhea) are widespread today. They are all 100% curable; however, outbreaks of these diseases happen frequently. The reason is that there is no vaccine against these diseases. Only a vaccine is capable of globally defeating the disease. It is a significant fact that the number of human lives saved thanks to vaccination is much larger than the number saved by medicine as a whole. Vaccination has saved hundreds of millions of people.

– Rakhim Musaevich, how would you estimate the time required to design the vaccine?

– It is difficult to give a prognosis, since there will also be phases III and IV. The latent period of the AIDS virus may be several years (from 2 to 10–20 years). It is a very sophisticated infection, so it is almost impossible to give any prognoses. What we need to do is to keep working.

– Is the research sufficiently supported by the Government?

– The Government of the Russian Federation has adopted a very good resolution pertaining to the struggle against infectious diseases, including AIDS. Our program has received financial support, which allowed us to design the vaccine. Unfortunately, the resolution has already expired.

– Is the level of Russian research in some areas superior to that of foreign research?

– I would say that Russia is behind Western countries – the U.S., Western European countries, etc. – in terms of the general level of biomedicine. There have been some breakthroughs in Russia, as well. For example, we have done a lot in studying the immune status. Foreign researchers have not dealt with it. We have developed a large number of immunomodulatory agents (dozens agents), whereas only several agents have been designed by foreign researchers. A breakthrough has also been achieved in the area of AIDS vaccines: we were the first to develop vaccines with immunostimulators. There are no analogues of these vaccines in the world. In the international series AIDS Vaccine Blueprint, which annually reports on promising vaccines, our vaccine is mentioned among the top ten.

In general, there has been a lot of interesting research done, not only in immunology, but in other fields of biomedicine, genetics, molecular genetics, and biochemistry, as well.

– Has the level of AIDS morbidity increased over the past decade in Russia and in the world?

– Unfortunately, it is increasing significantly in Russia. In some countries (such as the U.S., Canada, Western European countries, and Uganda), the increase in the rate of AIDS morbidity was stopped thanks to preventive measures.

– As a research supervisor, can you make special mention of some of your students?

– Quite a few scientists have defended their Candidate or Doctoral thesis papers under my guidance. Now many of them are professors; some of them are even members of Academies of Sciences. Many of my students have received Government and national awards. I do not want to mention any particular names. I respect all of them; they are all good people. If I single out a person or two, the others may be jealous. It is impolite.

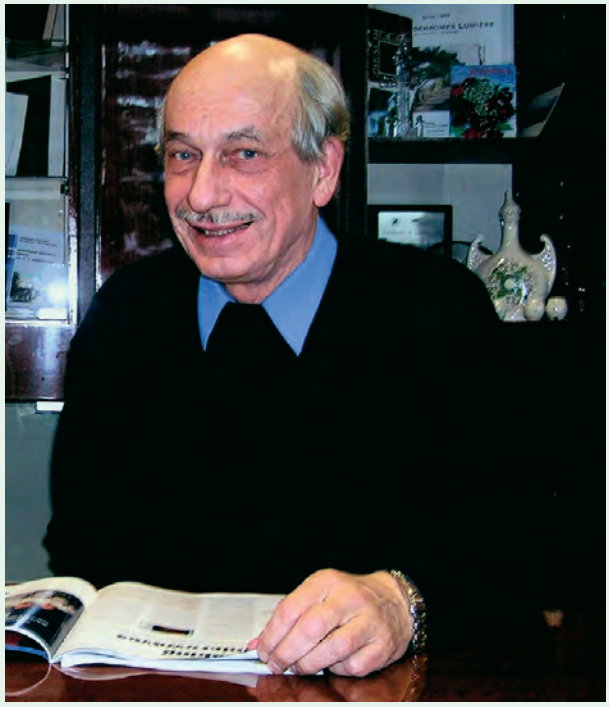
– What is your view about further developments in immunology?

– We have a lot of ideas at our institute; in particular, ideas in the fields of molecular immunology and nanoimmunology. We have good financial support and excellent equipment. The only thing that is needed is an inflow of young researchers. ●

Interview by Maria Morozova

The Chemistry of Life

Boris Aleksandrovich Trofimov, laureate of the Russian Federation National Award for substantial contribution to the development of new methods fine organic synthesis in order to design innovative drugs and modern materials, including special-purpose ones, tells us about his discoveries and further research plans.



Boris Aleksandrovich Trofimov, Director of the A.E. Favorsky Irkutsk Institute of Chemistry, Siberian Branch, Russian Academy of Sciences, Doctor of Science (Chemistry), Professor, and Academician of RAS. Laureate of the A.M. Butlerov Award and the Russian Federation National Award.

– Boris Aleksandrovich, you are the author of over 500 inventions. Which one is the most significant to you?

– I would say I am a co-author. Very few inventors work independently now. It is a relic of a phenomenon. Today, researchers typically work in collaboration, so serious findings are a result of the joint efforts of experts from different fields. Practically significant ideas most frequently emerge at the fault lines of sciences. Therefore, most of our inventions have a cross-disciplinary character and are a result of the efforts of my students, collaborators, and colleagues from related areas of science and technology.

Which invention is the most significant to me? I think it is a rather difficult question, similar to the situation when the father of a large family cannot say who his favorite child is. In addition, attitudes to inventions have changed as time has passed. Not all the inventions made 20 or even 40 years ago still seem important today. On the contrary, some findings that used to seem quite ordinary have turned out to be uncut diamonds. Of course, our recent inventions are the most important ones for me, since they are relevant to the demands of modern life. Still, there are some inventions that I regard as key ones. They laid the groundwork for new directions of chemistry and chemical engineering and became so-called "idea incubators," yielding new inventions. These inventions include the methods for pyrrole synthesis from ketones and acetylene; direct chlorine-free synthesis of phosphororganic compounds from elementary phosphorus and acetylene or its derivatives; and the synthesis of divinyl chalcogenides (e.g., divinyl sulfides, divinyl selenides, divinyl tellurides) directly from elementary chalcogens or their simplest derivatives (such as hydrogen sulfide) using acetylene or its derivatives. I would also attribute the "anchor" epoxides (e.g., Vinylox) used to produce many new brands of epoxy resins with special technical characteristics to the category of fundamental inventions.

– Pyrrole synthesis from ketones and acetylene is now known as the Trofimov reaction and has been included in a number of reference books, including the Encyclopedia of Chemical Technology (USA). Please tell us more about the reaction and what it is used for.

– Several approaches to pyrrole synthesis have been described so far; however, they either require expensive and complex starting

compounds or involve several reactions (chemists refer to them as multistage approaches), or the pyrrole yield is low, or these synthesis methods are insufficiently universal (i.e., they yield only a limited number of pyrrole derivatives). Our reaction (see the scheme), which was discovered in the early 1970s (jointly with junior researcher A.I. Mikhaleva (today, she is a professor)), requires only ketones, one of the most available and inexpensive classes of organic compounds, hydroxylamine, and acetylene, and even cheaper industrial products.

For example, the widely known acetone (the simplest ketone) reacts with hydroxylamine and acetone under mild heat treatment in the presence of a superbasic catalytic system, which consists of potassium hydroxide and dimethyl sulfoxide (used in medicine) and can be easily prepared, yielding α-methylpyrrole and its N-vinyl derivative. By the way, until our reaction was discovered, N-vinylpyrroles remained an unknown, although very promising, class of pyrrole compounds.

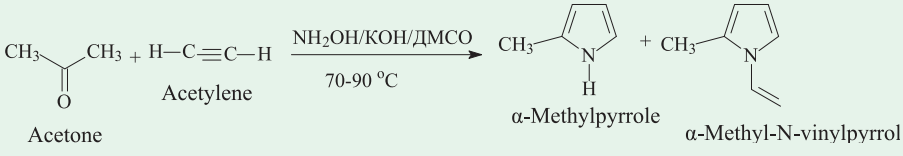
Pyrrole and its derivatives are the fundamental structures of key life-sustaining systems; namely, chlorophyll, hemoglobin, natural antibiotics, insect pheromones, and a number of drugs. Our method for pyrrole synthesis is currently finding increasing application for producing pharmaceutical agents, biosensors, light-sensitive metal complexes for the diagnosis and therapy of cancer, molecular systems for artificial photosynthesis, and materials for new technologies (organic semiconductors, electro- and photochromic materials, and optoelectronic devices). The reaction discovered by us enabled to develop the world's first technology to produce synthetic indole from cyclohexanone and acetylene. Pilot plant production of indole with a capacity of 200 tons per year is currently being planned. Our technology enables the simultaneous production of tetrahydroindole (today, 1 g of this compound costs over 200 euro), its vinyl derivative, and N-vinylindole, which are valuable intermediate products for the synthesis of pharmaceutical agents and are also unique monomers which are not produced anywhere in the worlds at the moment.

Indole is finding wide application in the synthesis of pharmaceutical agents and the important biologically active compounds regulating the vital activity of warm-blooded animals. Indole is the main structural element of such crucial compounds in nature as tryptophan (an essential amino acid), serotonin (one of the main neuromediators, the "happiness hormone"), and melatonin (the hormone regulating human circadian rhythms). Its structure can be found in other hormones and hallucinogens. Well known indole-based drugs include indopan, indometacin (anti-inflammatory, antipyretic, and analgesic agents), reserpine (a tranquilizer), and strychnine (a nervous system stimulant). Heteroauxin (β-indolyl acetic acid), an indole-based plant growth stimulant, is widely used in practice.

– So why has acetylene drawn your attention?

– Indeed, we have designed and further developed the scientific principles of original methods for organic and element-organic synthesis based on acetylene. Acetylene is the simplest high-energy hydrocarbon, an obligate product of gas, coal, and oil processing, the fundamental unit of the organic matter. This gas has even been detected in interstellar space. It has been conventionally used and is still being used as a starting raw material for industrial and fine organic synthesis. In the XX century, it was used to synthesize all major chemical products. It has been replaced by ethylene and propylene produced from oil (which remain cheaper raw materials). However, it is now assumed that acetylene will return in the chemical industry in the nearest future due to the steady rise in the prices of oil and gas, since acetylene can also be produced from coal, material that will remain available for hundred of years. Acetylene is currently being increasingly used in fine organic synthesis oriented towards the production of complex science-intensive molecules (biologically active substances, vitamins, drugs, agricultural fertilizers, and high-tech materials) due to the fact that it enables to take the shortest and simplest pathway to the obtaining of the target compounds.

In the early 1960s, when the Siberian Branch of the USSR Academy of Sciences was being established, Mikhail Fedorovich Shostakovsky, then a corresponding member of the USSR Academy of Sciences and favorite pupil of outstanding Russian organic chemist Aleksei Evgrafovich Favorsky, brought the research area and traditions of the school of his great teacher to the Irkutsk Institute of Organic Chemistry, Siberian Branch of the USSR Academy of Sciences. So a new and young branch of Academician Favorsky's school, whose scientific interests focused on acetylene chemistry and fine organic synthesis, was established and began to rapidly develop in Eastern Siberia. Most of the attention was always focused on the production and modification of biologically ac-



Scheme. Synthesis of α-methylpyrrole and α-methyl-N-vinylpyrrole from acetone and acetylene.

tive substances; namely, terpenoids, steroids, alkaloids, and saccharides. Our institute was later named after Academician Favorsky for the successful development of the scientific heritage of this outstanding founder of acetylene chemistry. My pupils and I are proud to belong to his school.

– You have developed methods for fine organic synthesis of various chemical compounds, including pharmaceutical agents, dyes, chemical additives, pesticides, surfactants, and synthetic enzymes. Which pharmaceutical agents produced via fine organic synthesis are the most noteworthy?

– To be more precise, we have fundamentally supplemented the fine organic synthesis procedure. We have discovered a number of new reactions and have substantiated new methodological principles (e.g., the use of superbasic catalytic media, reactants, and catalysts). As a result, previously unknown ways for producing useful substances have emerged. Like other synthetic chemists, we search for new laws of formation of chemical bonds in order to perform a targeted and simple construction of high-tech materials, innovative compounds, and drugs, without which modern civilization cannot exist. It is not by mere chance that the recent Nobel Prize in chemistry was awarded for the methods of formation of the carbon-carbon bond via the substitution of atoms (typically, halogens) in the presence of noble metals. Our methods are based on another type of reactions, addition reactions, which are catalyzed by natural ions (sodium and potassium ions, hydroxide anion) or light quanta; therefore, these methods are usually wasteless. In other words, they satisfy the principles of green chemistry to a significant extent, are environmentally friendly and energy- and resource-saving (chemists refer to these principles as atom-economic methods).

All this is valid for the new type of reactions discovered and developed by my colleagues, academicians V.N. Charushin and O.N. Chupakhin, both laureates of the same National Award. Their reactions involve hydrogen replacement in the aromatic or heteroaromatic ring. The hydrogen being released does not pollute the environment and can be used as the "purest" fuel and chemical reactant. The new chemistry has allowed my friends and colleagues to complete the registration of the new antitumor agent lisomustin. This drug has been introduced into medical practice and is successfully used in clinics. They have developed new industrial methods for the synthesis of antibacterial fluoroquinolone antibiotics (perfloxacin and levofloxacin). Preclinical studies of the original antiviral drug triazavirin, which is capable of providing 90% protection against a number of dangerous viral infections (e.g., H5N1 and H1N1influenza), were recently completed.

Acizolum, a high-efficiency antidote of carbon monoxide, has been designed in our institute on the basis of N-vinylimidazole obtained via direct vinylation of an essential heterocycle, imidazole, with acetylene. This antidote efficiently protects people against poisoning with this insidious gas and other combustion products. Acizolum has been included in Russia's Vital and Essential Drug List. This drug is produced by the MAKIZ Farma pharmaceutical company under the supervision of Acizol, a closed joint-stock company (Moscow). This drug is becoming more important as the number of fires increases all over the world. A number of additional valuable properties have recently been revealed for acizolum. In addition to being a unique antihypoxant, it is an active hepatoprotector (comparable to the best known drugs) and an antiarrhythmic agent. It allows one to cure the most severe pneumonias and even relieves hangover.

A high-efficiency antiseptic agent, anavidin, which was designed in our institute, is now produced by an especially established company on a setup with a capacity of 100 tons per year. The dynamically developing Russian pharmaceutical company Farmasintez in a few months intends to bring to market a new anti-tuberculosis agent, perchlozone, which is active against resistant mycobacterial species. This original drug took many years to design in our institute (starting from the first synthesis of the molecule in the laboratory headed by Professor G.G. Skvortsova) and, of course, is a product of fine organic synthesis. I would like to mention that no other anti-tuberculosis agents capable of efficient resistance to new aggressive strains of tuberculosis bacteria have appeared in pharmaceutical practice during the past decades.

– Boris Aleksandrovich, new environmentally friendly methods for synthesizing organic compounds of phosphorus and chalcogens have been developed in your institute...

– The synthesis of drugs (more frequently, drug precursors) and drug candidate molecules is significant in fundamental studies carried out in our institute. To be more precise, it is the applied stage of these studies. Our main purpose is to develop new methods for the synthesis of complex organic molecules and reveal the relationship between the structure of the synthesized compounds and their useful properties in order to increase efficiency and reduce the costs associated with the targeted synthesis of compounds that people currently need. The demand is not for drugs only. First of all, people need health. And it would be better if a person could be healthy without drugs. To achieve this, we need to reduce pollution of the environment, since a significant number of contemporary diseases are a result of our reckless and even crimi-

nal attitude to nature. The methods for synthesizing phosphorus- and chalcogen-organic compounds are more environmentally friendly than already existing ones (e.g., our chlorine-free method for producing phosphorus-organic compounds). These technologies are less harmful to human health; so the amount of drugs needed by people will decrease. Furthermore, high-efficiency hemosorbents for hemodialysis, which are currently used in clinical practice, have been developed based on our divinyl sulfide (the product of the addition of hydrogen sulfide to acetylene). The first Russian pesticide, Vindidat, which was designed in our laboratory and is based on acetylene and sulfur compounds, was produced in the USSR on a pilot setup and is still used to protect residential and nonresidential areas and in the food industry.

– What pharmaceutical agents obtained via functionalization of heterocycles have reached production? Please tell us about them.

– Functionalization of heterocycles has for a long time been a common conventional approach in synthesizing various drugs. This refers to the drugs synthesized by my colleagues, academicians O.N. Chupakhin and V.N. Charushin, as well as to the drugs synthesized in our institute. For example, the previously mentioned drug Acizolum is a naturally abundant heterocycle functionalized by a vinyl group via direct interaction with acetylene. The new anti-tuberculosis drug perchlozone jointly designed by the researchers of our institute, St. Petersburg Research Institute of Phthisiopulmonology, and the company Farmasintez is also a very common heterocycle (pyridine) with thiosemicarbazide moiety introduced via a double bond. By the way, pyridines can be easily obtained via single-stage synthesis from acetylene and the simplest nitrogen-containing compounds (ammonia, amines, hydroxylamine, etc.). We have designed a convenient and simple method for the synthesis of pyridines from hydroxylamine, acetylene, and ketones. The reaction takes place in an aqueous alkaline medium; catalysis with heavy or noble metals, which is typical of this type of synthesis, is not required in this case.

A new group of functionalized pyrroles containing aromatic and heteroaromatic substituents with various functional groups that can be easily obtained using our reaction turned out to be another promising class of anti-tuberculosis agents. It has drawn the attention of many synthetic chemists working in pharmaceutical companies. Italian researchers have already found several leading agents. Some of them will apparently be sold in pharmacies soon. We are also working jointly with the pharmaceutical company Farmasintez in this direction.

One of the most marketable drugs, Atorvastatin (also known as Liprimar), is based on pyrroles with several benzene substituents and functional groups, which can also be obtained from ketones and acetylene through ketoximes, which allows one to eliminate several complex and labor-intensive stages in the conventional synthesis procedures. Atorvastatin is a widely known hypolipidemic agent; i.e., a drug that reduces the level of cholesterol in the blood.

Doctor of Chemical Sciences L.N. Sobenina and I have recently discovered a new simple method for introducing various functional groups to pyrroles and indoles using acetylenic ketones, acids, or their esters. A functionalized acetylene substituent is introduced into the pyrrole or indole ring when the reactants interact on active surfaces (e.g., upon grinding on aluminum oxide at room temperature in air). These reactions (known as cross coupling reactions) are typically carried out in the presence of palladium catalysts and a number of additional reactants in an inert atmosphere upon heating. As I have already mentioned, the Nobel Prize was awarded in 2010 to R. Heck, E. Negishi, and A. Suzuki for the development of palladium-catalyzed cross coupling reactions between acetylenes and halogen arenes. It is an interesting fact that another Nobel Prize was awarded in 2007 to G. Ertl for reactions on active surfaces.

A large number of modern drugs belong to the class of functionalized imidazoles. Therefore, the targeted introduction of the desired functional groups into the imidazole ring always remains a topical task for synthetic chemists and pharmaceutists. Acetylene chemistry helped us again. An original, general and very efficient strategy for functionalization of imidazoles via zwitterionic intermediates, adducts of imidazoles with cyanacetylenes, has been in systematic development in our laboratory for several years. This strategy allows one to simplify the synthesis of certain known drugs or their precursors and to synthesize new promising pharmaceutical agents.

– For which discovery did you receive the A.M. Butlerov Award?

– My pupil, Doctor of Chemical Sciences A.G. Mal'kina, and I received this award for a series of studies devoted to the synthesis of functionalized iminodihydrofuranes, which are key structural units and analogs of ascorbic and penicillic acids, natural cardiac glycosides, and certain synthetic agents exhibiting anticancer, antiulcerogenic, antiallergic, and anti-HIV activity. Iminodihydrofurane derivatives find application as nonsteroidal anti-inflammatory agents and analgesics.

We have designed simple and original methods for the synthesis of these compounds on the basis of cyanacetylene alcohols, which in turn have become widely available due to our systematic research. They are

used in fine organic synthesis based on acetylene as convenient universal reactants. It is worth mentioning that Academician A.E. Favorsky, to whose school we belong, was the pupil closest to A.M. Butlerov. It was Butlerov who charged his PhD student Favorsky with developing the research area of acetylene. Thus, it is more correct to say that acetylene chemistry is the development of traditions, approaches, and methods of the traditional Russian Butlerov and Favorskys school.

– Boris Aleksandrovich, I would like to ask you about international collaboration. Are there any joint drug production projects?

– We have collaborated with a number of foreign universities and companies in the U.S., England, Germany, France, Spain, the Netherlands, Canada, Portugal, China, South Korea, and Taiwan. However, the collaboration is mainly oriented towards creating materials for modern technologies, including nanostructured materials, such as organic semiconductors, chemical current sources, highly sensitive sensors, electrochromic materials for thin-film flexible displays, field transistors, etc. International pharmaceutical companies prefer to negotiate on purchasing our patents or licenses for drugs (joint projects cannot be carried out because of the inertia of our bureaucracy). Until recently, an academic institute had no right to sell its final developments. Some points are becoming clearer today; however, a lot of obscure points still remain.

We are participating in an international project of the European Economic Community aimed at designing a new zinc-polymer accumulator based on ionic liquids. Research teams from universities and companies in England, France, Spain, Canada, Portugal, and the Netherlands also participate in the project. The accumulator is intended to be used for electric vehicles; i.e., to make the environment and mankind healthier. This project is not directly associated with drug synthesis; however, it is directed towards decreasing drug consumption. I think that its significance is no less than that of curing people who are suffocating in noxious exhaust gases emitted by cars, airplanes, motor ships, and diesel locomotives.

Very few people have given a thought to the fact that the combustion of 1 ton of carbon requires an additional 3 tons of oxygen, which is not replaced in time because of the cutting-down of trees by the timber industry. We are turning our planet into an artificial gas chamber; it is especially true for megalopolises.

– What works carried out by your pupils would you like to mention?

– I am very lucky to have many pupils who are really gifted people. Among those, there are 26 professors and doctors of sciences and more than 70 candidates of sciences. I am especially happy to be surrounded by young and talented pupils. One of them is Elena Yur'evna Schmidt. We recently discovered a new fundamental reaction of addition of ketones to acetylenes in the presence of superbases (there is a real chance that this reaction will be named after her in future). My other pupil is Andrei Viktorovich Ivanov, a young doctor of sciences who defended his doctoral thesis a year ago at only 30 (he apparently is the youngest doctor of sciences in the Siberian Branch of the RAS). He is one of my science deputies, he deals with innovations and youth policy. Olesya Aleksandrovna Shemyakina is one of my young pupils who has also defended her doctoral thesis this year (she is 32). Aleksandr Viktorovich Artem'ev, one of my youngest pupils, is now carrying out a breakthrough study devoted to the synthesis of organic compounds containing seleniumand phosphorus with consultations by Professor N.K. Gusarova. We hope that he will become Doctor of Science at the age of 27–28. So there are enough people who will develop our chemistry in future.

– Boris Aleksandrovich, how do you manage to combine research and administrative duties?

– It is pretty simple: when you have reliable and experienced deputies who have been tested through hard times, when you have highly qualified assistants: advisors, secretaries and research secretaries, who catch everything at once, who are proficient in several languages, who are on familiar terms with the computer, and who can type at the speed of sound and with high literacy (a rather rare phenomenon nowadays). All this is about my deputies and assistants. Thanks to them, I can visit the laboratory every day and grasp the details of almost each important experiment. I consider a day to be lost if I do not discuss current work with my colleagues next to a draft hood.

But speaking seriously, it is getting more and more difficult to combine research and administrative duties. Each day, the bureaucratic terror becomes worse. The number of documents (questionnaires, inquiries, reports, references, etc.) that should have been filled out yesterday or even two days earlier grows exponentially. In addition, new and even more convoluted documents are being constantly invented. In fact, this situation is rather sad. It makes experts do purposeless work (the same as driving nails with a microscope). A bureaucratic apparatus (even as huge one as ours) is incapable of processing this amount of documents. If this All-Russia paper deluge is not stopped, all our science will sink pretty soon.

But I would like to end our conversation on an optimistic note. I am sure that the paper deluge will be stopped because even the Flood ended in due time. ●

Interview by Maria Morozova

The Use of Cellular Technologies in Treatment of Liver Pathologies

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ABSTRACT **Cell techniques find increasing application in modern clinical practice. The II and III phases of clinical trials are already under way for various cellular products used for the restoration of the functions of the cornea, larynx, skin, etc. However, the obtainment of functional cell types specific to different organs and tissues still remains a subject of laboratory research. Liver is one of the most important organs; the problems and prospects of cellular therapy for liver pathologies are currently being actively studied. Cellular therapy of liver pathologies is a complex multistage process requiring a thorough understanding of the molecular mechanisms occurring in liver cells during differentiation and regeneration. An analysis of the current cellular therapy for liver pathologies is presented, the use of various cell types is described, the main molecular mechanisms of hepatocyte differentiation are analyzed, and the challenges and prospects of cell therapy for liver disorders are discussed in this review.**

KEYWORDS **cell transplantation; cellular therapy; differentiation; liver.**

ABBREVIATIONS **ES cells – embryonic stem cells; iPS cells – induced pluripotent stem cells; HSC – haematopoietic stem cells, MSC – mesenchymal stem cells; SP cells – side population cells.**

INTRODUCTION

The treatment of liver diseases is a significant problem of modern medicine. The statistical data tell us that more than 200,000 people are diagnosed with various chronic and acute liver diseases in the Russian Federation annually. Despite the progress achieved in modern medicine, conventional therapeutic approaches remain insufficient for treating chronic and acute liver pathologies; the mortality rate thus remains at the level of 80–90%.

Transplantation of liver or its parts remains the major method for treating severe pathologies. The shortage of donor material has spurred an active search for approaches of cell therapy for liver diseases. A large body of data accumulated over recent years attests to the fact that cell therapy can be considered as one of the priority areas in modern biomedicine and biotechnology.

Cell therapy has a number of significant advantages:

1. As opposed to sophisticated surgery, cell transplantation is technically a much simpler and less invasive procedure; it has no risk of rejection or other complications.

2. Donor material for cell therapy is easier to obtain; it can be prepared beforehand and cryopreserved for long-term storage.

3. Cell transplantation not only compensates for the organ dysfunction and facilitates restoration of the function of a patient's own cells, but it also impedes the emergence of fibrosis in damaged tissues by filling the missing cell niche.

4. The cells, upon autologous transplantation, are not eliminated by the immune system and can give a prolonged (or permanent) effect. In the case of allogeneic transplantation for inherited disorders, the donor material can compensate for the recipient's genetic defect as normal proteins are synthesized by donor cells.

The efficiency of substitution of tissue defects, ability to stimulate a recipient's own organ repair, the absence of a risk of emergence of fibroses mainly depend on the cells being used. It has been demonstrated in a number of studies that cells of different types can express hepatocyte-specific markers under certain growth conditions. However, the true functionality of particular cells still needs proof. The question that emerges is what criteria does a transplanted cell need to meet in order to provide efficient compensation for the dysfunction of the damaged liver? Firstly, that would be the ability to carry out synthetic and detoxication functions. The cells need to be capable of expressing hepatocyte-specific proteins, such as cytochromes P450

and albumin, as well as storing glycogen, synthesizing urea, binding bilirubin, etc. The search for the optimal cell sources and obtainment of functionally active types of cells in amounts sufficient for transplantation obviously remain among the main challenges of cell biology. The cells need to be easy to obtain and capable of rapid *in vitro* proliferation, endure long-term cryostorage, be immunocompatible and capable of differentiating into functionally active hepatocyte-like cells.

Repair success also depends on participation of the growth factors, cytokines and chemokines, which are part of the complex signalling system coordinating cell behavior. For this reason, the cells capable of identifying the proper growth factor combination can be proposed for the stimulation and correction of the repair of certain tissue defects. On the other hand, the cells being used may make a significant contribution (in many cases, the contribution is crucial) to the repair process due to transdifferentiation into target-differentiated and functional-tissue cells.

MECHANISMS OF LIVER CELL REGENERATION

The liver possesses a high degree of self-restoration and a considerable capability of repair even after resection of its largest part. These properties are provided by a complex regeneration system (*Fig. 1*). Its major features include the proliferative capability of differentiated hepatocytes, as well as their ability to produce mature hepatocytes and transdifferentiate into cholangiocytes [1]; regeneration from the reserve stem cells; repair with haematopoietic cells via fusion of myeloid cells with damaged hepatocytes and/or differentiation of bone marrow mesenchymal stem cells into hepatocyte-like cells [2, 3].

Hepatocytes are differentiated polyploid cells; however, their capability to proliferate and population maintenance makes them similar to stem cells. In adult liver, hepatocytes mostly remain in a dormant state (G0 phase of the cell cycle); however, if regeneration becomes necessary, hepatocytes start dedifferentiating, proliferating, and reproducing differentiated hepatocytes. For example, after biliary cells in rat liver were damaged, hepatocytes exhibited a certain degree of phenotypic plasticity and were capable of transdifferentiation into cholangiocytes [1]. The hepatocyte population increases without the participation of stem cells during the postnatal growth [4]. During the fetal and early postnatal periods, hepatocytes undergo mitosis, followed by the process of mitotic polyproidization, resulting in an increase in the number of hepatocytes and their ploidity. Cytotomy does not occur in the first cycle after DNA replication, giving rise to a binuclear hepatocyte. The next mitotic cycle after DNA duplication includes synchronous nuclear division; chromosomes

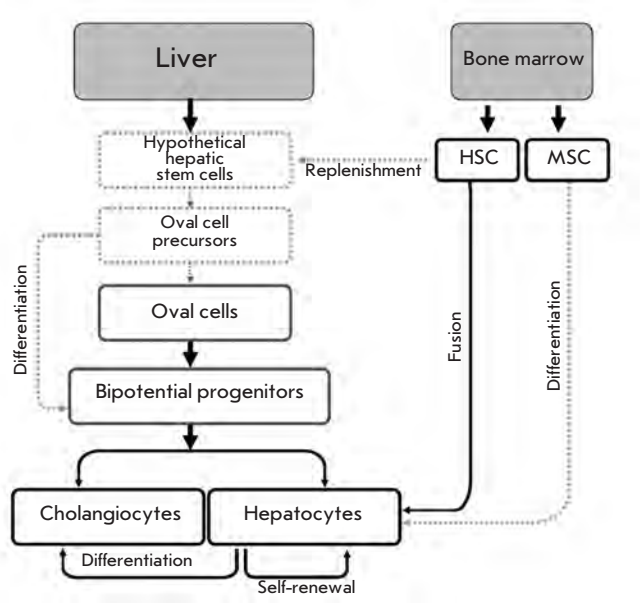

Fig. 1. Mechanisms of cellular regeneration of postnatal liver. Taken and modified from [2, 3]. The scheme is hypothetical.

aggregate to yield a single mitotic plate, giving rise to two mononuclear tetraploid cells. The alternation of these two cycles with a gradually increasing hepatocyte ploidity occurs subsequently [5]. In order to make possible postnatal growth of the liver, the initially diploid hepatocytes undergo five or six polyploidizing mitoses. However, in the cases requiring rapid regeneration (e.g., after exposure in toxic or infectious conditions, etc.) mitoses without cytokinesis are temporarily eliminated and cell fission proceeds via the conventional pathway. This protects liver cells against excessive polyploidization. The major factors regulating hepatocyte proliferation in liver regeneration include interleukin-6 (IL-6) and the tumor necrosis factor α (TNF-α) secreted by Kupffer cells, as well as the hepatocyte growth factor (HGF) secreted by stellate cells. These factors initiate hepatocyte transition from the G0 to the G1 phase. The transforming growth factor β (TGF-β) suppresses the entrance of hepatocytes into mitosis upon completion of regeneration. HGF, the vascular endothelial growth factor (VEGF), and the fibroblast growth factors 1 and 2 (FGF1, FGF2) secreted by endothelial cells play an important role in the replication and viability maintenance of hepatocytes as well [6, 7]. The major molecular mechanisms making possible hepatocyte proliferation are schematically shown in *Fig. 2*.

Hepatic stem cells also play a significant role in the regeneration process if the hepatocyte population proves incapable of repairing the damaged liver (after

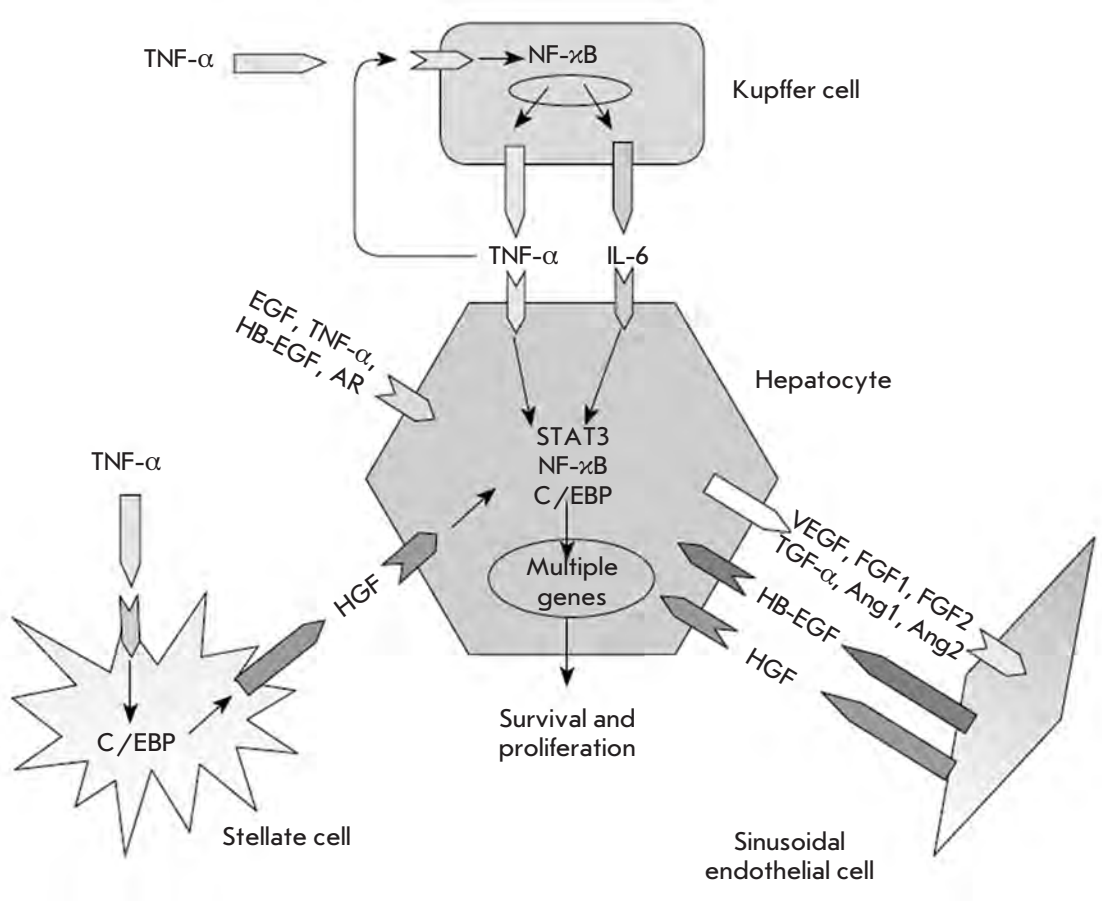

Fig. 2. Molecular mechanisms of hepatocyte population maintenance and initiation of hepatocyte proliferation. Taken from [7].

the resection of the critical part of the organ, upon extensive toxic, infectious, etc. lesions). The postnatal liver contains a number of stem cells whose hierarchical relationship is still under discussion [8]. Oval cells are the major precursors of hepatocytes and cholangiocytes. The term "oval cells" is usually used to refer to a population of small cells (about 10 μm) that possess bipotent differentiation potential and are characterized by a high nuclear-cytoplasmic ratio. Oval cells presumably originate from the canals of Hering, which are believed by some authors to exclusively consist of stem cells [9]. Oval cells express albumin, α-fetoprotein, cytokeratin 19, the specific surface marker OV6 (A6 in mice), and the embryonic marker Delta-like/Pref-1 that is also typical of hepatoblasts [10]. In addition, oval cells produce stem cell markers, such as c-Kit, Sca-1, nestin, and CD90 (Thy-1). In all likelihood, the population of these cells is heterogeneous and may contain cells of different origins. Some cells carry the CD45, c-Kit, CD90 markers and albumin. These cell populations presumably consist of haematopoietic stem cells that penetrate into the liver from the blood flow [11]. In general, the population of true oval cells expressing the markers OV6 and cytokeratin 19 is the population of committed, temporarily proliferating hepatic stem cells. An assumption was made that the adult liver has a compartment with less differentiated cells, the original stem cells of the postnatal liver. A population of stem cells expressing the epithelial cell adhesion molecule EpCAM was obtained in [12]. These cells were referred to as hepatic stem cells $EpCAM^+$ (hHpSCs); in the fetal liver, they act as hepatoblast precursors; in the postnatal liver, they reside in the canals of Hering. Hepatic stem cells also express NCAM, c-Kit, CD133/1, CD44H, cytokeratin 19 and are weakly positive with respect to albumin. Hepatic stem cells do not express α-fetoprotein, CD45, or mature hepatocyte markers (cytochromes P450, intracellular adhesion molecules ICAM-1, transferrin). With *in vitro* differentiation induced, the cells proved capable of synthesizing α-fetoprotein and ICAM-1. Transplantation of hepatic cells to NOD/SCID mice induced the synthesis of proteins typical of mature hepatocytes (albumin, transferrin). It was assumed that these cells are stem cells in the fetal and postnatal liver and may presumably be precursors of oval cells [12]. The general hierarchy of hepatic stem cells is shown in *Fig. 3*.

Bone marrow stem cells can also contribute to liver regeneration. The liver is known to serve as a haematopoietic organ during the fetal and early postnatal periods. In the adult liver, part of the population of oval cells is made up of haematopoietic cells that are CD34-, CD45-, and CD133-positive; the liver can become an organ of extramedullary haematopoiesis upon certain pathological processes. It has been demonstrated that if bone marrow from male mice is transplanted to lethally irradiated female mice, 1–2% of hepatocytes carry the Y-chromosome marker 6 months following the transplantation. These hepatocytes express albumin and can be either diploid or polyploid [13]. When studying the biopsy material obtained from the liver of six women who received haematopoietic cells obtained from the peripheral blood of male donors, the Y-chromosome was revealed in hepatocytes at an s frequency varying from 0 to 7% [14]. Haematopoietic stem cells were assumed to be capable of differentiating into hepatocytes; however, a number of studies have demonstrated that haematopoietic cells can fuse with a recipient's hepatocytes, thus preventing their death and stimulating regeneration [15, 16]. Myelocytary cells, granulocytes, and macrophages/monocytes also undergo fusion with hepatocytes [16]. The relative contribution of transdifferentiation and cell fusion to liver repair by haematopoietic stem cells is currently being discussed. There is a possibility that both these processes occur in the organism.

USE OF CELLS ISOLATED FROM DONOR LIVERS

Hepatocyte transplantation can serve as an alternative approach to the liver transplantation that is conventionally used in modern clinical practice. It is a commonly known fact that liver transplantation may include the substitution of either the entire liver with a donor organ or part of it. However, the shortage of donor organs, the poor implant survival rate, and significant complications due to rejection or insufficient functioning of the transplanted liver limit the applicability of this method to a significant extent. Furthermore, a sufficiently efficient procedure enabling long-term storage of the liver as a whole organ has not been elaborated thus far. Due to these reasons, transplantation of hepatocytes isolated from a donor liver becomes a promising direction of cell therapy for liver disorders. The advantages of this approach include the possibility of using both freshly isolated cells and cells subjected to long-term cryostorage; donor cells can compensate for the pathologies caused by genetic disorders and act as gene therapy vectors. Hepatocyte transplantation is a significantly less invasive procedure; it virtually has no risk of rejection. The transplanted hepatocytes fill the cell niches that remain

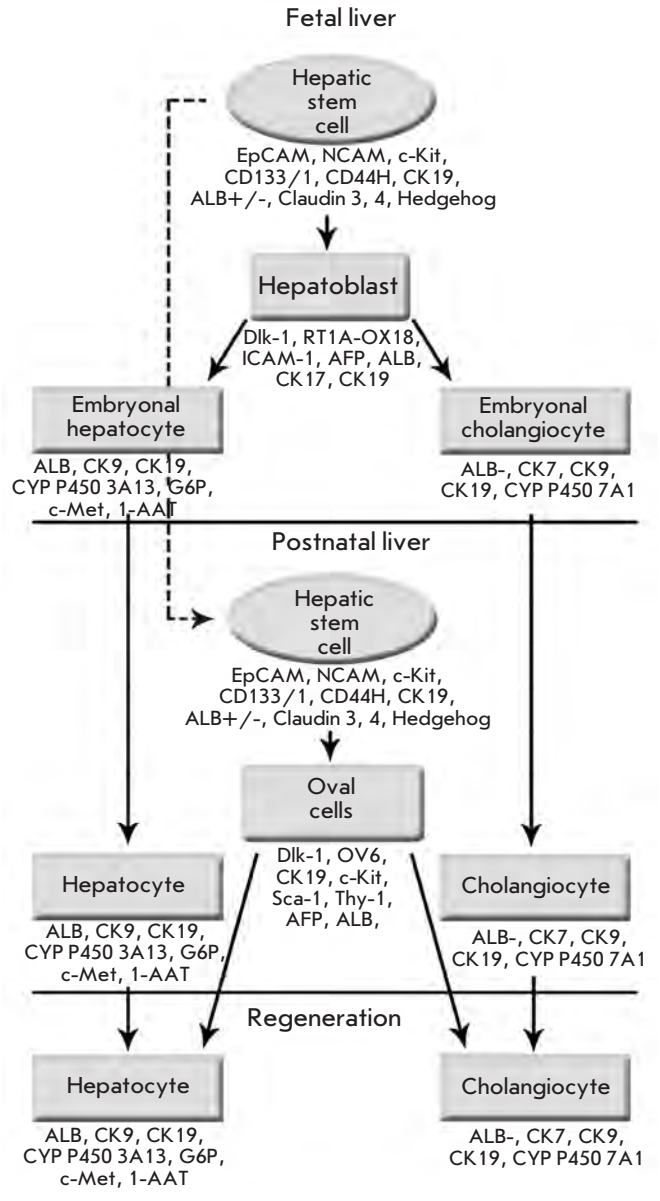

Fig. 3. The hierarchy of liver stem cells. Taken and modified from [2, 115]. The scheme is hypothetical.

empty as a result of mass death of the patient's own cells (e.g., after acute exposure to toxic or infectious conditions), which considerably reduces the risk of fibrosis formation. Moreover, hepatocyte transplantation does not require resection; thus, regeneration of the patient's own organ is possible (e.g., upon acute hepatic failure).

The hepatocyte transplantation procedure includes a number of conventional techniques elaborated in accordance with the GMP (Good Manufactured Practice) requirements [17]. A donor liver that cannot be

used for transplantation due to fatty dystrophy (over 40–50% of the organ), chronic ischemia, mechanical damage, liver capsule rupture, blood group mismatch, damaged blood vessels or biliary ducts can serve as a hepatocyte source [18–20]. Fetal liver can be used for transplantation in rare cases [21]. The cell sources may include the liver from non-heart-beating donors, liver affected with atherosclerosis or fibrosis. The standard hepatocyte isolation procedure includes liver perfusion, enzymatic treatment to disintegrate the intercellular substance, and washing of the resulting cell suspension. The isolated hepatocytes are typically characterized by an approximately 70–90% viability and (1—17) x 10^6 cells/g of tissue (hepatocytes with at least 60% viability are recommended for use for clinical purposes). The cells obtained are cooled to +4°C and immediately re-suspended in an infusion solution to be directly transplanted or in a freezing solution for subsequent cryostorage [22, 23]. The metabolic characteristics of the hepatocytes are checked based on the activity of the cytochromes P450 (CYP1A2, CYP2A6, CYP3A4, CYP2C9, and CYP2E1) and their ability to synthesize urea [24].

Hepatocytes are typically transplanted via the portal vein, the splenic vein, or via an intraperitoneal catheter. Direct transplantation into the peritoneal cavity, pancreatic gland, or hepatic parenchyma demonstrates a poorer survival rate of hepatocytes. Introduction via the portal vein is regarded as the best method of transplantation; however, when performing this procedure, one needs to control the pressure in the portal vein to prevent its obstruction [25, 26]. Introduction of hepatocytes into the spleen is typically used in patients with chronic liver disorders, when fibrosis impedes cell engraftment. The amount of cells required for transplantation depends on the type of pathology and is equal to about 5–10% of the theoretical liver weight ((2–4) x 10^8 cells/kg of body weight); however, no more than 1% of the amount of a patient's hepatocytes is introduced per procedure. The adult human liver contains approximately 2.8 x 10^{11} hepatocytes; therefore, the recommended amount of donor cells to be introduced per transplantation procedure is (2–4) x 10^9 [27]. According to some reports, the amount of cells to be transplanted can be lower in case of chronic disorders, whereas it needs to be increased for the therapy of inherited pathologies. A stable therapeutical effect is achieved on week 4–8 following transplantation and lasts for 6–9 months.

At the moment of writing, donor hepatocytes have been transplanted to more than 80 patients in 13 medical centres [18, 19, 28–30]. Among them, about 30 (including children) had inherited metabolic disorders of the liver, such as ornithine transcarbamylase deficiency or glycogenosis. Hepatocyte transplantation significantly improved the condition of patients with inherited disorders. It has also been demonstrated that hepatocyte transplantation can stabilize the condition of children awaiting donor liver transplantation [29, 31]. In a series of case reports, e.g., in patients with the Crigler-Najjar disease, the amount of cells required to achieve a stable clinical effect is equal to 12% of the patient's liver weight; therefore, repeated transplantations are needed because of the limited amount of cells that can be introduced per transplantation. Hepatocyte transplantation in patients with disorders of bilirubin metabolism can be a successful alternative to whole liver transplantation during a period of over 11 months [32–34]. Restoration of normal glucose levels has been observed in patients with glycogenosis (both children and adults) [19, 35].

The major drawback of this method is a shortage of donor material. The priorities in this field include the improvement of the quality of the isolated hepatocytes, optimization of cryostorage procedures, and enhancement of the efficiency of liver "accommodation." No optimal immunosuppressive procedures have been designed thus far as well: the transplanted donor hepatocytes are known to be eliminated from the liver in 6–9 months. Approaches may include selecting optimal populations of hepatic stem cells capable of proliferation and significant *in vitro* division followed by differentiation, and designing proper cell lines [36]. On the other hand, the search for an optimal alternative source of cells (including authologous sources) for the therapy of liver disorders remains a priority.

ALTERNATIVE SOURCES OF CELLULAR MATERIAL

The demand for alternative sources of cellular material for the therapy of liver disorders is mainly fuelled by the shortage of donor organs and low availability and insufficient amount of hepatocytes that can be used for transplantation. Moreover, cells obtained from alternative sources can be used for autologous transplantation. It has been demonstrated that different cell types are to a certain extent capable of differentiating into hepatocyte-lineage cells; however, no functionally active hepatic cells have been obtained thus far [37]. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells [38–41], as well as hepatic stem and progenitor cells, [12, 42] are the best studied both experimentally and clinically at this moment. Mesenchymal cells from bone marrow [43, 44] and adipose tissue [45–47], amniotic fluid cells [48–50], etc. have been studied as cells capable of differentiation into hepatocytes. However, only partial transdifferentiation has been observed in these studies; the functionally active state that is typical of hepatocytes has not been attained.

The major stages of differentiation of ES cells into hepatocytes [55, 56]

Differentiation stage	Duration, days	Major differentiation markers	Hepatocyte markers characteristic for this stage
Induction of endoderm formation	3–4	Activin A	Sox17. Hnf-3β
Cell commitment to the hepatocyte lineage	4–7	BMP2, FGF4	Hnf-3β, alpha fetoprotein
Proliferation of hepatoblast-like cells	5–10	HGF, KGF	Albumin, alpha fetoprotein, G6P, TAT
Maturation of hepatoblast-like cells	8–15	Oncostatin M, dexamethasone, N2, B27	Albumin, G6P, TAT, PEPCK, TDO, CYPP450, etc.

Pluripotent ES and iPS cells

The interest in embryonic stem cells is mainly rooted in their broad differentiation potential: embryonic stem cells isolated from the inner cell mass of blastocysts retain their pluripotent properties upon long-term *in vitro* cultivation and can produce cells of all three germ layers. At the time of writing, a large amount of studies have been devoted to the differentiation of ES cells into various cell types of the adult organism. Meanwhile, the practical use of ES cells can be limited by a number of unsolved problems, such as the risk of teratoma formation, ethical issues related to the destruction of embryos, long-run and labor-intensive differentiation protocols, etc. The low immunogenicity of human ES cells has been reported, which may also be of interest. However, it remains unclear whether these cells retain their low immunogenicity after differentiation into a certain lineage is induced [51].

The hepatocyte differentiation protocols of ES cells include several major stages imitating the processes occurring during liver development [52–54]. The major stages of the process are given in *Table*.

Various demethylating agents are used to enhance the differentiation efficiency. The idea of using demethylating agents is based on their ability to activate gene expression by DNA demethylation: demethylation of the promoter regions activates gene expression, which significantly broadens the differentiation potential of cells. However, since DNA demethylation is a random process, the combination of demethylating agents and growth factors or cytokines is used to commit cells into a certain lineage [32]. The differentiation efficiency of murine ES cells was successfully increased using valproic acid inhibiting histone deacetylase [57]. Hepatocyte-like cells capable of synthesizing albumin, cytochromes P450 and accumulating glycogen have thus been obtained. Differentiation without valproic acid yielded structures resembling biliary duct cells. However, in this case, the injection of ES cells differentiated into the hepatocyte lineage to Balb/c nude mice resulted in teratoma formation [57]. It should be mentioned that no teratomas have been observed after human ES cells differentiated into hepatocytes are injected to immunodeficient mice, whereas the injection of undifferentiated ES cells has resulted in teratoma formation [55, 58].

Another source of hepatocyte-like cells is iPS cells. iPS cells are induced pluripotent stem cells that are artificially obtained from the somatic cells of the human organism, into which certain genes and factors that are important to attain the pluripotent state are introduced [59]. Identically to ES cells, iPS cells can differentiate into cells of all three germ layers; however, opposite to ES cells, it is possible to obtain autologous iPS cells for substitutive cellular therapy and iPS cells from patients with various inherited disorders to simulate the pathological process *in vitro* and test therapeutic agents [60, 61].

In general, the hepatocyte differentiation protocols of iPS and ES cells are similar. *In vitro* differentiation of human iPS cells into hepatocyte lineage cells using cytokines and adenoviral vectors expressing the *Hex* gene, which plays a significant role in hepatocyte development, yielded hepatocyte-like cells expressing the endoderm markers Hnf-3β and Sox17, as well as albumin and cytochromes P450 [60]. It was also shown [54] that 60% of the cells start producing albumin and α-fetoprotein on day 7 of the differentiation of human iPS cells using the standard protocol; by day 20, the cells were capable of synthesizing urea (approximately 15% of the level of urea synthesis by hepatocytes) and storing glycogen [54], but the percentage of hepatocyte-like cells was low (about 10%). However, the absence of an oncogenic potential for using these cells has not been demonstrated.

Somatic cells

Hepatic stem and progenitor cells. Multipotent postnatal hepatic and progenitor cells can be an alternative source for cellular therapy. They actively proliferate *in vitro* (and/or *in vivo*), enabling one to obtain significant amounts of such cells from a small bioptate. These cells retain viability for a considerably longer time period and better endure cryostorage compared to mature hepatocytes; furthermore, they are characterized by a lower immunogenicity. Hepatic stem cells are both capable of *in vivo* differentiation into hepatocytes and population maintenance; this fact may prolong the therapeutic effect of their introduction. Stem cells are already committed to hepatocytes and require no additional time-consuming differentiation procedures. The major problem impeding the widespread use of these cells is the shortage of donor material.

Specific attention is given today to methods consisting in the isolation of hepatic stem cells and searching for optimal cell populations possessing the highest regenerative potential. Hepatic cells carrying the surface marker and epithelial cell adhesion molecule EpCAM were isolated by continuous-flow fluorometry. The percentage of such cells in donors of all ages is 0.5–2.5% of the hepatic parenchyma cells. These cells can undergo over 150 *in vitro* passages and are positive with respect to cytokeratins 8, 18 and 19, CD133/1, CD44H, and weakly positive with respect to albumin. Hepatic cells do not express α-fetoprotein, adult hepatocyte markers (cytochromes P450), intracellular adhesion molecules ICAM-1, markers of haematopoietic (CD45) and mesenchymal cells (desmin, VEGFRe). After differentiation is induced, these cells acquire the capability of synthesizing α-fetoprotein and ICAM-1. Transplantation of hepatic $EpCAM^+$ cells to NOD/SCID mice has resulted in the formation of hepatic structures from human cells and in the synthesis of proteins that are typical of mature hepatocytes. Thus, it has been assumed that these cells act as stem cells of the postnatal liver and can be used for substitutive cellular therapy [12]. In another study, a Thy-1 (CD90)-positive cell population was isolated from the adult donor liver via immunomagnetic sorting. In all likelihood, this population was heterogeneous and contained cells that were positive with respect to markers of progenitor cells, namely, haematopoietic cells – CD34, stem cells – CD117, CK19, duct cells – CK14, and oval cells – OV6. The population of Thy-1-positive cells possessed a higher differentiation potential compared to that of the Thy-1-negative population and was capable of differentiating both into hepatocytes and duct cells. The functional activity of these cells is supported by the expression of HepPar 1 and human albumin after they are injected to immunodeficient mice [42]. The isolation of the so-called SP (side population) cells via continuous flow fluorometry can be considered as another approach. A number of types of stem cells were shown to contain the ATP-dependent ABC transporters responsible for the elimination of various cytostatics and drugs, whose activity results in the development of the multiple-drug-resistance phenomenon, from the cell. Dye Hoechst 33342 is one of the compounds eliminated from stem cells; the use of this dye allows one to sort unstained small cells (referred to as SP cells) on a continuous-flow cytofluorimeter. CD45- and Hoechst 33342-negative SP cells capable of colony formation upon *in vitro* growth have been derived from the human liver. Large cells containing a large number of granules, intracellular lipofuscin and, rather frequently, the ambiguous nucleus emerged in the colonies after 2–3 weeks of cultivation. The cultured cells were positive with respect to human hepatocyte markers: namely, HepPar, cytokeratins 8 and 18, cytochromes P450 and albumin. Thus, SP cells isolated from an adult donor liver are capable of *in vitro* differentiation into hepatocyte lineage cells [62]/

HSC and MSC obtained from bone marrow, cord blood and adipose tissue. The interest towards bone marrow stem cells as a potential source of hepatocytes appeared in early studies carried out by Petersen *et al.* [63]. Donor cells were found in the liver of irradiated mice after transplantation of the bone marrow; these cells subsequently differentiated into hepatocyte-like cells. These experiments have cast doubt on the previous assumption that hepatocytes can be obtained exclusively from endodermal sources. It turned out that hepatocytes with a male karyotype could be detected in women transplanted with bone marrow derived from male donors [13]. It remains unclear whether hepatocytes are formed from bone marrow cells via transdifferentiation, fusion, or lateral gene transfer; this question remains a subject for discussion [64].

Haematopoietic stem cells (HSC) can be easily sorted based on CD31 and CD34 markers and isolated from the bone marrow, cord blood, or, in certain cases, from peripheral blood. It has been demonstrated that upon hepatic lesions, transplanted human HSC become capable of producing albumin-synthesizing cells in murine liver and repairing hepatic defects both via fusion [15] and without fusion with the host cells [65]. Yet, the cell fusion phenomenon has not been observed in bone marrow-derived mesenchymal stem cells (MSC) [66]. MSC derived from bone marrow, cord blood, and adipose tissue exhibit immunosuppressive and anti-inflammatory properties, can be easily grown *in vitro*, and synthesize a number of cytokines and growth factors capable of stimulating the repair of a patient's own cells. Because

of these properties, MSC are often regarded as a convenient cellular source for substitutive cellular therapy [67–69]. The condition of mice with acute hepatic failure induced by carbon tetrachloride was shown to improve after the transplantation of bone marrow MSC. A significantly higher survival rate of hepatocytes was observed in the experimental group compared to the control group, despite the fact that MSC engraftment had not occured by the time of the observation. The positive effect of MSC introduction is attributed to their stimulating and anti-inflammatory action [70]. Intact MSC from human cord blood were also introduced into fetal sheep liver; expression of human albumin was detected 56–70 days following the transplantation; the percentage of human cells in lamb liver varied from 2.6 to 12.5% [71].

MSC have been differentiated into hepatocyte-like cells in a number of studies. Expression of α-fetoprotein and albumin was achieved through treatment of MSC from human adipose tissue with HGF, oncostatin M, and dexamethasone [45]. In another study, a hepatocyte culture medium and a demethylating agent (20 μM 5-azacytidine) were used to differentiate rat adipose tissue-derived MSC into cells expressing albumin, α-fetoprotein, cytochromes P450 1A1, and cytokeratins 18 and 19 [46]. These cells were also capable of synthesizing urea. *In vitro* hepatocyte differentiation could not be induced upon differentiation of MSC derived from human bone marrow using FGF4, HGF, and dexamethasone. However, the addition of the demethylating agent trichostatin A (1 μM) inhibiting histone deacetylase yielded epithelium-like cells expressing cytokeratin 18. The cells also synthesized albumin, and they were characterized by enhanced cytochrome P450 activity and urea secretion [43].

Thus far transplantation of bone marrow cells for the therapy of liver disorders has been performed on several occasions [72]. The granulocyte colony-stimulating factor (G-CSF) was used in some of the transplantations to immobilize patients' own bone marrow stem cells and to stimulate liver regeneration without isolating bone marrow [73, 74]. Transplantation of autologous bone marrow-derived stem cells to 27 patients with chronic hepatic disorders or cirrhosis resulted in an increase in albumin secretion and a decrease in the bilirubin level [75–77].

Despite some degree of success in using bone marrow-derived stem cells in patients with liver diseases, the mechanism underlying their action remains unclear. The problems related to safety have not been solved, including those associated with possible MSC-induced fibrosis, which may worsen the course of the disease [78]. The impact of these cells on damaged liver and their mechanisms of action need elucidation prior to making any attempts at using them in clinical practice.

Amniotic fluid cells. Amniotic fluid contains a heterogeneous population of cells of fetal origin with stem cells positive with respect to mesenchymal markers (CD29, CD44, CD73, CD90, CD105), neutral markers (nestin, β-3-tubulin, NEFH), and certain pluripotency markers (Oct4, Nanog). These cells are of interest mostly due to their broad differentiation potential: they can undergo *in vitro* osteogenic, adipogenic, neutral, endothelial, hepatocyte, etc. differentiation [50, 79–82]. It has recently been demonstrated that amniotic fluid stem cells can express epithelial markers (keratin 19, keratin 18, and p63) simultaneously with the mesenchymal markers [83]. This fact has disproved the previous concept that amniotic fluid stem cells are MSC. Although the status of these cells is being actively discussed, an assumption can be made that the ability to form fibrous lesions upon introduction of amniotic fluid cells will be lower than that for cells of truly mesenchymal origin. The drawbacks of this cellular source include the low availability of these cells, the limited amount of donor material, and the requirement to collect cells at a certain stage of the pregnancy, which is not always possible.

The possibility of hepatocyte differentiation of amniotic fluid cells has been demonstrated. The cells were grown in matrigel- or collagen-coated plates in the presence of HGF, FGF4, insulin, oncostatin M, and dexamethasone. Cell morphology was altered by day 7 of differentiation: the cells acquired a polygonal shape without spikes. Synthesis of albumin, α-fetoprotein, Hnf-4α, and HGF receptor c-Met was observed on day 45. The level of synthesized urea increased from 50 ng/h per cell in the control culture to 1.21 x 10^3 ng/h per cell in the differentiated culture [49]. The differentiation abilities of human bone marrow-derived MSC and amniotic fluid stem cells were compared. Cells were grown in collagen I coated plates in the presence of differentiating agents: days 0–2 – FGF4, days 3–5– HGF, days 6–18 – HGF + insulin-transferrin-selenite + dexamethasone and trichostatin A (histone deacetylase inhibitor). Morphological changes were observed in both cultures starting on day 7: the cells became rounder and polygonal in shape. The shape of amniotic fluid cells subsequently changed to that of epithelial cells in a more rapid and stable fashion. It was demonstrated by quantitative PCR that the original expression of hepatocyte markers, such as α-fetoprotein, albumin, cytokeratin 18, Hnf-1α, C/EBPα, and CYP1A1, was either negligible or absent in both cell cultures. The expression of these markers remained virtually unaltered at the initial stage of differentiation. However, at the stage of

hepatocyte maturation expression of hepatocyte markers increased significantly; on day 14 of the differentiation, expression of all the markers in the amniotic fluid cell culture was considerably higher than that in the bone marrow-derived MSC culture. Expression of all markers, with the exception of α-fetoprotein, increased at the stage of hepatocyte maturation. Expression of α-fetoprotein reached a maximum by day 14 of the differentiation, followed by a decrease, whereas maximum albumin expression was observed by day 28 of the differentiation. Albumin expression in amniotic fluid cells was approximately 1.3 times higher than that in bone marrow-derived MSC. An immunophenotypic analysis revealed that the percentage of cells that are positive with respect to hepatocyte markers is reliably higher than that in the MSC culture. These cells were also capable of synthesizing urea and accumulating glycogen [50].

All these data attest to the high potential of using amniotic fluid stem cells in cellular therapy; however, a better understanding of their differentiation status and fibrosis formation ability is required.

Cells of endodermal origin. The possibility of cell transdifferentiation within the same developmental germ layer lineage is currently being actively studied. The advantages of this approach are obvious: cells of close histogenetic origin exhibit a considerably higher phenotypic plasticity within the same developmental germ layer lineage; they can be more rapidly and deeply transdifferentiated into other cell types of the same developmental germ layer lineage without time-consuming and labor-intensive differentiation protocols.

A sufficient body of data pertaining to *in vitro* and *in vivo* transdifferentiation of endodermal cells has been accumulated. Pancreatic ductal cells transplanted into the rat liver differentiate into hepatocytes [84]. Oval cells can also differentiate into endocrine and exocrine pancreatic cells [85]. Islet cells in an *in vitro* culture can differentiate into hepatocytes if the seeding density increases [87]. Thus, endodermal cells are capable of mutual transdifferentiation and can compensate for the functional insufficiency of another tissue within the endoderm germ layer. However, the problem of shortage of donor material exists both for hepatic and pancreatic cells. For this reason, the search for an optimal source of endodermal cells for substitutive cellular therapy remains rather topical.

Salivary gland cells are one of the potential sources of endodermal cells. The salivary gland is usually formed during the embryonic stage as an ectodermal bud; cells of endodermal origin subsequently migrate into it [88]. Since salivary gland cells are functionally identical to exocrine pancreatic cells, they can be used as a convenient source of endodermal cells for substitutive therapy in patients with hepatic and pancreatic disorders. A sufficiently large body of data pertaining to *in vitro* cultivation of salivary gland cells isolated from humans and animals has been accumulated. The *in vitro* cultured salivary gland cells represent an actively proliferating culture that is capable of undergoing a significant number of passages [89]. Salivary gland cells in humans and animals (mouse, rat, pig) are positive with respect to cytokeratins 18 and 19 and often with respect to α-fetoprotein [90, 91]. Salivary gland cells become capable of synthesizing albumin under certain conditions [92]. However, this source of cellular material remains relatively poorly studied. Thorough elucidation of the mechanisms of hepatocyte differentiation of salivary gland cells and their contribution to the treatment of liver diseases is still to be performed.

Direct differentiation technique: the use of genetic constructs for somatic cell reprogramming

The direct technique of cellular differentiation is based on using genetic constructs for the re-programming of various cell types directly into the target cells, bypassing the return to their pluripotent state. One of the major advantages of this approach over using pluripotent ES and iPS cells consists in the absence of risks of teratoma formation. Being a relatively new approach, it requires thorough understanding of the molecular and genetic mechanisms of a certain cellular differentiation and has been recently undergoing active development.

A number of studies have been carried out that demonstrate that direct re-programming of cells of different origins is possible [93]. For instance, functioning β-cells can be obtained from murine exocrine pancreatic cells. The minimum gene set (*Ngn3*, *Pdx1* and *Mafa*) required to re-program differentiated cells derived from an adult organism into cells exhibiting the properties of endocrine pancreatic cells has been determined experimentally by the *in vivo* re-expression of key regulatory genes. These cells are identical to endogenous β-cells in terms of their size, shape, and ultrastructure; they express the genes required for β-cell function and can reduce hyperglycemia by actively secreting insulin and facilitating the rearrangement of local blood vessels [94].

As for hepatic cells, there are only very few studies devoted to the obtainment of functionally active hepatocyte-like cells via direct differentiation. This can be mainly attributed to the complexity and multistageness of hepatocyte differentiation, which impedes the search for the key differentiation genes. However, the first success in this area has already been achieved. The lentiviral transfection of 14 genes playing a key role in liver development was used to induce hepato-

cyte differentiation of fibroblasts obtained from mouse tail-tip [95]. After the analysis of the published data, two gene sets inducing the epithelial phenotype in fibroblasts and expression of hepatocyte markers were selected. The first set consisted of six genes: *Foxa2*, *Foxa3*, *Hnf-1α*, *Hnf-4α*, *Hnf-6*, and *Gata4*; the second one contained eight genes, including *Foxa1* and *Hlf* [96, 97]. A significant increase in the number of epithelial-like colonies was observed after *Hnf-6* was eliminated from the gene set, whereas elimination of *Hnf-4α* promoted the formation of epithelial-like colonies to an even greater degree. The remaining genes were also divided into two sets: *Gata4*, *Hnf-1α*, *Foxa3* and *Gata4*, *Hnf-1α*, *Foxa2*; the former set showed a better result. It is interesting to note that the use of the *Gata4*, *Hnf-1α* and *Foxa3* gene set provided endogenous Foxa2 and Foxa3 expression, whereas the elimination of any gene from this set blocked hepatocyte re-programming. The induced hepatocyte-like cells were called iHep. These cells were positive with respect to E-cadherin and the tight-junction protein Tjp1. On day 14, 23% of the epithelial-like cells were albumin-positive. iHep were also positive with respect to α-fetoprotein, cytokeratins 18 and 19, Hnf-4α, and cytochromes P450. No pancreatic differentiation markers were detected; iHep did not exhibit the properties of cell types other than hepatocytes. iHep were also capable of accumulating glycogen and secreting albumin into the medium. An intrasplenic injection of iHep cells to $Fah^{-/-}$ mice with disturbed tyrosine metabolism, which can survive only if their food contains 2-(2-nitro-4-trifluoromethylbenzene)-1,3-cyclohexandione, resulted in considerable liver re-population (from 5 to 80%). These mice could survive without receiving 2-(2-nitro-4-trifluoromethylbenzene)-1,3-cyclohexandione, whereas an injection of the intact fibroblasts caused death of mice and did not result in liver re-population [95]. All these data attest to the efficiency of the direct differentiation of murine fibroblasts into hepatocyte-like cells via the regulatory factors Gata4, Hnf-1α, and Foxa3. Nevertheless, this approach requires further investigation, since the use of re-programmed fibroblasts is associated with an increased risk of fibrosis formation in the culture. There can be another optimal set of regulatory genes if cells with a minimum tendency to develop fibrosis are used.

Another approach to stimulating liver regeneration is to use genetic vectors carrying the key genes enhancing hepatic cell proliferation (*Fig. 2*), reducing apoptosis, or compensating for the gene defects of the liver function [7]. However, this approach requires thorough investigation; including designing optimal and safe vectors for gene transfer, elaborating methods for the delivery of vectors to the liver, etc.

MOLECULAR AND GENETIC MECHANISMS OF HEPATOCYTE DIFFERENTIATION

The definitive endoderm spawns most digestive tract organs, including the liver [53]. Prior to the activation of the organo-specific genes, only several early endoderm markers (including Otx2, Hesx1, Hex, Cdx2) are activated. Mesoendoderm cells in the primitive streak subsequently begin producing a number of factors, such as GSC, Hnf-3β, Cxcr4, Sox17a/b, Brachyury, E-cadherin, VEGER2, VE- cadherin, PDGFRa, Gata4, and Gata6 determining the differentiation of the cells of the definitive endoderm and the mesodermal precursors. The liver emerges from the lateral endoderm of the developing ventral compartment of the fore intestine (approximately at the E8.5 stage of mouse embryo development and week 3 of human pregnancy) [97]. The growth factors secreted by cardiac mesoderm and the mesenchyme of the transverse septum (FGF, BMP) stimulate further differentiation of the underlying endoderm into hepatocyte-like cells. Expression of the *Hnf-3* (*Foxa*) genes triggers hepatocyte differentiation in endoderm, which is induced by FGF signals [98]. However, Wnt and FGF4 expression in the mesoderm of the dorsal intestine compartments at this stage inhibits hepatocyte differentiation [99]. Contrariwise, at the late stages (upon formation of hepatocytes and cholangiocytes), Wnt stimulates proliferation and differentiation. HGF, which is required for further growth and proliferation of cells of the liver bud, plays a crucial role for fetal hepatic cells. This type of regulation is performed via the HGF receptor c-Met. HGF impedes hepatoblast commitment into cholangiocytes via blockage of Notch signalling. Endothelial cells have been shown to stimulate liver development (among other factors, due to HGF secretion) [100]. The *Tbx3* gene promotes hepatoblast development via suppression by $p19^{ARF}$ [101]. During hepatoblast formation, their shape changes from a cubic to a prolonged one; a pseudo-multilayered epithelium is subsequently formed. This process is regulated by the *Hex* gene. The basement membrane is subsequently destroyed, and the cells proliferate in the surrounding stroma. These and the later morphological changes are regulated by the *Prox1*, *Hnf-6/OC-1*, and *OC-2* genes. Hnf-6 and OC-2 are regulated by E-cadherin, trombospondin-4, and Spp1, which control cell adhesion and migration in a number of tissue types [102]. Notch provides switching of hepatoblast development from the hepatocyte direction towards bile duct formation [103]. Haematopoiesis also plays an important role in the hepatocyte maturation process. After the liver bud begins to protrude from the endodermal canal, haemopoietic cells secreting oncostatin M and IL-6 migrate into it [104]. Oncostatin M stimulates the expression of hepatocyte

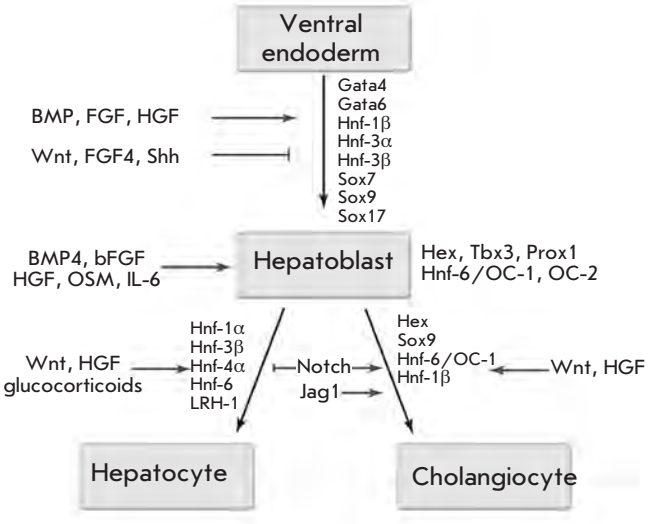

Fig. 4. The main stages of liver cell development. Taken and modified from [97, 116].

differentiation markers, induces morphological changes in cells of the liver bud, promotes activation of the synthetic and detoxication properties of the liver, and controls cell adhesion. Glucocorticoids also promote liver maturation and maintain the proliferation and functioning of differentiated hepatocytes. It has been demonstrated that physiological concentrations of dexamethasone (a synthetic glucocorticoid) in fetal liver suppress α-fetoprotein production, initiate albumin synthesis [104], and promote glycogen accumulation [105]. Figure 4 shows the major stages of development of hepatic cells.

A specific transcription profile that is typical of hepatocytes is maintained by a number of genes, including the *Hnf* family, which encodes hepatocyte nuclear factors. The key genes of this family include *Hnf-1*, a member of the family of POU homeobox genes; *Hnf-3*, a DNA-binding domain; *Hnf-4*, a member of the superfamily of steroid hormones; and *Hnf-6*.

The Hnf-1α and Hnf-1β (or vHnf-1) variants of the Hnf-1 factor interact with homo- or heterodimeric DNA. These proteins have identical DNA-binding domains, but they activate the transcription of different genes. Hnf-1β is expressed in the endoderm of the fore intestine (at stage E5–E6 in mice), whereas Hnf-1α is activated later (at stage E11 in mice), when the parenchyma of the liver is formed. Hnf-1α expression in the fetal liver is lower compared to that of Hnf-1β; however, the Hnf-1α expression level increases after birth. Hnf-1 activates over 1,000 liver-specific genes containing the binding site of this factor in the promoter region; meanwhile, Hnf-1 negatively regulates its own expression. Hnf-4 is a positive regulator of *Hnf-1*, which is capable of activating the expression of this gene; however, the expression of the target genes is independently regulated by these factors [106].

The Hnf-3 subfamily consists of three proteins: Hnf-3α, Hnf-3β , and Hnf-3γ (or Foxa1, Foxa2, and Foxa3, respectively), which bind to monomeric DNA. The members of this subfamily are characterized by strict homology in the area of DNA-binding domains; they can recognize the same nucleotide sequences. Hnf-3α and Hnf-3β regulate gene expression in hepatocytes and in gastric, intestinal, and bronchial epithelium. Hnf-3γ also plays a significant role in gene expression in hepatic, intestinal, and testicular cells. Hnf-3β is formed in the primitive streak on day 7 of mouse embryo development, and Hnf-3α has a similar expression dynamics; however, its concentration is lower. Hnf-3γ expression starts at stage E12 of mouse embryo development.

Hnf-4 consists of three major members (Hnf-4α, Hnf-4β, and Hnf-4γ) and numerous transition variants. Hnf-4 belongs to the superfamily of nuclear steroid hormone receptors; it binds to homodimeric DNA. Hnf-4β has a lower DNA-binding activity and is a weaker transactivator compared to Hnf-4α. Hnf-4α is expressed in the liver, kidney, and pancreas. Hnf-4β is also expressed in these organs, as well as in the stomach, intestine, lungs, ovaries, and testicles, whereas Hnf-4γ is expressed in the kidney, pancreas, testicles, but not in the liver. Hnf-4 is the key regulator of tissue-specific gene expression in visceral endoderm, which is required for normal expression of the secreted factors, such as α-fetoprotein, apolipoproteins, the retinol-binding protein, etc. Some researchers believe that Hnf-4α plays a key role by triggering a reaction cascade and maintaining hepatocyte-specific transcription. Hnf-4α binds to approximately 12% of the genes expressed in hepatocytes, whereas the other transcription factors can bind to no more than 2.5% of the promoter regions [106]. Being one of the earliest endodermal markers, Hnf-4α emerges in mouse embryos on day 5 of development. Prior to stage E9, Hnf-4α expression is confined to the extra-embryonic visceral endoderm; then it is formed in the liver and intestine. In an adult organism, Hnf-4α is expressed in the liver, kidney, intestine, and pancreas.

Hnf-6 belongs to the family of Onecut transcription factors (also known as OC-1). Hnf-6 binds to the CREB-binding protein (CBP) and is expressed in the liver, pancreas, and the nervous system. Hnf-6 is detected on day 6 of fetal development. Between days 12.5 and 15 it disappears from the mouse embryonic liver to emerge there again after day 15. In an adult organism, Hnf-6 is expressed in the liver, pancreas, encephalon, and

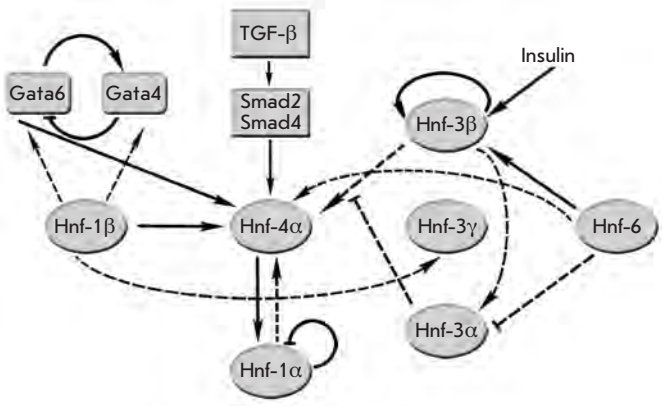

Fig. 5. Transcriptional hierarchy of hepatocyte nuclear factors. The interconnections possessing the most universal character are denoted by continuous lines; the dotted lines show the regulation revealed at certain stages of development. Taken from [108].

testicles. It is an interesting fact that Hnf-3β and Hnf-6 potentially regulate the expression of the same genes; they are crucial to hepatocyte functioning. Hnf-6 recognizes the −138 to −126 region of the *Hnf-3β* promoter; it is required to activate this promoter. Meanwhile, Hnf-3β is capable of binding to the Hnf-6 promoter and represses it [107]. Hnf-6 promotes hepatoblast differentiation into biliary duct cells, whereas Hnf-3β plays a key role in hepatocyte differentiation and functioning.

Expression of nuclear hepatocyte factors and other markers of visceral endoderm during the fetal development is induced by Gata6. TGF-β family growth factors are also capable of inducing the expression of these markers. The hypothetical regulatory pathways ensuring the maintenance of the pattern of liver-specific gene expression are shown in Fig. 5 [108].

PROBLEMS AND PROSPECTS

One of the major problems emerging upon transplantation of donor hepatocytes is poor engraftment and elimination by the immune system within several months after the transplantation. The extracellular matrix has been shown to play an important role in the survival and engraftment of transplanted cells. Either collagen or fibronectin was intraportally injected into rat liver prior to the transplantation of donor cells in a study investigating the effect of the extracellular matrix on the engraftment of donor hepatocytes. Four days following the transplantation, the percentage of hepatocytes that survived in the liver had increased more than tenfold when either collagen or fibronectin was preliminarily introduced [109]. Simultaneous introduction of hepatocytes and transformation growth factors (TGF-α) can also increase the number of survived cells [110]. Cell survival is known to depend on the presence of a corresponding "cellular niche." Temporary blockage of the portal vein inducing ischemia and the partial death of hepatic cells was used in experiments on macaque monkeys. Upon subsequent transplantation of donor hepatocytes, their percentage in the recipient's liver was about 10% of its weight [111]. However, it remains disputable whether it is reasonable to use this approach in clinical practice.

In order to increase the lifespan of transplanted hepatocytes in a patient's organism, ABO and HLA compatible donors are selected. Immunosuppression is now frequently achieved using anti-IL-6 monoclonal antibodies in combination with small doses of the drugs tacrolimus and sirolimus [112]. Natural killer (NK) cells play an important role in the elimination of introduced hepatocytes. It has been shown that blockage of NK cells in the liver by specific or local immunosuppression enhances the survival rate and proliferation of the transplanted hepatocytes [113]. Moreover, the use of hepatic stem cells can have a longer-term effect due to the fact that frequently stem cells are not eliminated by the recipient's immune system and can maintain their population for a long time, giving rise to a population of differentiated cells.

Improving the quality of the isolated hepatocytes is another topical problem. The use of a Celsior® solution to store and transport liver bioptates prevents hepatocyte degradation and death [22]. Perfusion of the donor tissue with N-acetylcysteine has also made it possible to improve the quality of the isolated cells [114].

Biological safety and efficacy remain the key issues in modern cellular therapy. A reliable protocol for the elimination of undifferentiated cells from the transplant is required when pluripotent (ES and iPS) cells are used, since pluripotent ES and iPS cells are capable of teratoma formation. The assessment of the risk of fibrosis formation when certain cell types are used remains problematic, as well. The percentage of donor MSC and their degree of hepatocyte differentiation have been determined in a study of the ability of bone marrow MSC to restore hepatic cells upon acute and chronic lesions. The contribution of the donor cells four weeks following the transplantation turned out to be low (about 0.08% and 3–4% of the total number of hepatic cells upon acute and chronic lesion, respectively); only 5–10% of them had a hepatocyte phenotype. A significant percentage of donor cells (about 35%) had a myofibroblast phenotype; most of the cells resided within septal fibrosis areas [78]. It becomes obvious that a quantitative assessment of the efficiency of hepatocyte differentiation is required prior to using certain cell types, as well as elucidating whether there is a risk

in fibrosis formation by these cells. One of the key tasks of cellular biology is to search for an available source of cells with a low pro-fibrogenic potential and high hepatocyte differentiation ability. Moreover, there should be an opportunity to use these cells for both allogenic and autologous transplantation.

Direct cell differentiation seems to have a high potential; however, one needs a thorough understanding of the molecular mechanisms of the processes occurring upon hepatocyte development and differentiation to elaborate standard protocols. One of the key tasks in this field is to determine the key differentiation genes that would be optimal for the transdifferentiation of cells of various histogenetic origins.

CONCLUSIONS

A number of fundamentally different approaches to the therapy of liver disorders are currently being developed. Various cell types are being tested *in vitro* and *in vivo*, and the optimal differentiation procedures are being selected. Despite some encouraging results obtained on laboratory animals, a sufficiently safe and efficient method is still to be found. A shortage of donor liver and donor hepatocytes stimulates the search for alternative sources of cellular material; however, no cells that could be able to perform hepatocyte functions to an adequate degree have been obtained thus far. A search for the optimal cell type and development of differentiation procedures that would satisfy the biological safety and functional efficiency criteria is needed. ●

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Receptor Properties and Features of Cytokinin Signaling

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ABSTRACT **Cytokinins belong to one of the most important and well-known classes of plant hormones. Discovered over half a century ago, cytokinins have retained the attention of researchers due to the variety of the effects they have on the growth and development of vegetable organisms, their participation in a plant adaptation to external conditions, and the potential to be used in biotechnology, agriculture, medicine and even cosmetics. The molecular mechanism by which cytokinins function remained unknown for a long time. Things started to change only in the 21st century, after the discovery of the receptors for these phytohormones. It appeared that plants found ways to adapt a two-component signal transduction system borrowed from prokaryotic organisms for cytokinin signalling. This review covers the recent advances in research of the molecular basis for the perception and transduction of the cytokinin signal. Emphasis is placed on cytokinin receptors, their domain and three-dimensional structures, subcellular localization, signalling activity, effect of mutations, ligand-binding properties, and phylogeny.**
KEYWORDS **cytokinins; receptors; sensor histidine kinases; two-component systems; signal transduction.**
ABBREVIATIONS **HK – histidine kinase; HP – phosphotransmitter; RR – response regulator; ER – endoplasmic reticulum.**

INTRODUCTION

Along with auxins, gibberellins, abscisic acid and ethylene, cytokinins belong to the group of classical plant hormones. Cytokinins were discovered by F. Skoog and co-workers in 1955 [1]. The hormone received its name because of the ability to activate *in vitro* division (cytokinesis) of plant cells. In terms of structure, natural cytokinins are adenine derivatives with a small substituent at the N^6 position (*Fig. 1*). Most cytokinins (e.g., zeatin, isopentenyladenine) have the isopentenyl group at this position; however, there can be an aromatic substituent (N^6-benzyladenine, kinetin) as well. Certain synthetic derivatives of phenylurea (e.g., thidiazuron) also exhibit cytokinin activity. Cytokinins affect a number of physiological processes: they stimulate cell division and expansion, plastid differentiation, they retard the ageing process in leaves, activate metabolite inflow and shoot formation from calluses in culture [2–5]. Cytokinins are widely used in bioengineering and agricultural production to grow plant cell cultures in bioreactors, to carry out micropropagation (cloning) of cultivated plants, to obtain transgenic plants, to control plant sex, for cotton defoliation, etc. [4, 5]. Cytokinins participate in the inorganic nutrition of plants and in the formation of nitrogen-fixing root nodules, affect the cereal grain size (i.e., the crop capacity) and the plant resistance to adverse factors [6–8]. Cytokinins and related compounds have recently been finding increasing application in medicine and cosmetology; they are used as anti-tumor agents and inhibitors of neurodegenerative processes and as an active agent in liniments that prevent age-related changes in the skin [4, 9, 10].

During the past 15 years there has been substantial progress in elucidating the molecular mechanism of cytokinin action; sequencing of the genome of the model plant *Arabidopsis thaliana* played a significant role [11]. Discovery of receptors, the key components of hormone signal reception and transduction, was of particular significance. Four papers devoted to the identification and characterization of cytokinin receptors in *Arabidopsis thaliana* were published in 2001 [12–15]. A receptor named CRE1 (Cytokinin Response 1), or AHK4 (Arabidopsis Histidine Kinase 4), has been characterized.

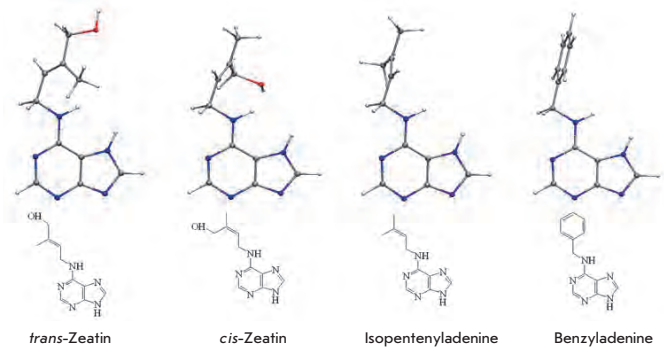

Fig. 1. Structures of typical cytokinins. The most favoured conformations of cytokinins are shown in the upper line; their chemical structures are shown in the lower line.

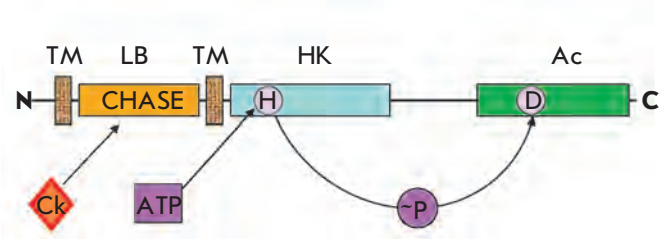

Fig. 2. Domain structure of cytokinin receptor (exemplified by CRE1/AHK4 from Arabidopsis). Protein domains: TM – transmembrane; LB – ligand-binding (CHASE); HK – histidine kinase; Ac – acceptor; Ck – cytokinins; H – conserved histidine; D – conserved aspartate; **N** and **C** denote the N- and C-termini of the protein. The rightwards arrows indicate the sites for phosphorylation and the transfer of high-energy phosphates (~P).

A mutation that manifested itself in a shortening of the Arabidopsis root in the absence of phloem (*wooden leg*, or *wol*) was identified before that. This mutation affects the same gene referred to as *WOL* [16]. In addition to the *CRE1/AHK4/WOL* gene, two of its paralogues which became known as *AHK2* and *AHK3* have also been identified in the Arabidopsis genome sequence [13, 14, 16, 17]. Thus, three cytokinin receptors have been identified in Arabidopsis; these receptors are transmembrane proteins with a similar structure and a molecular weight of over 100 kDa.

This review is focused upon the major questions pertaining to cytokinin recognition and signalling, such as the domain structure of receptors, the biochemical basis of signal perception and transduction, subcellular localization, ligand-binding characteristics and the effect of mutations on receptor properties, the three-dimensional structure of receptors, and the emergence and evolution of receptors in plants.

THE DOMAIN STRUCTURE OF CYTOKININ RECEPTORS

Cytokinin receptors belong to the group of catalytic receptors. They have a complex multidomain structure (*Fig. 2*). The so-called CHASE domain (Cyclase/Histidine kinase Associated Sensory Extracellular) located at the N-terminus of a receptor molecule possesses hormone binding activity [18, 19]. There are two or more transmembrane domains at the two sides of this sensor domain. The last transmembrane domain is followed by a catalytic domain with histidine kinase activity. The core component of this region consists of a dimerization domain and the ATP/ADP binding phosphotransfer domain. The dimerization domain (A-domain) consists of two antiparallel helices that are adjacent to each other (two-stranded coiled-coils). The A-domains of two receptors can interact thus forming a four-helix bundle. According to current concepts, each histidine kinase subunit in the dimer is phosphorylated by the other one (*in trans* reaction) [20]. The phosphotransfer domain contains a conserved site (H-box) of the general structure – **A**TV**SH**E**IR**TP – with the histidine residue being phosphorylated in its centre.

Four conserved motifs (N-, G1-, F-, and G2-boxes) participate in ATP binding. They probably participate in the catalysis and transfer of the phosphate moiety as well. The C-terminus of the receptor contains the receiver domain with the conserved acceptor aspartate residue in the sequence denoted as DD-**D**-K. Cytokinin receptors contain a pseudo-receiver domain which is structurally similar to the receiver domain but cannot receive a phosphate from the conserved histidine residue. The pseudo-receiver domain resides between the regions of histidine kinase and the receiver domains [21, 22]. The function of the pseudo-receiver domain has not been elucidated.

Thus, cytokinin receptors belong to the group of membrane sensor histidine kinases in terms of their general structure and are homologous to some other sensor proteins from plants (ethylene receptors and phytochromes) [22, 23].

MOLECULAR BASIS OF CYTOKININ SIGNAL TRANSDUCTION

Cytokinin receptors are structural and functional relatives of sensor histidine kinases belonging to two-component signal transduction systems that are common among prokaryotes, and have also been found in a number of eukaryotes except for animals [20, 24]. A classical prokaryote two-component system consists of two proteins, namely, a sensor histidine kinase and a re-

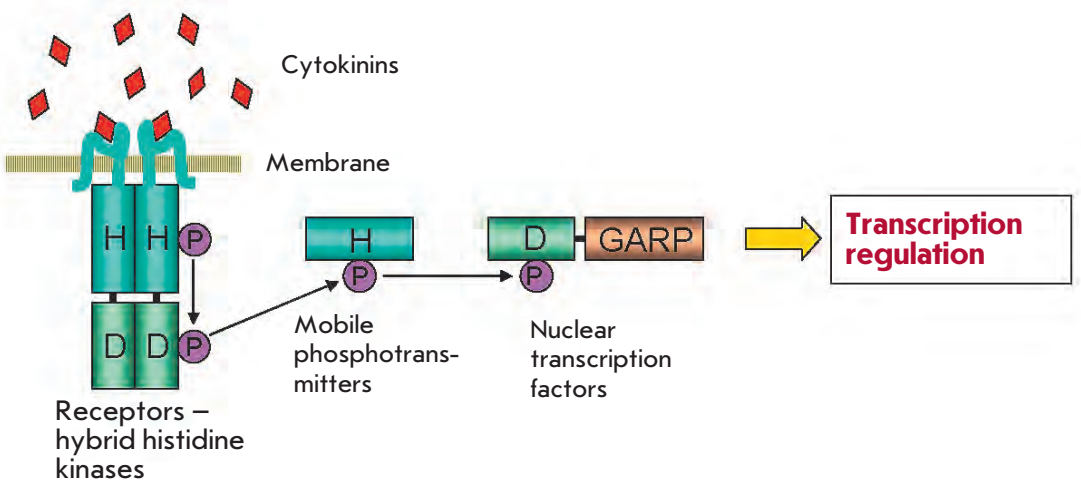

Fig. 3. Scheme of cytokinin signal transduction based on the principle of multistep His-Asp-His-Asp phosphorelay. The phosphorylation of nuclear transcription factors (ARR-B-type response regulators) leads to their activation and subsequent alteration of the primary response gene transcription.

sponse regulator (usually a transcription factor). Under the influence of external factors the histidine kinase is activated and autophosphorylated. The high-energy phosphate then passes to the response regulator. In two-component systems, phosphate is transferred from the conserved histidine residue of one protein molecule (histidine kinase) to the conserved aspartate residue of another molecule (the receiver domain of the response regulator). This process is referred to as phosphorelay. The phosphorylation of the response regulator results in its activation, which in turn triggers transcription of a particular gene or gene set [25].

The scheme of signal transduction is more complex in the case of cytokinin perception, since the receiver domain is part of the sensor histidine kinase (a type of chimeric protein). The signal is transduced according to the principle of multistep (His-Asp-His-Asp) phosphorelay (*Fig. 3*). Hormone binding to the CHASE sensor domain yields phosphorylation of the conserved histidine residue in the resulting histidine kinase dimer. This phosphate moiety is subsequently intramolecularly transferred to the conserved aspartate residue of the receiver domain of histidine kinase and then transferred to the conserved histidine residue of the mobile low-molecular-weight protein-phosphotransmitter (HP), which permanently migrates between the cytoplasm and the cell nucleus [26]. When a phosphorylated phosphotransmitter enters the nucleus, it passes its high-energy phosphate to the conserved aspartate residue in the receiver domain of the response regulator. This protein (which is typically a transcription factor) is activated by phosphorylation and acquires the ability to regulate (typically, activate) the transcription of the primary response genes [27–29]. The cytokinin regulation of the biosynthesis of mRNA of the primary response genes is dependent only on a nontranscribed promoter; i.e., it occurs at the stage of transcription initiation [4].

THE FEATURES OF CYTOKININ SIGNALING IN ARABIDOPSIS

The first cytokinin receptors were identified in Arabidopsis; their ability to recognize the hormonal signal has been confirmed in experiments with transformed bacteria and yeast. Expression of cytokinin receptors from plants in these unicellular organisms resulted in the emergence of a response to low (hormonal) cytokinin concentrations [12–14, 30, 31]. The *in vivo* role of these proteins as receptors has been verified by studying the insertional mutants of Arabidopsis. In general, the mutation in a single receptor does not result in any noticeable changes of plant phenotype. However, mutation in two and, in particular, all three receptors has serious effects. When all three receptors were inactivated, the triple mutant was insensitive to cytokinins and represented a sterile dwarf plant with reduced viability [32–34].

The receptors functionally complement each other, although they are not redundant in a number of processes. The CRE1/AHK4 receptor is mainly expressed in roots, whereas AHK3 prevails in leaves. In accordance, the effects of cytokinin on the aerial and underground parts of a plant depend to a larger extent on the AHK3 and CRE1/AHK4 receptors, respectively [4]. Five typical phosphotransmitters (AHP) and 22 response regulators have been identified among the elements of the two-component system in Arabidopsis. Phosphotransmitters are small proteins up to 17 kDa [35]. Similarly to receptors, AHP proteins are redundant and participate in the transduction of the cytokinin signal additively;

the mutant with respect to all five genes exhibited abruptly reduced sensitivity to cytokinins and phenotypically resembled the receptor triple mutant [36–39]. AHP 1, 2, 3 and 5 play the key role in cytokinin signal transduction. According to current concepts, AHP proteins permanently migrate between the nucleus and the cytoplasm; the pattern of their localization is independent of phosphorylation [26, 35, 40].

AHP6 is another Arabidopsis protein that structurally resembles the phosphotransmitter. However, this protein belongs to pseudo-AHPs since it does not contain the conserved histidine residue required for the phosphorelay. The AHP6 binds to both receptors or response regulators inhibiting their interaction with typical phosphotransmitters and thus acting as a negative regulator of cytokinin signal transduction [41].

Arabidopsis response regulators can be classified into three groups (A, B, and C); there is also a group of pseudo-regulator proteins [42]. The B-type response regulators which contain both the phosphorylated N-terminal receiver domain and a special B-motif including the DNA-binding GARP-domain and the glutamine-rich domain are the real transcription factors [43–46]. Due to the nuclear location signals (NLS) B-type response regulators are localized in the nucleus. The total number of *ARR-B* genes is 11; however, B-type response regulators are not identical in terms of cytokinin signal transduction. The *ARR1, 10* and *12* genes play the key role: the triple mutant with knocked out genes is phenotypically similar to the cytokinin receptor triple mutant [47–49]. The expression of the genes of B-type response regulators is not regulated by cytokinins [28, 29, 50, 51]. It should be mentioned that direct evidence of the interaction between the proteins that are components of the signal transduction circuit and their ability to donate and accept phosphate according to the scheme shown in *Fig. 3* have been obtained [13, 36, 38].

As opposed to ARR-B, the genes of A-type response regulators (ARR-A) can be promptly activated by cytokinins and belong to the primary response genes for these hormones [27–29, 52]. ARR-A consist of the typical receiver domain and a small C-terminal fragment. The A-type response regulators may accept phosphate from phosphotransmitters similar to the B-type regulators; however, they cannot induce the typical transcription response.

A body of observations allows to conclude that ARR-A act as negative regulators of signal transduction, the conserved aspartate residue being required to implement their inhibitory effect [53–55]. The multiple mutant with respect to the genes of A-type response regulators is characterized by increased sensitivity to cytokinin. It is assumed that the A-type response regulators are capable of suppressing cytokinin signal transduction from the AHP proteins by competing with the B-type regulators for the high-energy phosphate. Thus, the participation of ARR-A in the system of cytokinin signal transduction provides negative feedback. Although the structure of C-type response regulators is similar to that of ARR-A, they are not induced by cytokinins and seem not to play a significant role in cytokinin signal transduction [42, 56]. In the absence of cytokinin the CRE1/AHK4 receptor acts as phosphatase and removes a phosphate group from the AHP proteins, thus deactivating signal transduction from the other cytokinin, receptors too [57]. In general, a large number of studies carried out using Arabidopsis plants have persuasively demonstrated that the cytokinin signal is transduced via the two-component pathway, with hybrid histidine kinases acting as receptors.

SUBCELLULAR LOCALIZATION OF RECEPTORS

The cytokinin receptor is an integral transmembrane protein with the CHASE domain located to one side of the membrane and the histidine kinase and receiver domains located to the other side. Cytokinin receptors were believed to be localized on the plasma membrane; it was assumed evident that the CHASE domain has to be localized extracellularly, whereas the remaining part of the protein has to be intracellular. This assumption was partly based on the computational prediction of subcellular localization [12, 14, 16] and on the analogy with a bacterial cell, where the CHASE domain of sensor proteins is extracellular (this fact is evident from the domain name). This belief was further bolstered when the localization of the cytokinin receptor in the plasmalemma revealed by expression of the *AHK3-GFP* construct in Arabidopsis protoplasts was reported [58]. The localization of cytokinin receptors on the plasma membrane assumes that the cytokinin signal enters the cell from the environment due to extracellular cytokinins. On the other hand, it was demonstrated by determining the pH-dependence of cytokinin binding to receptors that the binding is optimal in neutral and alkalescent media, which are typical of the cytoplasm, and that it decreases abruptly under acidified conditions, which are typical of the extracellular space (the apoplast) [59]. This fact attests on the contrary to the intracellular localization of the receptor. Therefore, studies of the subcellular localization of cytokinin receptors were continued.

Three articles claiming that the receptors (or at least their majority) are localized inside the cell on the membranes of the endoplasmic reticulum (ER) have recently been published [60–62]. Sites of ^{3}H-*trans*-zeatin high-affinity binding in the fraction containing membranes

(microsomes) but not in fractions containing mitochondria or chloroplasts have been revealed in experiments with subcellular organelles [60]. After the microsomes had been separated in an aqueous two-phase polymer system into the plasmalemma and endomembranes, it turned out that the high-affinity sites were mostly confined to the endomembrane fraction both in Arabidopsis [61] and corn [60]. Taking into account the predominance of endomembranes in the cell, it was assessed that over 90% of the hormone binding sites are localized intracellularly.

By studying the localization of the Arabidopsis receptor-fluorescent protein fusions expressed in tobacco leaves [61, 62] and the corn receptor ZmHK1 in protoplasts from corn leaves [60] it has been demonstrated that fluorescence distribution corresponds to the endoplasmic reticulum network. For the AHK3 receptor, the fluorescence pattern coincided with the pattern for the ER marker but not the plasmalemma marker [61, 62]. In addition, the AHK3 protein was *in vivo* glycosylated at the sites that are sensitive to glycosidase endoH, which attests to localization in ER [62]. The same glycosylation was recorded in control experiments for the ethylene receptor ERS1 integrated into ER [63, 64], whereas the potential endoH-sites in histidine kinase AHK1 localized in the plasmalemma were not glycosylated [62].

It should be mentioned that the intracellular localization of cytokinin receptors, which was revealed via fluorescence, was observed under various conditions of expression of the inserted genes using promoters of different strengths. However, the most convincing result was obtained by analysis of the localization of the receptors expressed under natural conditions. This approach was implemented via immunoblotting with antibodies against the corn receptor ZmHK1. The membrane fractions obtained upon separation in a sucrose gradient in the absence or presence of magnesium cations were analyzed [60]. In the absence of magnesium, ribosomes dissociate from the ER, resulting in the shift of the ER towards the top of the gradient. This shift is not observed if magnesium is present in the medium. This effect referred to as the Mg-shift is typical of ER but not the other membranes unbound to the ribosomes. It was demonstrated by the analysis of fractions from corn cells that the ZmHK1 protein undergoes a Mg-shift and is co-localized with the ER marker protein (BiP) [60].

The stable Arabidopsis transformants expressing AHK2 or AHK3 receptor genes under their own promoters and with the Myc peptide at the C-terminus of the protein were obtained . The expression of these constructs compensated for the phenotype of the *ahk2 ahk3* double mutant of Arabidopsis, attesting to the functionality of these modified receptors. The typical Mg-shift and correlation with the ER marker were also revealed when analyzing the membrane fractions via immunoblotting with anti-Myc antibodies [61].

All these data allow one to conclude that cytokinin receptors are mostly localized in the endoplasmic reticulum. Along with the data pertaining to the ability of ER-localized receptors to bind cytokinins and the pH-dependence of this binding typical of cytoplasmic proteins, this result may attest to the fact that cytokinin signal perception occurs mainly inside the cell and that intracellular cytokinins play the key role in this process. However, the presence of a small number of receptors on the plasma membrane should not be left without consideration. These receptors can be responsible for the perception of the signal from extracellular cytokinins. Further research is needed to assess the functional properties of each pool of cytokinin receptors.

LIGAND-BINDING PROPERTIES OF RECEPTORS

Along with gibberellines, cytokinins are represented by a variety of isoforms in plants (*Fig. 1*); among those *trans*- and *cis*-zeatins, isopentenyladenine, dihydrozeatin (bases), their N^9-ribosylated derivatives (ribosides) and N^9-riboside phosphate derivatives (nucleotides) are prevalent. Aromatic cytokinins such as N^6-benzyladenine and its derivatives, topolin, etc. occur as well [4, 5, 65]. Cytokinins migrate within a plant along transport channels: in the upward direction from the root into the shoot via xylem and in the downward and other directions via the phloem. Cytokinin compositions in the xylem differ from those in the phloem: *trans*-zeatin-type cytokinins (mostly, *trans*-zeatin-riboside) are the prevailing isoforms in the xylem, whereas isopentenyl-type cytokinins are prevalent in the phloem [66–68].

The physiological role of each cytokinin isoform is determined by its affinity to the receptor; therefore, the investigation of cytokinin–receptor interaction and ligand specificity of the receptors is of high importance. The ligand-binding properties of cytokinin receptors have been studied mostly using heterologous model systems upon expression of the receptor genes in transformed bacterial (*Escherichia coli*) or yeast cells. Plant receptors turned out to be capable of functional replacement of mutant sensor histidine kinases with a similar (hybrid) structure in these unicellular organisms [12, 13, 30].

Both functional tests [13, 15, 30, 69, 70] and hormone-receptor binding assays [59, 60, 71, 72] have been carried out based on the aforementioned model systems. In general, as was expected, the affinity of the hormone to the receptor positively correlated with the hormone's ability to induce a biological response [59, 71]. *trans*-Zeatin is one of the most active ligands for most of the receptors studied; K_d of the hormone-receptor complex

Table 1. Rows of cytokinin affinity for the receptors from Arabidopsis and corn

Species	Receptor**	Cytokinin affinity rows**
Zea mays	ZmHK1	iP ≥ BA >> tZ ≥ cZ >> DZ >> Ade
Arabidopsis thaliana	CRE1/AHK4	iP ≥ tZ > BA > DZ > cZ >> Ade
Zea mays	ZmHK2	tZ ≥ iP > DZ > BA > cZ >> Ade
Arabidopsis thaliana	AHK3	tZ > DZ > iP > cZ > BA >> Ade
Zea mays	ZmHK3a	iP > tZ > BA > cZ >> DZ >> Ade
Arabidopsis thaliana	AHK2	iP > tZ > BA > cZ > DZ >> Ade

* Orthologous receptors are grouped pairwise.
** Cytokinins: iP – isopentenyladenine; BA – N^6-benzyladenine; tZ – *trans*-zeatin; cZ – *cis*-zeatin; DZ – dihydrozeatin. Ade – adenine.

varies within a range of 1–10 nM. Such values of the constants are typical of high-affinity hormone-receptor interactions. Let us note that these values of the constants are close to the measured concentrations of *trans*-zeatin in living plants [34, 66, 68, 73]. A Scatchard analysis has revealed the single receptor-ligand binding site without any signs of cooperative interaction [59, 74]. Meanwhile, natural (N^6-adenine derivatives) and synthetic (thidiazuron, a phenylurea derivative) cytokinins bound to the same receptor site [59].

Yet, the receptors differ in their preference of cytokinin isoforms [59, 60, 75]. The Arabidopsis receptors CRE1/AHK4 and AHK2 have the same high affinity to *trans*-zeatin and isopentenyladenine and a considerably lower affinity to dihydrozeatin. On the contrary, the AHK3 receptor is characterized by a relatively high affinity to dihydrozeatin and a lower affinity to isopentenyladenine. All three Arabidopsis receptors are capable of binding, though with low affinity, *cis*-zeatin, too. Cytokinin glucosylation at the N3 or N7 nitrogen atoms and at the oxygen atom of the side chain blocks the hormone-receptor binding [30, 59].

The ligand specificity of cytokinin receptors has also been studied for corn, a monocotyledonous plant, three receptors of which are orthologous to those of the dicotyledonous plant Arabidopsis: ZmHK1 orthologous to CRE1/AHK4; ZmHK2 orthologous to AHK3; and ZmHK3 orthologous to AHK2 [76]. The corn receptors were partly similar to, partly different from, their Arabidopsis counterparts [60, 76]. In general, the order of relative ligand activity turned out to be rather similar for corn and Arabidopsis orthologues (*Table 1*). Whereas isopentenyladenine exhibited higher activity than *trans*-zeatin with respect to ZmHK1 and ZmHK3, the opposite was observed for ZmHK2. A stronger difference between corn receptors was observed upon their interaction with dihydrozeatin: the affinity of ZmHK2 to this cytokinin is more than two orders of magnitude higher compared to its affinity to ZmHK1 and ZmHK3. A relatively high affinity to *cis*-zeatin is a characteristic feature of corn receptors, ZmHK1 demonstrating almost identical affinities to *trans*- and *cis*-zeatins. This feature of corn receptors is in accordance with an increased concentration of *cis*-zeatin in this plant species [77, 78].

The regularities of the receptor preferences to certain ligands can be interpreted with allowance for their possible role in long-range signalling in plants. The Arabidopsis receptors AHK3 and their orthologues ZmHK2 in corn are mainly expressed in shoots and control the metabolic processes occurring in leaves. These receptors are "tuned" primarily to *trans*-zeatin-type cytokinins; i.e., to the cytokinins transported to the shoot from the roots. In turn, the CRE1/AHK4 and ZmHK1 receptors that are prevalent in roots actively respond to isopentenyladenine, the major cytokinin in phloem, which is translocated from the shoot to the roots with the phloem sap (*Fig. 4*). Thus, signal exchange can occur between different parts and organs of a plant organism, when the cytokinin signals of a remote organ turn out to be more significant for the cell compared to the signals from the closer located tissues [4, 59, 79].

EFFECT OF MUTATIONS ON RECEPTOR ACTIVITY

The identification of the Arabidopsis mutation named *wooden leg* (*wol*) resulted in the discovery of cytokinin receptors. The mutant plants were different from the wild-type plants by a shorter length and a disturbed development of the vascular system of the main root. The latter consisted of protoxylem only (metaxylem

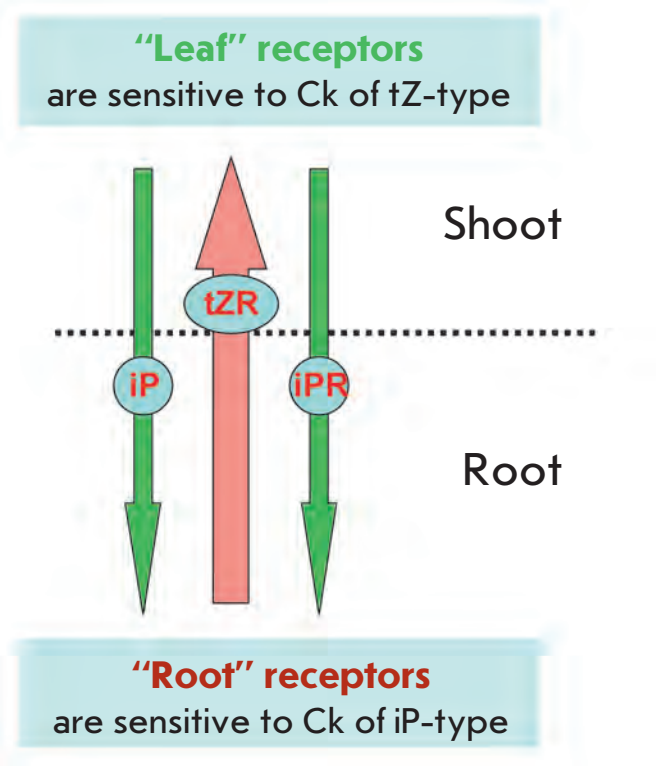

Fig. 4. A model for long-distance cytokinin action. The arrow in the middle denotes the translocation of cytokinins (Ck) of *trans*-zeatin type from the root to the shoot via xylem. The lateral arrows denote the translocation of cytokinins of the isopentenyladenine type from the shoot to the root via phloem.

and phloem have not been developed); the total number of cells was significantly lower. Moreover, the plants had no lateral roots and exhibited enhanced formation of adventitious roots. The phenotypic manifestation of this mutation was first described in 1995 [80].

The *wol* mutation was subsequently found to be localized in the gene of sensor histidine kinase CRE1/AHK4 and to consist in the substitution of threonine 278 (threonine 301 according to the modern numeration) with isoleucine in the hormone-binding CHASE domain [12, 15, 16]. The *wol*-2 and *cre1-1* mutations were later obtained via chemical mutagenesis. These mutations consisted in replacing leucine 529 with isoleucine [82] and glycine 490 with aspartate [12], respectively. All these mutations also resulted in the typical *wol* phenotype caused by underdevelopment of the vascular system due to a reduced number of meristem initial cells because of blockage of cell division [16]. The defects in the vascular system impeded auxin transport to the pericycle; as a result, lateral roots were not formed. Meanwhile, the disturbance in the vascular system of the main root resulted in auxin accumulation in the hypocotyl bottom region, which in turn stimulated the formation of adventitious roots. It is of interest to note that normal development of the vascular system in adventitious roots, as opposed to the main root, was observed in the *wol-3* mutants [82].

The radioligand-binding technique demonstrated that the receptor loses its cytokinin-binding capacity upon *wol* mutation [15]. However a stop codon introduced to the mutant *CRE1/AHK4* gene recovered the wild-type phenotype in *wol* plants [57]. Therefore, it was reasonable to assume that the mutant receptor CRE1/AHK4 not only stops participating in cytokinin signal transduction but also suppresses the transduction of this signal from the other receptors, AHK2 and AHK3. Some bacterial histidine kinases have been ascertained to possess phosphatase (in addition to kinase) activity, resulting in dephosphorylation of phosphoproteins [83]. It has been demonstrated in *in vitro* experiments and in experiments on transgenic yeasts that CRE1/AHK4 also has a constitutive phosphatase activity, whereas its histidine kinase activity is manifested only in the presence of cytokinins [57]. Thus, the *wol* mutation which makes CRE1/AHK4 incapable of cytokinin binding blocks its histidine kinase activity, whereas phosphatase activity is retained. Consequently CRE1/AHK4 harboring the *wol* mutation dephosphorylates the phosphotransmitter proteins phosphorylated with the AHK3 and AHK2 receptors, thus blocking the cytokinin signal transduction. CRE1/AHK4 predominates in root cells [17, 32]; therefore, the *wol* mutant phenotype is mainly manifested in roots.

The same mutations in the CHASE domain of AHK3 and AHK2 receptors have not resulted in the emergence of the *wol*-like phenotype [31]. This attests to the fact that the AHK2 and AHK3 receptors do not have phosphatase activity.

Thus, a number of mutations in the CRE1/AHK4 receptors resulting in the emergence of the typical *wol*-phenotype have been revealed. It has been demonstrated that the receptor, upon all these mutations, no longer transduces cytokinin signals despite the fact that only the *wol* (*wol-1*) mutation is localized in the CHASE domain. The *wol-3* mutation is localized in the region between the second transmembrane and histidine kinase domains; the *wol-2* and *cre1-1* mutations are localized in the histidine kinase domain.

In general, the analysis of mutations in cytokinin receptors has enabled to confirm and refine our understanding of the roles of the defined parts of receptors. The isolated CHASE domain with the adjacent transmembrane domains retains the ability to high-affinity cytokinin binding, whereas the receptor without the CHASE domain lacks such ability [84]. Hormone-recep-

tor binding was also suppressed by the other mutations in this domain [84]. Thus, the role of the CHASE domain as a hormone-binding one is beyond question.

Although the mutations in the CHASE domain disturbed receptor functioning in the absolute majority of cases, a mutation (called *ore12-1*) in this domain resulting in constitutive activity of histidine kinase was found in the AHK3 receptor [58]. Upon this mutation proline 243 located in the middle of the CHASE domain was replaced by serine. It was assumed that this substitution of amino acids could result in an alteration of the CHASE domain structure similar to that caused by cytokinin binding [58].

Conserved histidine and aspartate residues which undergo phosphorylation during the signal transduction are known to play a special role in the molecules of sensor hybrid histidine kinases. Substitution of these residues (His482Gln and Asp996Asn) resulted in a loss of both histidine kinase activity and the ability of CRE1/AHK4 to respond to cytokinins [12]. The substitution of Asp996Asn also resulted in a total loss of phosphatase activity, whereas substitution of histidine caused only a slight reduction in the activity [57]. Note that the His482Gln replacement did not alter the cytokinin-binding capacity of the receptor [84].

A number of mutations in CRE1/AHK4 have been obtained using PCR: Gly435Cys, Phe436Ser, Met447Thr in the second transmembrane domain; Val471Ala in the region between the second transmembrane and histidine kinase domains; and Met494Leu in the histidine kinase domain. All these mutations were localized in a short region of approximately 60 amino acid residues between the ligand-binding domain and the conserved histidine residue which plays a significant role in protein autophosphorylation [31]. These mutations led to the emergence of constitutive histidine kinase activity in CRE1/AHK4; i.e., this receptor acquired the ability to send a signal whether cytokinins were present in the media or not. Meanwhile, the mutant receptors retained their cytokinin-binding capacity, which has been confirmed in experiments of tritium-labelled isopentenyladenine binding by these receptors within the membranes of *Schizosaccharomyces pombe*. It is interesting to note that the CRE1/AHK4 receptor with the Phe436 mutations retained its constitutive histidine kinase activity even after the *wol* mutation was additionally introduced, despite losing its cytokinin-binding capacity. Thus, in the presence of these constitutive mutations the cytokinin-binding capacity of the receptor plays no role in signal transduction [31].

The introduction of mutations into the same regions of the other cytokinin receptors may also yield the same results. For instance, substitutions of conserved hydrophobic amino acids in the AHK2 (Ile586Ala) and AHK3 (Val449Ala) receptors, similar to the Val471Ala substitution in the CRE1/AHK4 receptor, resulted in the emergence of constitutive histidine kinase activity in the receptors [31]. The replacements of amino acids in the second transmembrane domain and in the downstream region could result in conformation changes in the protein molecule similar to those emerging upon cytokinin-receptor binding, thus stimulating histidine kinase activity in the absence of hormone.

Based on the structure of the cytokinin receptor, it is reasonable to expect that mutations removing the receiver domain or disturbing its structure will result in receptor inactivation. Indeed, plants *A. thaliana* carrying mutations in the CRE1/AHK4 receptor gene (called *cre1-3* and *cre1-7*) where the triplets encoding Trp1026 and Gln475, respectively, were replaced with stop codons have been obtained [85]. It is evident that these mutations result in the synthesis of a truncated receptor lacking the entire or part of the receiver domain. In the *cre1-6* mutant, the replacement of nucleotides resulting in Gly493Ala substitution apparently leads to splicing disturbances and to the formation of the truncated receptor. Thr1008Ile and Ala1032Thr substitutions occurred in the mutants *cre1-4* and *cre1-9*, respectively. They resulted in the formation of full-size proteins carrying mutations in the receiver domain [85]. The response to phosphate starvation, which is suppressed by cytokinins under normal conditions, was examined in the resulting mutant plants. As opposed to the controls, the mutant plants almost did not respond to cytokinin in this biotest. Thus, mutations leading to the formation of truncated CRE1/AHK4 receptors and mutations in the receiver domain resulted in the suppression of cytokinin sensitivity of plants in the phosphate starvation biotest [85].

Similar mutations in the MtCRE1 cytokinin receptor have been obtained and studied in lucerne *Medicago truncatula* [86]. These mutations affect the histidine kinase domain of the receptor. In the case of the *mtcre1-1* mutation, the Trp573-encoding triplet localized in the middle of the domain was substituted with a stop codon, resulting in the formation of a truncated protein. The mutation in *mtcre1-2* consisted in the replacement of Thr642Ile in the conserved G2 motif of the domain. Upon mutation in *mtcre1-3*, the substitution Gly545Glu was localized in the variable region of the domain. It has been demonstrated in the biotest for root growth suppression that the *mtcre1-1* and *mtcre1-2* mutants, as opposed to the *mtcre1-3* mutant, lose their sensitivity to cytokinin. Nodule formation upon exposure to symbiotic bacteria was disturbed in the *mtcre1-1* and *mtcre1-2* mutants [86]. All these facts underscore the significant role of each conserved domain in the normal functioning of the receptor.

THREE-DIMENSIONAL STRUCTURE OF THE RECEPTOR

To understand the structural and functional features of the receptor, it is important to know the three-dimensional structure of the protein under study. X-ray crystallography is the most common technique for studying the three-dimensional structure; a protein monocrystal is required to carry out this type of analysis. However, crystallization of cytokinin receptors is complicated since they are high-molecular-weight transmembrane proteins. Therefore, thus far structural studies have not been completed for any of these receptors.

It is more realistic to shed light on the structure of a domain of the receptor. Research in this area has been done for the ligand-binding [79, 87–89] and receiver [90, 91] domains. An attempt to predict the tertiary structure of the CHASE domain of the CRE1/AHK4 receptor was made back in 2004 [87]. Homology modeling of the CHASE domain based on the X-ray structures of the ligand-binding regions of the sensor histidine kinases from bacteria *E. coli* (PDB ID: 1OJG) and *Klebsiella pneumoniae* (PDB ID: 1P0Z) was used in this study. Molecular docking studies of the cytokinins *trans*-zeatin and kinetin into the putative binding site of this model were subsequently carried out. The results showed that the CHASE domain corresponds to the so-called PAS-type domain; the binding site covered the entire cytokinin molecule. A number of amino acid residues responsible for protein-ligand binding have been identified [87] (including Thr278, whose substitution with Ile - the *wol* mutation - resulted in receptor inactivation). However, the proposed model turned out to be generally incorrect presumably due to the too-distant relationship between the template proteins and the CHASE domain of CRE1/AHK4.

The investigation of the tentative structure of the hormone binding site in the CHASE domain was continued using the evolutionary proteomics approach; i.e., the search for the conserved amino acids of the CHASE domain required for ligand recognition and binding [84]. Several amino acid residues that may participate in the interaction with hormone have been found; five of them were substituted with alanine in the CRE1/AHK4 receptor. Upon expression of these mutant receptors in *E. coli*, two out of five substitutions (Phe281Ala and Thr294Ala) led to complete elimination of the hormone-binding capacity of the receptor. In two cases (Trp221Ala and Arg282Ala) binding decreased considerably as compared to the intact CRE1/AHK4 receptor. The Lys274Ala mutation had no effect. It has been noted that most efficient mutation sites are localized near the predicted central β-sheet structures of the domain, which assumes that these β-strands play a significant role in hormone binding. These results were essentially confirmed by subsequent identification of the X-ray structure of the CHASE domain in complex with the hormone [89]: the amino acid residues Thr294, Phe281, and Arg282, indeed, were in contact with cytokinin, whereas Lys274 did not form direct contacts with the hormone.

Decisive success in determining the three-dimensional structure of the CHASE domain was achieved in 2011, when a research team from the Salk Institute (USA) obtained a crystal of the CHASE domain of the CRE1/AHK4 receptor suitable for X-ray crystallographic study [89]. This allowed to determine the structure of the ligand-binding CHASE domain of the CRE1/AHK4 receptor in complex with various cytokinins (PDB ID: 3T4J, 3T4K, 3T4L, 3T4O, 3T4Q, 3T4S, 3T4T; resolution 1.53–2.30 Å). According to the data obtained (*Fig. 5*), the N-terminus of the CHASE domain forms a long α-helix neighboring two PAS domains connected by helical linkers. The β-strand closer to the C-terminus of the PAS domain is covalently linked to the N-terminal α-helix via a disulfide bridge, which makes the domain structure more rigid and compact. It is interesting to note that similar tertiary structures of the sensor domains were previously identified in the histidine kinases of certain bacteria (*Bacillus subtilis*, PDB ID: 2FOS, 4DBJ; *Sinorhizobium meliloti*, PDB ID: 3E4P; *Shewanella oneidensis*, PDB ID: 3LIC) despite the low similarity between the sequences of the bacterial receptors and CRE1/AHK4 [92]. The sensor domains of both CRE1/AHK4 and their bacterial homologues crystallized in the form of homodimers. It has been ascertained that for cytokinin recognition CRE1/AHK4 uses the PAS domain located at a significant distance from the membrane. The ligand-binding cavity of the receptor completely embraces the ligand, as shown for a number of the best-known cytokinins: isopentenyladenine (3T4J), N^6-benzyladenine (3T4K), *trans*-zeatin (3T4L), and kinetin (3T4S); differences between the structures of the receptor CHASE domain in complex with various hormones were negligible. The "floor" component of the cytokinin-binding site is formed by the central β-sheet of the PAS domain and is lined by small hydrophobic amino acid residues. Substitutions of these residues with bulkier amino acids block the cavity for cytokinin binding, thus inactivating the receptor. That just occurs upon the most common mutation *wol*, where the small Thr278 residue is substituted with Ile having a bulkier side chain. Two short β-strands form the hydrophobic "ceiling" of the active site. The hydrogen bonds are formed between the adenine component of cytokinin and the Asp262 residue (these bonds play the crucial role in binding), Leu284, Tyr250, and Thr286. The two latter hydrogen bonds are mediated by water molecules, which in turn interact with cytokinin atoms. The remaining residues

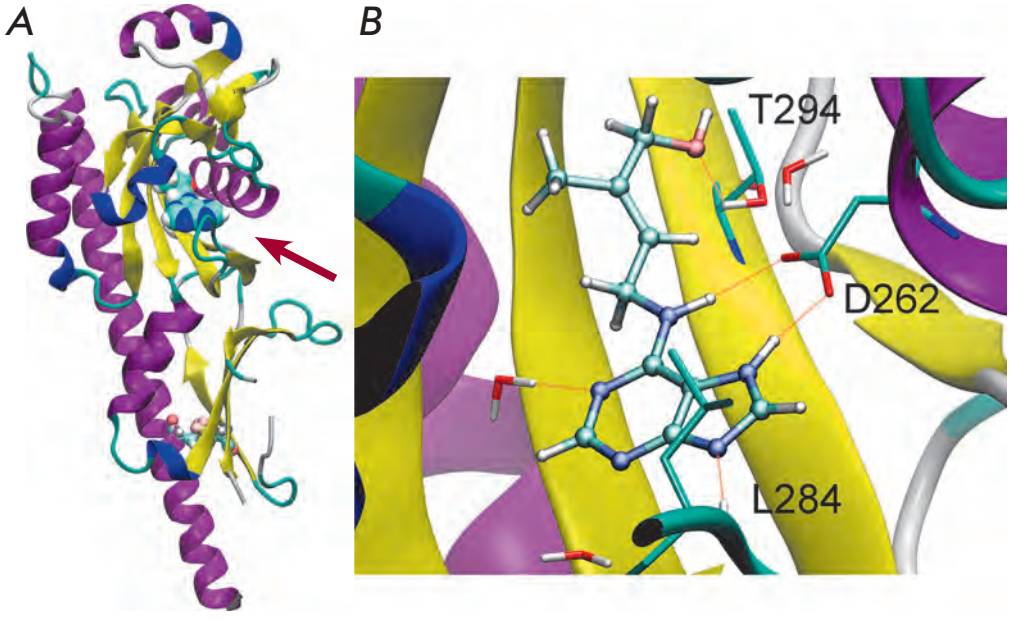

Fig. 5. Three dimensional structure of the CHASE domain of the Arabidopsis cytokinin receptor CRE1/AHK4. The general view (A) and structure of the binding site with a *trans*-zeatin molecule (B). Cytokinin molecule is shown in spacefill representation, cystine bridge is shown as the ball-and-stick model. The arrow indicates the location of the bound cytokinin.

participate in hydrophobic interactions with both the adenine- and, in particular, the tail component of cytokinin (*Table 2*). The total number of amino acids forming the ligand binding pocket is approximately 20 ([89] and Hothorn M., personal communication).

The amino acids that play a significant role in binding appeared to be appreciably conserved in different cytokinin receptors; substitution of these conserved residues in the CHASE domain of CRE1/AHK4 typically resulted in receptor inactivation [89].

In plants, cytokinins can be glycosylated at nitrogen atoms of the adenine moiety, whereas the OH group of the isoprenoid component of the ligand can be acylated or glycosylated. As previously mentioned, all these modifications render cytokinins inactive [30, 59]. The X-ray structure of the receptor supports these results, since the limited volume of the ligand-binding cavity is not sufficient to enclose cytokinins carrying additional glycosyl or other groups.

As opposed to *cis*-zeatin, *trans*-zeatin forms an additional hydrogen bond with Thr294 via the OH group of the side chain. This fact makes it clear why CRE1/AHK4 binds *trans*-zeatin with higher affinity than *cis*-zeatin. The binding mode for cytokinins carrying more bulky aromatic tail components was demonstrated by the example of kinetin and benzyladenine. The furfuryl moiety of kinetin, similar to the isoprenyl group of *trans*-zeatin, forms a hydrogen bond with Thr294 in the case of kinetin via water molecule. Using thidiazuron (3T4T structure) as an example it has been confirmed that the CRE1/AHK4 receptor uses the same site for binding synthetic and natural cytokinins, synthetic cytokinins forming hydrogen bonds with the same amino acids as cytokinins - N^6-adenine derivatives.

The general principles used to design compounds with cytokinin activity are as follows: such compounds need to have a planar ring structure occupying the "adenine" part of the ligand binding cavity, with a linker

Table 2. Amino acid residues forming the cytokinin binding site of the CRE1/AHK4 receptor.

Region in contact with hormone	Amino acid residues in the CHASE domain cavity surrounding the bound N^6-isopentenyladenine*												
Adenine component	G200	M226	V248	Y250	L251	D262	F281	R282	L283	L284	T286	V292	A322
	1	1	2	H*H*	3	HH	1	1	3	H; 2	H*	3	3
Tail component	A202	A204	V241	M256	I266	T294	Y318	G320	G321				
	3	3	3	3	3	3	3	3	3				

* 1, 2, and 3: relative strength of hydrophobic interactions between iP and AHK4/CRE1; H, and HH: 1–2 hydrogen bonds, H*: hydrogen bond via water molecule

that is capable of forming hydrogen bonds with Asn262 and attaching a small planar aliphatic or aromatic tail group [89].

PHYLOGENETIC ANALYSIS OF CYTOKININ RECEPTORS

Until recently, cytokinin receptors had been studied in detail only in two plant species (although phylogenetically rather distant): Arabidopsis and corn. Therefore, it became of interest to elucidate the features of the cytokinin perception apparatus in other plants species and trace back the formation of the cytokinin signalling system during plant evolution. Such research became possible thanks to the complete genome sequencing of a number of plant species.

The results of the phylogenetic analysis of a number of genomes has enabled researchers to conclude that the pathway of perception and transduction of the cytokinin signal based on two-component system emerged in metaphytes after transition to terrestrial life as one of the aspects of their biochemical adaptation to new living conditions [93]. The genes encoding sensor histidine kinases with the CHASE domain and A-type response regulators in the genomes of studied species of lower and higher plants have been found starting from mosses and spikemosses. In higher organized plants the number of components of the cytokinin signalling system is usually higher compared to that in more primitive plants. In particular, this applies to phosphotransmitters and response regulators. It has been noted that the cytokinin receptors of all flowering plants analyzed fall into three individual branches of the phylogenetic tree, corresponding to the Arabidopsis receptors CRE1/AHK4, AHK3, and AHK2. In the evolutionary tree the receptors in archegoniates (moss, spikemoss) keep aloof, attesting to the fact that three major types of receptors presumably emerged together with flowering plants but before their split into monocotyledonous and dicotyledonous plants [93].

A broader phylogenetic analysis based on the sequenced genomes of 30 species of multicellular land plants provided further insight into the evolution of cytokinin receptors. Among the annotated genes 112 were revealed which encode proteins with a typical for cytokinin receptors domain organisation, including the CHASE domain, histidine kinase, and receiver domains (*Fig. 6*). The genes of these sensor histidine kinases are present in the genomes of all higher plants that have been sequenced. The number of sensor histidine kinases comprising the CHASE domain varies from one in potato *Solanum tuberosum* and the common monkey-flower *Mimulus guttatus* to eight in cultured soybean *Glycine max*. Several branches of closely related genes have been revealed in flowering plants via a phylogenetic analysis. Three branches corresponding to the Arabidopsis receptors AHK2, AHK3, and CRE1/AHK4 have turned out again to include the largest number of genes. A subdivision into groups of monocotyledonous and dicotyledonous orthologues has been observed in these branches. Moreover, certain small branches kept aloof; in particular, the group of monocotyledonous orthologues ZmHK3. In general, cytokinin receptors can be phylogenetically subdivided into three and four groups for dicotyledonous and monocotyledonous plants, respectively. The receptors of one plant species belonging to different groups are more similar to the group orthologues from other species than to each other within the same species. These receptor groups are non-identical in different plant species. As mentioned above, only one receptor belonging to the orthologues CRE1/AHK4 and AHK3, respectively, has been found in each of the dicotyledonous plants: potato and the common monkey-flower. If additional genes are identified in the genomes of these species, it might be that these species contain the other receptors, too. The StHK4 receptor from potato contains the phenylalanine residue instead of the conserved Tyr318. However, no direct evidence of a significant role played by this residue in receptor functioning has been offered [89]. It is of interest that tomato, a close relative of potato, carries the normal representatives of receptors belonging to all three major evolutionary branches. The CRE1/AHK4 orthologue is duplicated in the Leguminosae; four orthologues of CRE1/AHK4 (two in each duplication group) have been identified (*Fig. 6*). The two other branches contain two representatives of the soybean receptors, each. In lucerne *Medicago truncatula* the only orthologue of CRE1/AHK4 belongs to either one of the two duplication groups. The common bean *Phaseolus vulgaris* and *Lotus japonicus* have two representatives of the orthologue of CRE1/AHK4, each, but they do not have orthologues of AHK3 or AHK2, respectively. However, the highly conserved leucine in the PvHK4a from the common bean is substituted with tryptophan, which raises some doubt as to whether this protein can act as a cytokinin receptor. Few substitutions of the conserved amino acids have also been revealed in some other dicotyledonous species (sweet orange *Citrus sinensis*, cucumber *Cucumis sativus*, and cassave *Manihot esculenta*). The common feature of all the dicotyledonous species (with the exception of the common monkey-flower) is the mandatory presence of orthologues of the CRE1/AHK4 receptor.

The monocotyledonous species rice and corn also has representatives of two evolutionary branches of receptors, the orthologues of AHK3 and AHK4. The AHK4 group can be divided into two subgroups, corresponding to ZmHK1a and ZmHK1b. In corn, two receptors belong to each of these groups/subgroups. However,

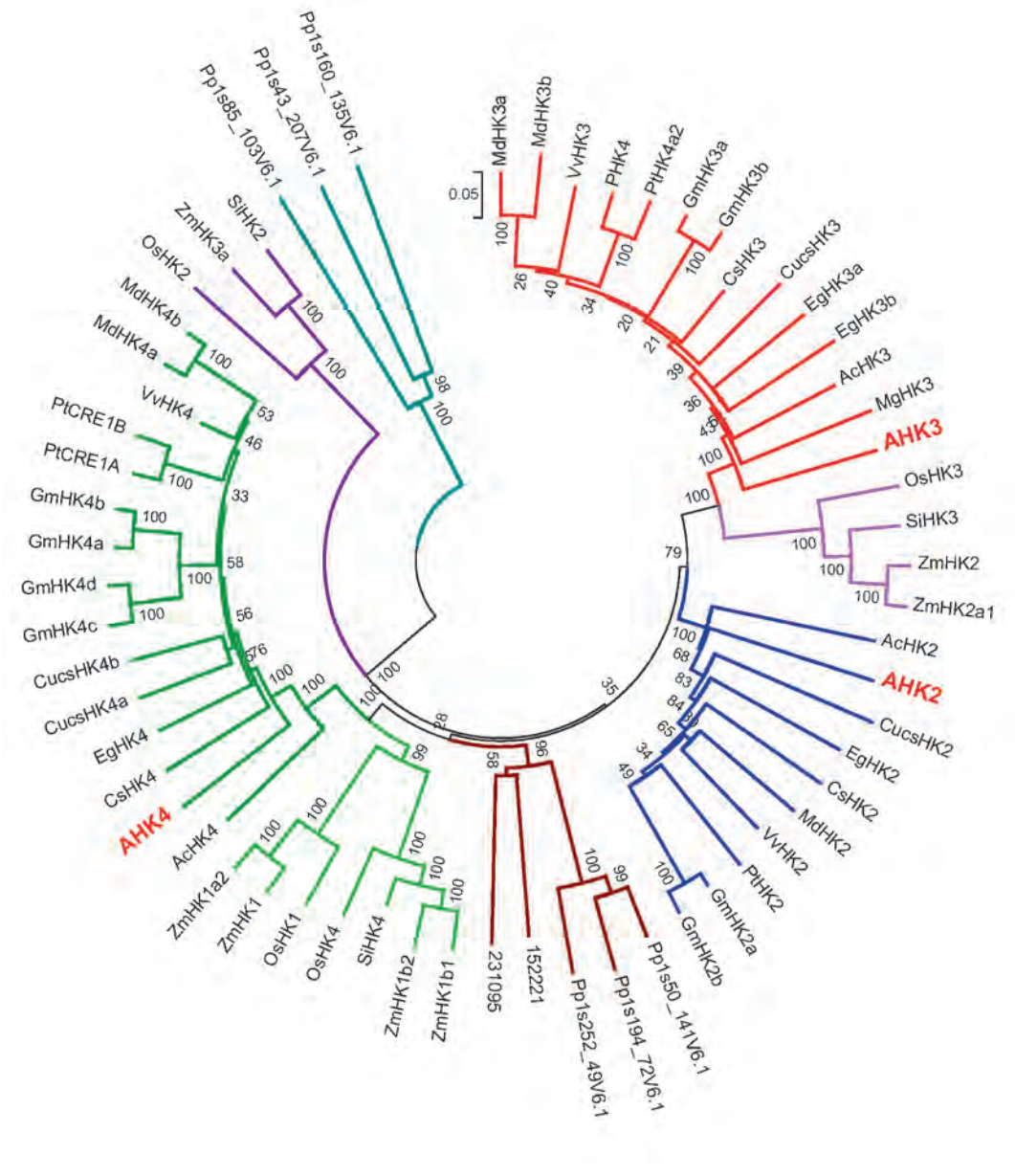

Fig. 6. Phylogenetic analysis of cytokinin receptors. The sequence alignment was performed using the ClustalW program. The phylogenetic tree was built using the MEGA 5.05 software; the bootstrap analysis includes 1,000 replicates. Bootstrap supports for the individual branches are given as a percentage based on 1,000 bootstrap trials.

the foxtail millet (*Setaria italica*) has no orthologues of CRE1/AHK4 in one of the subgroups (ZmHK1a), whereas sorghum (*Sorghum bicolour*) and *Brachypodium distachyon* have no orthologues of ZmHK3a (*Fig. 6*). Thus, all the known genomes of monocotyledonous plants encode at least one of the representatives of CRE1/AHK4 orthologues. It should be mentioned that the orthologues of CRE1/AHK4 are present in the sequenced genomes of almost all monocotyledonous plants in two versions. However, it is not improbable that this feature is typical only of the family Gramineae (for which the genomes have already been sequenced), whereas the other monocotyledonous families may contain a different number of CRE1/AHK4 isoforms. But in either case, orthologues of CRE1/AHK4 appear to be the most important cytokinin receptors in flowering plants at this point.

CONCLUSIONS

Although the major plant hormones have been known since the mid-20th century, just in the past decades research in phytohormones has undergone a renaissance. This is due primarily to the elucidation of the molecular mechanism of their action on a cell; i.e., the discovery of the receptors and genes that encode them, and to the fact that it is now possible to clone and insert genes for

cytokinin perception, biosynthesis and signal transduction, as well as to obtain targeted mutations [94–101]. The fundamentals for intracellular signalling of phytohormones are similar to those for the signalling of animal and human hormones. The role of receptors is to recognize the hormone; and some of the receptor properties are altered upon formation of the hormone-receptor complex, which results in signal transduction to the primary cellular target via the corresponding signal transduction system. Similarly to animals, the receptors in plant cells are mostly localized in two compartments: anchored on membranes or inside the nucleus (soluble receptors). The main cellular target for hormone signalling in plants and animals is a set of primary response genes, which is specific for each hormone. However, the molecular mechanisms of intracellular signal transduction have been found to be considerably different in plants and animals. Therefore the results of plant studies have significantly contributed to molecular hormonology as a field of science.

For cytokinin signalling, plants use a bacterial-type analogue of the two-component system of signal transduction, which presumably was borrowed from cyanobacteria [20, 24, 94, 102]. It is believed that the symbiosis of cyanobacteria and eukaryotic cells allowed plants to acquire chloroplasts and to use bacterial genes for this purposes [103, 104]. The landfall was a powerful stimulus for multicellular plants to form new hormonal regulation systems, including the cytokinin system. As animal cells contain no chloroplast-type organelles, animals lack the two-component signal transduction system, evidently because of the absence of symbiosis with the corresponding bacterial progenitors (cyanobacteria).

It is not by mere chance that the significant progress in revealing the molecular mechanisms of the action of phytohormones has been achieved in "post-genomic" 21st century. This was due to whole-plant genome sequencing, the Arabidopsis genome being the first study of the kind in 2000 [11]. As a result, Arabidopsis is as yet the only species whose cytokinin signal perception and transduction system has been thoroughly characterized. However, a host of questions remain unanswered even as regards this plant. In this respect, it is worth mentioning that studies devoted to the investigation of the cytokinin regulatory system are currently under way using various models and ultra-modern methods of molecular biology, hormonology, genetic engineering, bioinformatics, etc. The fact that we stand to soon witness new discoveries in this intriguing and promising field of natural sciences is beyond question. ●

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Lipopolysaccharide of *Yersinia pestis*, the Cause of Plague: Structure, Genetics, Biological Properties

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ABSTRACT The present review summarizes data pertaining to the composition and structure of the carbohydrate moiety (core oligosaccharide) and lipid component (lipid A) of the various forms of lipopolysaccharide (LPS), one of the major pathogenicity factors of *Yersinia pestis*, the cause of plague. The review addresses the functions and the biological significance of genes for the biosynthesis of LPS, as well as the biological properties of LPS in strains from various intraspecies groups of *Y. pestis* and their mutants, including the contribution of LPS to the resistance of bacteria to factors of the innate immunity of both insect-vectors and mammal-hosts. Special attention is paid to temperature-dependent variations in the LPS structure, their genetic control and roles in the pathogenesis of plague. The evolutionary aspect is considered based on a comparison of the structure and genetics of the LPS of *Y. pestis* and other enteric bacteria, including other *Yersinia* species. The prospects of development of live plague vaccines created on the basis of *Y. pestis* strains with the genetically modified LPS are discussed.
KEYWORDS lipopolysaccharide; lipid A; plague; *Yersinia pestis*; immune response; antibiotic resistance
ABBREVIATIONS CAMP – cationic antimicrobial peptides; LPS – lipopolysaccharide; NHS – normal human serum; Ara4N – 4-amino-4-deoxy-*L*-arabinose; Gal – galactose; Glc – glucose; GlcN, GlcNAc – glucosamine, *N*-acetylglucosamine; DD-Hep, LD-Hep – *D*-*glycero*-, *L*-*glycero*-*D*-*manno*-heptose; Kdo – 3-deoxy-*D*-*manno*-oct-2-ulosonic acid; Ko – *D*-*glycero*-*D*-*talo*-oct-2-ulosonic acid; PEtN – phosphoethanolamine; UndP, UndPP – undecaprenyl phosphate, diphosphate.

INTRODUCTION

In the past decade, significant progress has been achieved in the study of the chemical structure, biosynthesis, and biological role of the lipopolysaccharide (LPS) as one of the pathogenicity factors of the bacteria *Yersinia pestis*, the cause of plague. Significant progress in this area has been achieved in the past decade after the September 2001 terrorist attacks, horrific events that prompted thorough research in dangerous pathogens that could potentially find application in the realm of biological terrorism.

The *Yersinia* genus belongs to the Enterobacteriaceae family. Unlike other representatives of this family, including two enteropathogenic *Yersinia* species, *Y. pseudotuberculosis* and *Y. enterocolitica*, which cause chronic intestinal infections, *Y. pestis* cannot exist under ambient conditions for a long time. The plague microbe circulates in natural foci, including populations of over 200 species of rodent hosts (ground squirrels, marmots, gerbils, voles, pikas, *etc.*) and insect vectors (over 80 flea species) [1–5]. A high mortality rate due to plague in rodents is a prerequisite for the continuous transmission of *Y. pestis* in nature.

The *Y. pestis* species contains both genotypically and phenotypically different variants [5, 6]. Strains of the main subspecies, *Y. pestis* subsp. *pestis*, belonging to biovars antiqua, medievalis, orientalis, and intermedium are virulent for humans and guinea pigs. Three plague pandemics are believed to have been caused by strains of each of the first three biovars. It has been proposed that strains of biovars altaica, caucasica, hissarica, ulegeica, talassica, xilingolensis, ginghaiensis, and angola, which are highly virulent in rodent hosts (various vole species belonging to the genus *Microtus*) and white mice, while avirulent in guinea pigs and humans, should be attributed to the second subspecies of *Y. pestis* subsp. *microtus* [6, 7]. This terminology already exists in popular usage [8], and in this review, we will adhere to this variant of intraspecies classification of the causative agent of plague.

Plague is transmitted to humans predominantly via bites of infected fleas, as well as via direct contact with damaged skin and mucous membranes or via the inhalation of aerosolized respiratory secretions from animals or humans with the pneumonic form of the infection [2–5]. In humans, plague occurs as an acute infectious disease manifesting itself as an extremely severe intoxication, fever, lesions in lymph nodes, lungs, and other internal organs, which is frequently complicated with sepsis [4].

The high pathogenicity of *Y. pestis* is to a significant extent due to the unique ability of the bacteria to overcome the defence mechanisms of both mammals and insects, thus ensuring their survival during the entire transmission cycle. Lipopolysaccharide (LPS, endotoxin), the major component of the outer membrane of the cell wall which forms the outer layer of the LPS-phospholipid bilayer, contributes significantly to this feature of the plague microbe. The lipid component of the LPS (the so-called lipid A) acts as an anchor that keeps the LPS molecule bound to the membrane, whereas its carbohydrate chain is oriented towards the environment. A number of pathogenic rough-colony-forming bacteria, including *Y. pestis*, produce R-type LPS with its carbohydrate moiety being limited to an oligosaccharide (pentasaccharide or higher), which is named the core. The S-type LPS, which is typical of most smooth-colony-forming bacteria, in addition contains a polysaccharide chain (O-antigen) consisting of oligosaccharide repeating units, whereas the core is an intermediate region between the O-antigen and lipid A.

The biosynthesis of the O-antigen and the core-lipid A region proceeds via the independent, but convergent pathways [9]. The initial stages, i.e. the synthesis of lipid A, the transfer of core components to it, and the assembly of the O-antigen repeating unit on an undecaprenol carrier, are performed on the cytoplasmic side of the inner membrane. These stages are followed by the transmembrane transfer. The subsequent stages (polymerization of the repeating unit via the O-antigen-polymerase-dependent pathway, which is the most common pathway in enterobacteria; the possible further modifications in the core-lipid A region and O-antigen; and the linking of both parts into a single molecule) occur on the periplasmic side of the membrane.

LPS plays a significant role in the resistance of bacteria to antibiotics, complement, and other defence systems of the host organism; thus, it can be considered as one of the pathogenicity factors of Gram-negative bacteria. The fine structure of the carbohydrate moiety of LPS determines the specificity of the interaction between a bacterial cell and other biological systems, including the immune system and bacteriophages. Lipid A is responsible for the majority of the physiological effects caused by LPS in animals and humans. In mammals, the molecular mechanisms of these effects include the activation of specialized host cells, such as monocytes and macrophages, via the toll-like receptor TLR4, with the participation of the LPS-binding protein and co-receptors CD14 and MD-2. The activated cells secrete nitrogen monoxide, vasoactive lipids and bioactive mediators– pro-inflammatory cytokines. Low concentrations of cytokines are required for triggering the innate immune system of the host; however, their excessive concentration leads to the septic (endotoxic) shock.

Reviews devoted to structural features of the LPS of the plague microbe [10], as well as the immunological properties of its antigens, including LPS [11], have been recently published. This review presents the most up-to-date data pertaining to the chemical composition, structure, genetics and biosynthesis of the LPS of *Y. pestis*, as well as the biological function of the LPS, considered in the context of its structural features. The review also contains a discussion on the possible ways by which the accumulated data pertaining to LPS could be applied in health care.

CHEMICAL COMPOSITION AND STRUCTURE

The LPS of *Y. pestis* is composed of a short carbohydrate (oligosaccharide) chain bound to lipid A [10, 12, 13]. This chain contains a conserved pentasaccharide moiety, the so-called inner core, which is typical of all wild strains of enterobacteria. It consists of three residues of *L-glycero-D-manno*-heptose (LD-Hep) and two residues of 3-deoxy-*D-manno*-oct-2-ulosonic acid (ketodeoxyoctonic acid, Kdo) (*Fig. 1*). The inner core of *Y. pestis*, and certain other enterobacteria (*Serratia, Klebsiella, Proteus, Providencia*) [14], also contains a *D*-glucose residue bound to the heptose residue proximal to lipid A (LD-HepI). The aforementioned bacteria form the group with the so-called non-salmonella core type, whereas the salmonella core type contains phosphate, diphosphate, or diphosphoethanolamine, instead of a glucose residue, at the same position of LD-HepI [14]. The inner core region functions as a receptor of most of the bacteriophages specific to the LPS of *Y. pestis*, including bacteriophage φA1122 belonging to the T7 group [15, 16], which is used by the U.S. Center for Disease Control and Prevention for phage diagnostics of *Y. pestis*. The glycosidic bond of the Kdo residue located at the reducing terminus of the inner core binds the core to lipid A.

The structure of the *Y. pestis* LPS varies depending upon certain environmental factors; it has been thoroughly studied on preparations isolated from bacteria cultured at different temperatures, serving to imitate the conditions in warm-blooded mammals (37°C),

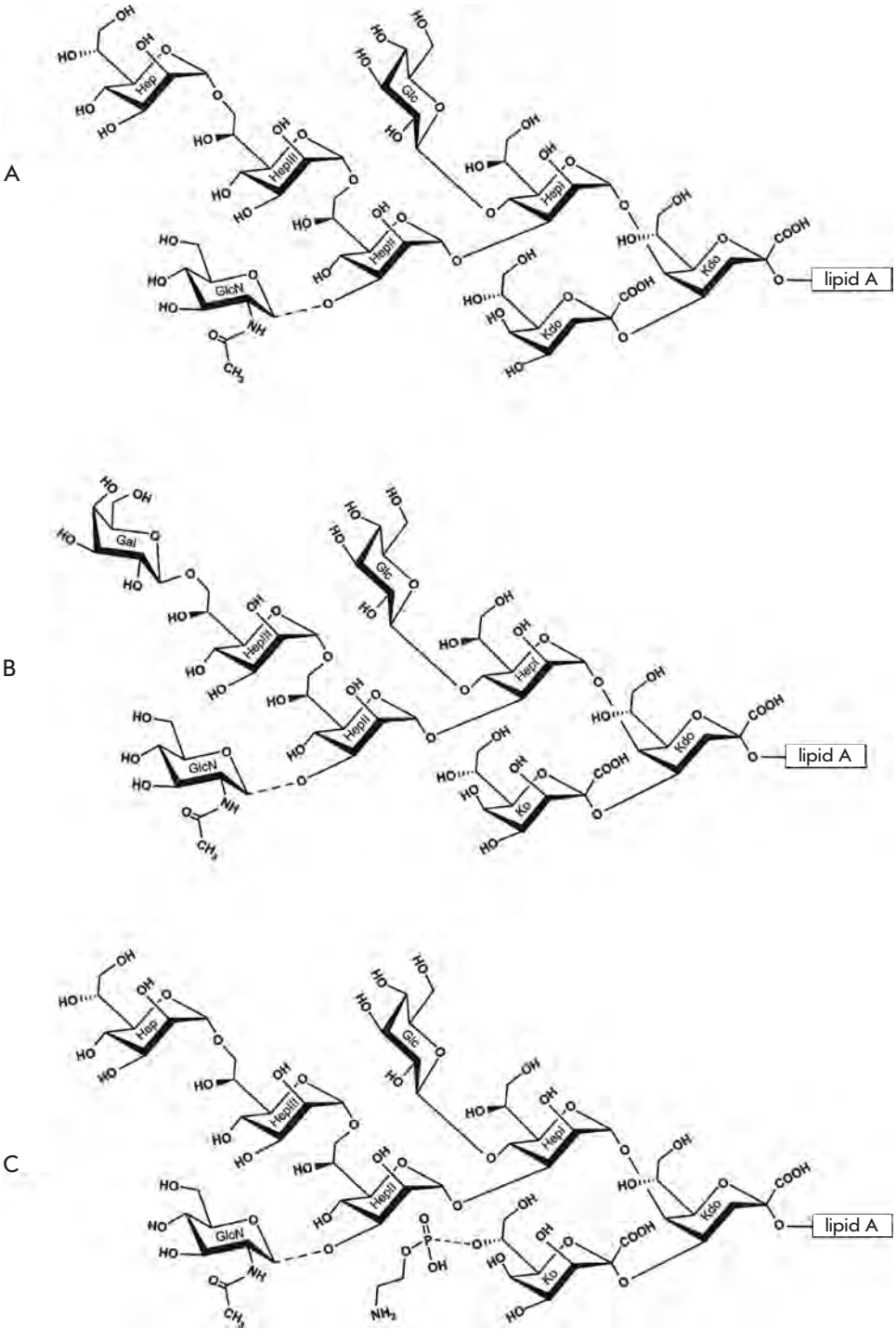

Fig. 1. Structural variants of the LPS core of *Y. pestis* [11–13, 17]. (A) DD-Hep+Kdo glycoform synthesized as the major variant at 37°C and one of the variants produced at 20–28°C. (B) Gal+Ko glycoform synthesized as one of the variants at 20–28°C; in addition, DD-Hep+Kdo, DD-Hep+Ko and Gal+Kdo glycoforms are present. (C) DD-Hep+KoPEtN glycoform synthesized at 6°C; in addition, Gal+Ko PEtN-lacking glycoform is present. Dotted lines indicate nonstoichiometric substitution. Glycine located on LD-HepI in some strains is not shown.

poikilothermic insects (20–28°C), and hibernating animals (6°C). The complete inner core is synthesized by both *Y. pestis* subspecies cultured both at 20–28°C and 37°C. However, the described structure is the sole (or almost sole) glycoform only at 37°C. (*Fig. 1A*), whereas a decrease in temperature results in the partial replacement of the Kdo residue in the side chain by its isosteric 3-hydroxy derivative, the residue of *D-glycero-D-malo*-oct-2-ulosonic acid (Ko) [12] (*Fig. 1B,C*). The Ko-containing glycoform is predominant at 6°C [17].

Y. pestis lacks the outer oligosaccharide region that would be present in the salmonella core type. Its inner region is decorated with several monosaccharides and noncarbohydrate components that are typical of the *Yersinia* species. Thus, the heptose residue that is most distal from lipid A (LD-HepIII) carries a residue

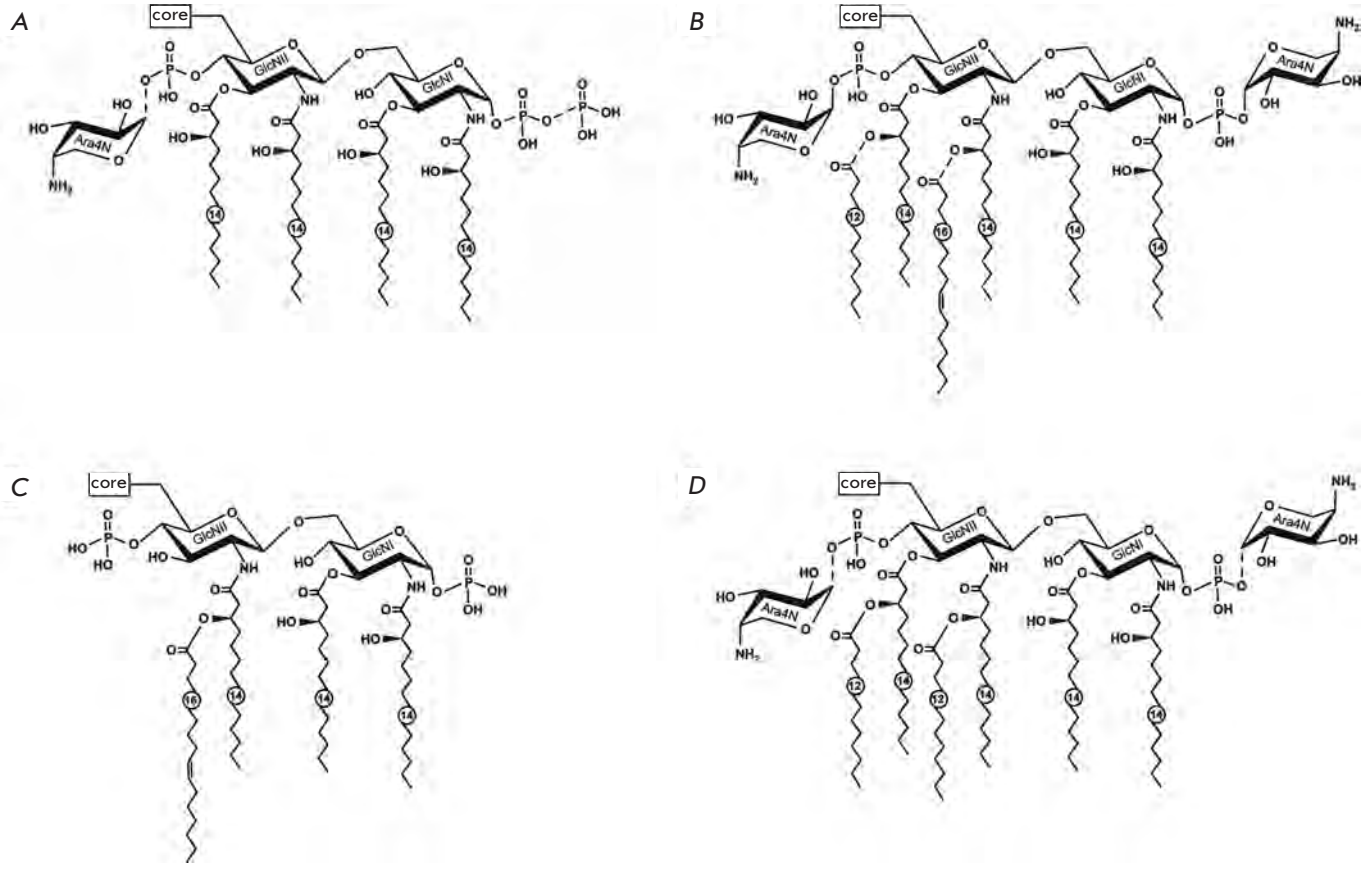

Fig. 2. Structural variants of lipid A of *Y. pestis*. (A) Tetraacyl form synthesized by wild-type strains at 37°C [11, 12, 23, 25]. One of the variants is shown; in the other variants, a diphosphate group may occur at position 4′ and Ara4N-1-phosphate at position 1; Ara4N-1-diphosphate may replace each of these groups [25]. (B) Hexaacyl form [11, 12, 20, 23] and (C) tetraacyl form [17] synthesized by wild-type strains at 20–28°C and 6°C, respectively. (D) Hexaacyl form synthesized by a recombinant strain of *Y. pestis* bearing the *lpxL* gene of *Escherichia coli* at 37°C and 26°C [27]. Dotted lines indicate nonstoichiometric substitution.

of *D-glycero-D-manno*-heptose (DD-Hep) or *D*-galactose. The former is typical of the high-temperature LPS variants (*Fig. 1A*), whereas both variants are synthesized at ambient and decreased temperatures [12] (*Figs. 1A,B*). Strains of certain biovars (caucasica, altaica) of *Y. pestis* subsp. *microtus* are incapable of incorporating DD-Hep into the LPS; as a result, most residues of LD-HepIII in the high-temperature LPS forms do not carry any monosaccharide substituents [12, 18, 19].

The central heptose residue (LD-HepII) is substituted with a residue of *N*-acetyl-*D*-glucosamine, which is present in nonstoichiometric amounts. One of the heptose residues (according to the authors' unpublished data, it is LD-HepI) can be partially acylated with glycine, the content of which decreases with increasing cultivation temperature [12]. The Ko residues are nonstoichiometrically phosphorylated with phosphoethanolamine (PEtN) at 6°C [17] (*Fig. 1C*). PEtN is also present in certain strains grown at 25°C [18, 19].

Y. pestis lipid A has a carbohydrate backbone that is typical of enterobacteria and consists of two 1,4′-biphosphorylated glucosamine residues acylated with four residues of 3-hydroxymyristate, which are known as primary acyl groups. Two residues bind to the amino groups, and the two others, to the hydroxyl groups of glucosamine residues (*Fig. 2A*). Secondary acyl residues, laurate and palmitoleate, bind to the hydroxyl groups of primary fatty acids in the glucosamine residue carrying the core oligosaccharide (GlcNII) [12, 20] (*Fig. 2B*). An additional acyl residue, decanoate, has been detected in *Y. pestis* lipid A; however, its position remains unknown [21, 22].

The content of different acylated forms of lipid A depends to a significant extent on cultivation conditions: it

is a mixture of tetraacyl, pentaacyl, and hexaacyl forms at 20–28°C; the triacyl form is also fairly common. A rise in temperature results in a decrease in the degree of acylation of lipid A. Thus, palmitoleate is not bound at 37°C; therefore, the hexaacyl form is not synthesized at all, and the pentaacyl form with laurate occurs only in a small amount [12, 18, 21, 23].

The high-temperature tetraacyl form (the so-called lipid IV_A) contains four primary residues of 3-hydroxymyristate (*Fig. 2A*), whereas another tetraacyl form with three residues of 3-hydroxymyristate, one of which carries palmitoleate [17] (*Fig. 2B*), is produced at a decreased temperature (6°C), along with the hexaacyl form (*Fig. 2B*). Another feature of *Y. pestis* lipid A is the cold shock-induced oxidation of one or two of the acyl groups [17]. However, it remains unclear which fatty acids are oxidized and which hydroxylated derivatives are formed during this process.

The phosphate groups of lipid A are glycosylated with residues of a cationic monosaccharide, 4-amino-4-deoxy-*L*-arabinose (Ara4N). In low-temperature LPS variants, the glycosylation of both phosphate groups is almost stoichiometric (*Fig. 2B*), whereas the Ara4N content decreases in high-temperature forms [12, 21], and one of the phosphate groups is additionally phosphorylated giving rise to a diphosphate group [18, 19, 24, 25] (*Fig. 2A*). In tetraacyl lipid A of bacteria cultured at 37°C, the diphosphate group can be located at any of two possible positions. Its presence in the pentaacyl variant at position 4' has been confirmed [25], but its location at position 1 also cannot be excluded. By approximate estimation based on mass spectrometry data, the total diphosphate content in the tetraacyl form is 5–6%. Similarly to monophosphate groups, the diphosphate groups at both positions of lipid A can be partially glycosylated with Ara4N [25]. LPS with the PEtN residue in the core, which is produced at 6°C, contains no Ara4N in lipid A [17].

The lack of any O-antigen polysaccharide chain is a feature of the *Y. pestis* LPS that distinguishes it from the LPS of other yersiniae. Meanwhile, similarly to other enterobacteria, *Y. pestis* can produce the enterobacterial common antigen polysaccharide composed of trisaccharide repeating units comprising one residue of each of the following compounds: *N*-acetyl-*D*-glucosamine (GlcNAc), 2-acetamido-2-deoxy-*D*-mannuronic acid (ManNAcA), and 4-acetamido-4-deoxy-*D*-fucose (Fuc4NAc), the GlcNAc residue being partially O-acetylated and partially N-deacetylated. From the two known forms of this polysaccharide (the linear form bound to phospholipid or lipid A and the lipid-free cyclic form), in *Y. pestis* the most thoroughly characterized is the cyclic form [26] (*Fig. 3A*).

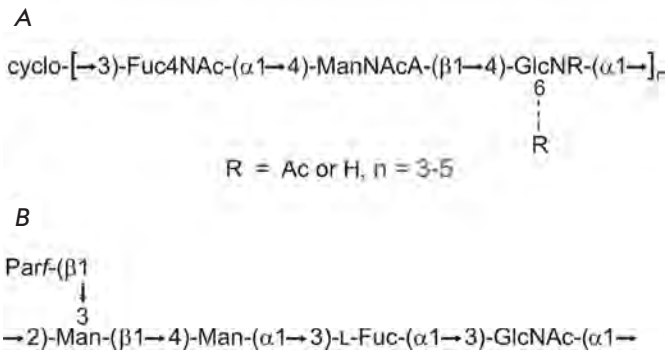

Fig. 3. Structure of the polysaccharide antigens of *Y. pestis* (A) and *Y. pseudotuberculosis* O:1b (B). (A) The cyclic form of the common enterobacterial antigen of *Y. pestis* [26]. The glucosamine residue is N-acetylated by ~50 % and 6-O-acetylated by ~20 %; n = 4 (major variant), 3 or 5 (minor variants). (B) The pentasaccharide repeating unit of the O-antigen of *Y. pseudotuberculosis* O:1b [28]. A nonfunctional gene cluster for biosynthesis of this polysaccharide is present in the genome of *Y. pestis* [29]. Par represents 3,6-dideoxy-*D*-*ribo*-hexose (paratose). All monosaccharides have the *D* configuration; paratose occurs in the furanose form, and the other monosaccharides occur in the pyranose form.

GENETICS AND BIOSYNTHESIS

The tetraacyl biphosphorylated precursor of enterobacterial lipid A (lipid IV_A) in *Y. pestis* is presumed to be synthesized via the same pathway as that of *E. coli* and *Salmonella enterica* [9], which have been studied most thoroughly in this context. Homologues of the *E. coli* genes that encode the enzymes mediating the late acylation of lipid A (myristoyltransferase LpxM (MsbB), palmitoleyltransferase LpxP and palmitoyltransferase PagP but not lauroyltransferase LpxL (HtrB)), have been identified in the *Y. pestis* genome [22, 30–32].

The functional *lpxM* and *lpxP* genes participate in the synthesis of the hexaacyl lipid A of *Y. pestis* (*Fig. 4*). Their expression level increases as the cultivation temperature decreases from 37 to 21°C. Regardless of temperature, the mutant at both genes synthesizes tetraacyl lipid A, which is similar to lipid IV_A (*Fig. 2A*) and is identical to that synthesized by wild-type *Y. pestis* strains at 37°C [22]. Meanwhile, the transcription level remains low under all conditions. The temperature dependence of the catalytic activity of the enzymes or other post-transcription effects can also affect the lipid A acylation pattern.

E. coli acyltransferase LpxM can use either myristate or laurate as a substrate; however, since its activity is higher with the former substrate, it links myristate to 3-hydroxymyristate at position 3' of GlcNII. This proc-

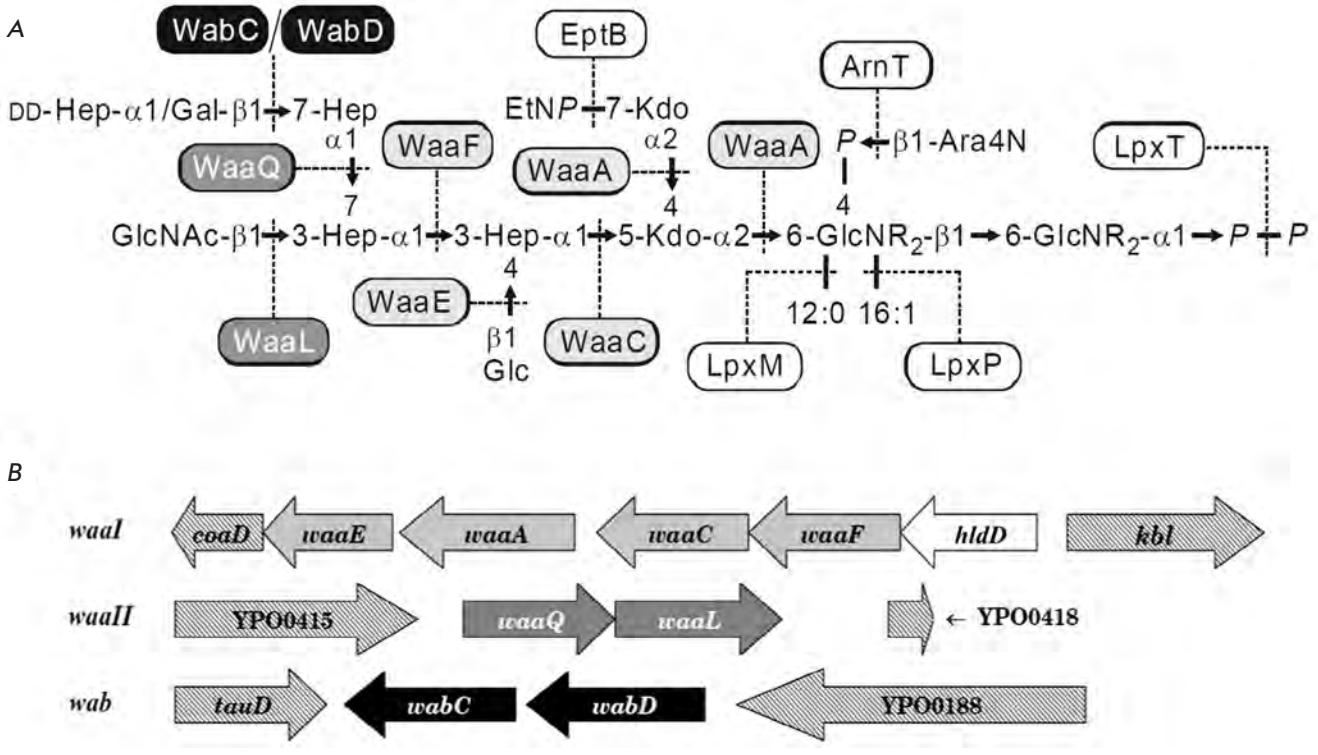

Fig. 4. Biosynthesis of the LPS of *Y. pestis* [22, 32–34]. (A) Enzymes of the LPS core synthesis and the late stages of the lipid A synthesis. R represents 3-hydroxymyristoyl. (B) Organization of the gene clusters for the LPS core synthesis. The genes in the clusters *waaI* (YP0054-YP0058), *waaII* (YP0416-YP0417), and *wab* (YP0186-YP0187) and the corresponding glycosyl transferases are shown in light gray, dark gray, and black, respectively. The functions of the glycosyl transferases genes are assigned based on the analysis of available *Y. pestis* full genome sequences and the data on LPS structure in knockout mutants at each gene. The figure is reproduced from the paper of the authors [32] with the permission of the Nauka Publishing Company.

ess is preceded by the transfer to 3-hydroxymyristate at position 2' of GlcNII of the secondary acyl group: laurate at 30–42°C catalyzed by LpxL [9] or palmitoleate at cold shock temperature (12°C) with the participation of LpxP [35]. The *Y. pestis* homologues LpxM and LpxP transfer laurate and palmitoleate to 3-hydroxymyristate residues at positions 3' and 2' GlcNII, respectively. Temperature control was observed only for LpxP, transferring palmitoleate to position 2' GlcNII prior to the binding of the secondary acyl group at position 3' (laurate in *Y. pestis* or myristate in *E. coli*), whereas LpxM activity is temperature-independent. Since *Y. pestis* lacks the *lpxL* gene, 3-hydroxymyristate at position 2' remains unsubstituted at an increased temperature, which deteriorates the efficiency of the laurate transfer with LpxM and results in the synthesis of mostly the tetraacyl form of lipid A and only a negligible amount of the pentaacyl form. The evidence for this is that the *Y. pestis* recombinant strain KIM5-pLpxL carrying the *E. coli lpxL* gene produces the hexaacyl form of lipid A with two secondary laurate residues both at 37 and 26°C [27] (*Fig. 2D*).

Acyltransferase PagP in *E. coli* and *S. enterica* transfers palmitate from position sn-1 of the glycerophospholipid [36], which distinguishes it from the early and other late acyltransferases that use a substrate bound to the acyl-carrier protein as a donor. Furthermore, palmitoylation of lipid A occurs on the outer rather than the inner membrane [36]. The palmitoylated forms of lipid A are also typical of *Y. pseudotuberculosis* and *Y. enterocolitica* [19–21] but have not been found in wild-type *Y. pestis* strains, despite the presence of the *pagP* homologue in the genome, which is 99% identical to the *Y. pseudotuberculosis* gene [31, 36]. The reason for this is the inactivation of this gene due to the substitution of a single nucleotide, resulting in the conversion of a tryptophan-200 codon into a stop codon [31].

The genes encoding the synthesis and transfer of Ara4N are components of the operon *arn* (*pmrHFIJKLM*) [9, 37]. An undecaprenyl phosphate (UndP) de-

rivative synthesized with the participation of Ara4N-transferase ArnC (PmrF) is a donor of Ara4N for its subsequent binding to the phosphate groups of lipid A. The transfer of Ara4N to lipid A, catalyzed by the product of the *arnT* (*pmrK*) gene, occurs on the periplasmic side of the inner membrane [38]. A complete inner core is required to ensure the most efficient binding of Ara4N, whereas the presence or absence of core monosaccharides distal from lipid A (GlcNAc, Gal and DD-Hep) has virtually no effect on this process [32–34]. Similar to that in *E. coli* and *S. enterica*, the *arn* operon in *Y. pestis* is regulated by two-component signal transduction systems, PhoP/PhoQ and PmrA/PmrB [21, 39]. However, in *Y. pestis* the mechanism of regulation by the PhoP/PhoQ system differs as it occurs without the participation of the PmrD protein, which is absent in this bacteria [39].

The synthesis of the *E. coli* core oligosaccharide begins with the attachment of two Kdo residues to lipid IV_A, a process that is catalyzed by bifunctional Kdo transferase WaaA (*Fig. 4A*). Kdo transfer precedes the late acylation of lipid A [9]. The subsequent assembly of the core takes place on a completely acylated lipid A and is followed by the transfer of LPS consisting of the core and lipid A through the inner membrane, with the aid of the ABC transporter MsbA. Meanwhile, neither core oligosaccharide nor Kdo (Ko) residues are required for the transmembrane transfer, since LPS without any core (i.e., lipid A) is expressed in the Kdo-deficient mutants of *E. coli* [40, 41] and *Y. pestis* [32, 33, 42].

The core biosynthesis genes in *E. coli*, *S. enterica*, and a number of other enterobacteria are clustered in a chromosomal region, forming the *waa* cluster [9]. Two clusters (*waaI* and *waaII*) with four and two homologues of the *waa* genes and one cluster with two *wab* genes, which also encode core biosynthetic enzymes, have been identified in the *Y. pestis* genome [32–34] (*Fig. 4B*).

The *waaI* cluster containing most of the genes for the synthesis of the inner core comprises the genes of Kdo transferase WaaA; heptosyltransferases WaaC and WaaF to transfer LD-HepI and LD-HepII, respectively; and glucosyltransferase WaaE. In addition, it includes the gene of heptose 6-epimerase HldD catalyzing the synthesis of ADP-LD-Hep from its biosynthetic precursor ADP-DD-Hep. Yet another heptosyltransferase gene, *waaQ*, is located in the *waaII* cluster. The enzyme encoded by this gene transfers LD-HepIII to LD-HepII; glucose must be present on LD-HepI in order to accomplish the transfer. In turn, the glucose transfer requires the prior attachment of LD-HepII.

The second gene in the cluster *waaII* is a homologue of the gene of ligase WaaL, which attaches the O-antigen to the core [9]. Unlike monosaccharide LPS components transferred by glycosyltransferases in the form of proper nucleoside diphosphate derivatives or (in case of Kdo) a nucleoside monophosphate derivative, the undecaprenyl diphosphate (UndPP) derivative acts as a ligase substrate. In the absence of any O-antigen in *Y. pestis*, WaaL attaches the GlcNAc residue to the core; therefore, this residue is not a true component of the core [12]. Ligase nonstrictly depends on the attachment of Glc and LD-HepIII; without them, the efficiency of the GlcNAc transfer decreases and the LPS of the *waaE* and *waaQ* knockout mutants contains only a small amount of this monosaccharide [32, 33]. In *Y. pestis* and other enterobacteria, the *wecA* gene participating in the synthesis of the UndPP-bound GlcNAc is located in the gene cluster of the enterobacterial common antigen [43], whose biosynthesis (in the same manner to that of GlcNAc-containing O-antigens) is initiated by the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to UndP.

The *wab* cluster contains the genes of glycosyltransferases WabC and WabD for the transfer of DD-Hep and Gal, respectively. It has been demonstrated using BLAST search that in strains of *Y. pestis* subsp. *microtus* bv. caucasica Pestoides F and bv. xilingolensis 91001, the *wabC* gene contains mutations resulting in disturbance of the synthesis of the corresponding protein [32]. Similar mutations are presumably present in the other DD-Hep-defective strains of *Y. pestis* subsp. *microtus* bv. caucasica and bv. altaica. The expression of the *wabD* gene and/or the activity of the WabD enzyme are temperature-dependent, and the transfer of Gal proceeds inefficiently at increased temperatures. The inability of the *phoP* mutant to incorporate Gal into the core demonstrates that galactosylation is controlled by the PhoP/PhoQ two-component signal transduction system [44]. Meanwhile, the attachment of DD-Hep does not require the functional PhoP/PhoQ system.

As mentioned above, the terminal Kdo residue at low temperature is partially substituted for the Ko residue. The latter is synthesized via the oxidation of the 3-deoxy group of Kdo with a unique Fe^{2+}/α-ketoglutarate/O_2-dependent Kdo-3-hydroxylase (KdoO) [45]. Its substrate specificity has not been studied; however, taking into account the fact that KdoO is a peripheral membrane protein, it can reasonably be assumed that 3-hydroxylation of Kdo occurs on the cytoplasmic side of the inner membrane after two Kdo residues have bound to lipid A. The molecular mechanism of the modulation of the temperature-dependent Ko content in the core has as yet to be elucidated.

Gene homologues of transferase EptB (YhjW) transferring PEtN from phosphatidylethanolamine to Kdo

[32, 33, 46] and phosphatase LpxT (YeiU) transferring phosphate from UndPP to lipid A giving rise to diphosphate [32, 33, 47] have also been found in the genome of *Y. pestis* [32, 33]. These genes, in an identical manner to the *kdoO* gene encoding Kdo-3-hydroxylase, as well as the genes of the late stages of lipid A synthesis (acylation and glycosylation with Ara4N), are spread over the chromosome as individual nonclustered genes. No gene of acyltransferase, which participates in glycine transfer to the LD-HepI residue, has thus far been identified in the genome of *Y. pestis*.

There is 100% homology of the proteins participating in the LPS biosynthesis (with the exception of the *wabC* gene that is mutated in a number of representatives of the nonmain subspecies; see above) within the *Y. pestis* species and 98–100% homology inside the *Yersinia* genus [32, 33]. This correlates with the high degree of similarity between the core and lipid A structures of LPS in various *Yersinia* species [19, 20]. In distantly related bacteria, the homology of the proteins WaaA, WaaC, WaaE, WaaF, EptB, LpxM, LpxP, and ArnT is beyond 70%. Meanwhile, in the enzymes WaaQ, WabC, WaaL, and KdoO, it is lower than 64%, whereas the homology between galactosyltransferase WabD that is unique to the *Yersinia* species and glycosyltransferases of other bacteria is less than 43%. The high homology of most of the proteins implicated in the LPS biosynthesis in *Y. pestis* and the bacteria belonging to various phylogenetic groups, in combination with the dispersed location of the corresponding genes in the chromosome of the plague microbe, suggests a multi-stage horizontal transfer of these genes to the genome of the *Yersinia* progenitor.

A nonfunctional O-antigen gene cluster was identified in the *Y. pestis* genome [29, 48]. At the nucleotide sequence level, it was 98.9% identical to the O-antigen cluster in *Y. pseudotuberculosis* O:1b [29] (the O-antigen structure is shown in *Fig. 3B*). Therefore, *Y. pseudotuberculosis* O:1b is considered as the most plausible progenitor clone of *Y. pestis*. Of the 17 biosynthetic genes that have been identified in the O-antigen gene cluster of *Y. pseudotuberculosis* O:1b, five genes in the *Y. pestis* cluster are inactivated by insertions or deletions. These genes include those responsible for the synthesis of the nucleotide-activated derivatives of *L*-fucose and 3,6-dideoxy-*D*-*ribo*-hexose (paratose), precursors of the O-antigen components, in the absence of which the O-antigen synthesis is rendered impossible. It is noteworthy that while 16 genes in the clusters of two bacteria are 99–100% identical, the *wzx* gene is only 90.4% identical. This gene encodes flippase Wzx, which mediates the transmembrane transfer of the UndPP-bound pentasaccharide repeating unit of the O-antigen in *Y. pseudotuberculosis* O:1b. After the loss of this function, *Y. pestis* flippase presumably changed and centered on the transfer of a single UndPP-bound GlcNAc residue through the inner membrane, which subsequently is bound to the LPS core by ligase WaaL at the same position as the polysaccharide O-antigen in *Y. pseudotuberculosis*.

BIOLOGICAL PROPERTIES AND ROLE IN PLAGUE PATHOGENESIS

Production by macrophages and other immune cells of the key pro-inflammatory cytokines (including the tumor necrosis factor α (TNF-α), the major mediator of septic shock (endotoxemia) that is induced by the action of LPS) plays a significant role in overcoming infectious diseases. In *Y. pestis*, as in the other Gram-negative bacteria, the cytokine-inducing activity of the LPS mediated by the TLR4 receptor is determined by the lipid A structure [49]. Thus, the production of TNF-α by human and mouse macrophage cell lines considerably falls with a decreasing degree of acylation of lipid A, in particular, with the absence of the hexaacyl form and a significant decrease in the content of the pentaacyl form [18, 23]. These structural changes in lipid A are observed when the temperature of bacterial cultivation increases from 21–28°C to 37°C, which imitates a transition from the temperature conditions in poikilothermic fleas (< 30°C) to those in warm-blooded mammals (37°C) [21, 23, 32, 33]. In terms of the TNF-α inducing activity at 25°C, LPS of *lpxM* knockout mutants occupies an intermediate position between the LPS of the parental strains cultivated at 25 and 37°C, which is in good agreement with the degree of acylation of lipid A [50].

The limited biological activity of the high-temperature low-acyl form of *Y. pestis* LPS may play a crucial role in overcoming the defence mechanisms of warm-blooded animals by bacteria. Whereas the innate immune system is efficiently stimulated by high-acyl LPS forms, the low-acyl forms are not recognized by the TLR4 receptor and, as a result, do no activate the innate immunity via the MD-2-TLR4-dependent pathway. Moreover, in the experiments with human macrophage cell lines [51] and dendritic cells [52], LPS from *Y. pestis* cells cultivated at 37°C behaved as an antagonist actively suppressing the TLR4-dependent pro-inflammatory response. The significance of this feature of LPS as a pathogenicity factor of the plague microbe has been convincingly supported by the study of the recombinant *Y. pestis* strain KIM5-pLpxL carrying the *E. coli lpxL* gene [27]. The LPS of this strain with the 'unnatural' hexaacyl lipid A (*Fig. 2D*) under all temperature conditions, including 37°C, stimulates the signalling via TLR4 and the induction of cytokines (TNF-α, interleukines-6 and -8) much more efficiently as compared to LPS of wild-type strains.

Remarkably, the *Y. pestis* recombinant strain KIM5-pLpxL could not cause bubonic plague in mice, despite the fact that the other pathogenicity factors, such as the type III secretion system, resistance to the bactericidal action of normal serum, and the proteolytic activity of Pla, were not affected. These findings provide a good illustration of the fact that the active (endotoxic) LPS form also plays a positive role in a host, ensuring prompt pathogen recognition and activation of the innate immune system at the early stages of infection. It has been demonstrated on mice model that attenuated *Y. pestis* strains with the immunostimulating LPS form can be regarded as a prototype for a new, efficient live vaccine against plague [27, 53].

It should be mentioned that the production of higher acylated low-temperature LPS forms is not a prerequisite for the survival of *Y. pestis* in flea intestine. Thus, the *lpxP/lpxM* double mutant with tetraacyl lipid A could colonize the digestive tract and block the proventriculus of rat flea *Xenopsylla cheopis* with the same efficiency as the wild-type strain, which is distinguished by a high level of expression of hexaacyl lipid A in a flea's organism [22].

A decrease in the degree of acylation of lipid A with increasing cultivation temperature [22] or inactivation of the *lpxM* gene [20] resulted in a moderate or negligible reduction in the lethal toxicity of LPS preparations on the model of mice sensibilized with actinomycin D. The incapacity of the *lpxM* mutant of *Y. pestis* wild-type strain 231 to synthesize hexaacyl lipid A had no effect on its virulence, whereas the same mutation in the attenuated vaccine strain of *Y. pestis*, EV line NIIEG, reduced its ability to cause lethal infection in mice and guinea pigs [49, 54]. Importantly, a decrease in the virulence of the *lpxM* mutant of the vaccine strain was accompanied by a significant increase in its protective activity against bubonic plague as compared with the parental vaccine strain [49, 54]. This phenomenon can presumably be attributed to the pleiotropic effects of mutation, including changes in the biosynthesis and the exposure character of the major immunoreactive antigens of the bacterial cell surface [55]. If the changes between the mouse and human LPS receptor do not level these changes, inactivation of the *lpxM* gene can be used for development of a live plague vaccine with reduced adverse effects.

An increase in the cultivation temperature of *Y. pestis* and *Y. pseudotuberculosis* from 26 to 37°C resulted in an increase in the permeability of the outer membrane for the hydrophobic agent *N*-phenyl-1-naphthylamine, which correlated with a decrease in the number (and as a result, with an increase in the flexibility) of the LPS acyl chains [56]. The absence of laurate and palmitoleate made the *lpxP/lpxM* double mutant sensitive to the deoxycholate detergent, with no effect on its resistance to the hydrophobic antibiotics rifampin and vancomycin [22]. Controversial data have been obtained using cationic antimicrobial peptides (CAMPs), one of the key factors of innate immunity: a decrease in the degree of acylation did not affect the resistance to polymyxin B, but it increased the sensitivity to cecropin A [22].

Yet, the resistance to CAMPs depends on the Ara4N content in *Y. pestis* LPS. This correlation, which is also typical of *S. enterica* and a number of other bacteria [37], can be attributed to the electrostatic repulsion of CAMPs by the cationic monosaccharide, which impedes the binding between the antibiotic molecule and the negatively charged (e.g., phosphate) groups on the outer membrane. A high resistance of the wild-type *Y. pestis* strains with a near-stoichiometric content of Ara4N in the LPS (two Ara4N residues per molecule), which is attained by culturing bacteria at 20–28°C, has been demonstrated using the polymyxin B model. A decrease in the resistance to CAMP correlates with a noticeable drop in the content of Ara4N as the temperature is increased to 37°C [21, 57]. Mutants with knockout genes *galU* encoding the pathway of Ara4N synthesis [58], *arnT* [32–34] encoding Ara4N-transferase, or *phoP* [21, 44] modulating the binding of Ara4N to lipid A are sensitive to CAMP independently of the cultivation temperature. The role of Ara4N is also supported by a marked increase in the content of this monosaccharide in the LPS of bacteria cultivated at 37°C in the presence of polymyxin B [12]. An increase in the Ara4N content in LPS and, as a result, in the resistance of *Y. pestis* to CAMPs with a decreasing cultivation temperature is undoubtedly of adaptive character. High resistance to polymyxin B at a temperature characteristic of insects can presumably be attributed to a greater contribution of CAMPs to the innate immunity defence mechanisms of insects as compared to that of mammals, which have a complement system in addition to CAMPs.

Another cationic component of LPS, glycine [57] located in the core, can contribute to a certain extent to the resistance to CAMPs, whereas uncharged core components do not seem to play a significant role. An increase in the sensitivity to polymyxin B, which was observed for a set of knockout mutants at glycosyltransferase genes producing a truncated core could presumably be attributed to the simultaneous decrease in the Ara4N content in lipid A due to the inefficiency of the Ara4N transfer to LPS molecules with an incomplete carbohydrate moiety [32–34].

The LPS core plays a significant role in the resistance of *Y. pestis* to the complement-mediated bactericidal effect of normal blood serum [32–34], another important component of the innate immune system. Wild-type strains of *Y. pestis* subsp. *pestis* are resistant

to normal human serum (NHS) both at 25°C and at 37°C [57]. The *waaL*, *wabC*, *wabD,* and *arnT* mutants lacking the terminal core substituents GlcNAc, DD-Hep, Gal or the cationic monosaccharide Ara4N, respectively, are characterized by an almost identical resistance. On the contrary, the mutants with an incomplete inner core region are highly susceptible to NHS [32–34]. The molecular mechanism, by which the core contributes to the serum resistance, has not been elucidated thus far. Possibly, it is mediated by the effect of LPS on the folding correctness and, as a result, on the functional activity of the outer membrane protein Ail (OmpX) [59], which plays a crucial role in the resistance of *Y. pestis* to serum [59, 60]. Studies of the recombinant *E. coli* strain carrying the *Y. pestis ompX* gene and its three mutants with a truncated core revealed that the size of the LPS core impacts not only the resistance to NHS, but also the OmpX-mediated virulence factors, such as the adhesive ability and the invasiveness of bacteria [60].

As opposed to strains of the main subspecies, the sensitivity of strains of *Y. pestis* subsp. *microtus* bv. caucasica to the action of NHS is temperature-independent, which correlates with the absence of documented cases of a human plague caused by strains of this biovar [57]. Meanwhile, these strains are resistant to mouse serum and probably to that of their main host, the common vole, ensuring their survival in rodent blood, which is required for circulation of *Y. pestis* in the natural plague foci. The only distinguishing feature of the LPS of biovar caucasica is that the core contains no DD-Hep [12]. However, a strain of another representative of *Y. pestis* nonmain subspecies, biovar altaica [57], whose LPS also contains no DD-Hep, is as highly resistant to the bactericidal effect of NHS as that of the *wabC* mutant of the main subspecies with a DD-Hep-deficient LPS [32–34]. This finding suggests that the adaptive changes, which made strains of *Y. pestis* subsp. *microtus* bv. caucasica sensitive to NHS, affected not only LPS, but also another factor(s) involved within the interaction between a bacterial cell and the complement system.

With no effect on the growth rate of *Y. pestis* cells [32, 33], a decrease in the size of the LPS core affects the *in vivo* formation of a biofilm, the polysaccharide-containing extracellular matrix, as well as the proventriculus blockage in fleas, which depends on this process [61]. A *Y. pestis* KIM6+ mutant with the knockout *gmhA* gene, which encodes one of the enzymes of the LD-Hep biosynthetic pathway, is characterized by a reduced ability to form the biofilm on the cuticle of nematode *Caenorhabditis elegans* and to block the proventriculus in *X. cheopis* with a moderate decrease in the level of *in vitro* biofilm formation [61]. This indirect effect of the absence of the major part of the core, including the heptose region, can be attributed to the interaction between the LPS core components and the outer membrane proteins participating in the synthesis, processing, or export of the biofilm.

A significant decrease in the virulence of *Y. pestis* 231 was observed in subcutaneously infected guinea pigs after the LPS core was shortened to five monosaccharide residues and the full loss of virulence for both guinea pigs and mice after the subsequent shortening of the LPS core to three monosaccharides [32, 33]. However, these observations were carried out for a period of 21 days, and it cannot be excluded that a prolongation of the experimental time could have led to the generalization of the infection, resulting in animal death at later stages. Attenuation of the mutants of the virulent CO92 strain of *Y. pestis* with a truncated core was also observed in BALB/c mice, whereas the absence of the core in mutants at the *yrbH* gene of the Kdo synthesis pathway or the *waaA* gene encoding Kdo transferase made them completely avirulent [15]. These data prove directly the exceptional significance of the LPS for the pathogenicity of the plague microbe, since an identical dramatic attenuation of *Y. pestis* strains was observed only when the main components of the type III secretion system [4], the gene cluster of synthesis and reception of the siderophore yersiniabactin [4], or the lipoprotein NlpD-encoding genes [62] were lost.

The biological significance of temperature-dependent variations in the monosaccharide composition of the core (replacement of the terminal Kdo and DD-Hep residues for the Ko and Gal residues, respectively, with decreasing ambient temperature from 37 to 28°C and lower) remains to be elucidated. It is reasonable to assume that the hydroxylation of Kdo at a reduced temperature (i.e., the conversion of Kdo into Ko) compensates for the decrease in the hydrophilicity of the LPS as a result of the acylation of the hydroxyl groups of primary fatty acid residues, which also occurs at low temperatures.

The investigation of the plasminogen activator Pla of *Y. pestis* (an outer membrane protein belonging to the omptin family and exhibiting functions of protease/adhesin) provided an answer (which may not be the only answer) to the intriguing question of which other advantages (in addition to the trivial energy saving advantage) was acquired by the plague microbe after it had eliminated the necessity for synthesizing the O-antigen. Pla converts plasminogen into plasmin, a key fibrinolytic enzyme, destroys the circulating α_2-plasmin inhibitor (antiplasmin), induces an uncontrollable tissue proteolysis, and facilitates the dissemination of *Y. pestis* in a macroorganism, thus playing a significant role in a plague pathogenesis. Studies of the activity of *Y. pestis* Pla in recombinant *Y. pseudotuberculosis* strains with

different levels of the O-antigen expression have demonstrated that the O-antigen sterically impedes the interaction between Pla and the high-molecular-mass substrate, thus impeding both plasminogen activation and α_2-antiplasmin inactivation [63, 64]. This fact leads to the conclusion that the loss of the O-antigen, which is necessary for the enhancement of the enzymatic activity of Pla, has enhanced the invasiveness of *Y. pestis*. On the other hand, the Pla activity depends on a specific interaction with the phosphate groups of lipid A [65] and requires the presence of LPS with a core that contains at least two LD-Hep residues [32, 66]. Such an unusual LPS dependence can be attributed to the fine conformational changes in the active site of omptin due to the binding with LPS [67].

CONCLUSIONS

A comparison of data pertaining to the structure, biosynthesis, and the biological properties of the LPS in *Y. pestis* and *Y. pseudotuberculosis* demonstrates that inactivation of the O-antigen gene cluster and the *pagP* gene, as well as the loss of the *lpxL* gene, which results in the synthesis of the R-form of LPS with a short carbohydrate chain and a loss of the ability to produce high-acyl forms of lipid A at 37°C, were the most significant events during the evolution of *Y. pestis* associated with changes in the LPS structure. These changes play a significant role in the plague pathogenesis, being an important part of the strategy by which the bacteria overcome the host's defence mechanisms. Thus, the absence of the polysaccharide chain ensures the functioning of such a significant pathogenicity factor of *Y. pestis* as the plasminogen activator. The temperature-mediated decrease in the degree of LPS acylation resulting in a reduction of the cytokine-inducing ability is considered to be one of the mechanisms for the prevention of pathogen recognition by the host's immune system at the early stages of infection. These data agree with the conception that the pathoadaptation of *Y. pestis* to a new ecological niche included the loss of the functionality of a number of genes required for saprophytic existence [36, 68]. The mutations in the *wabC* gene encoding DD-Hep-transferase, which remained in representatives of bv. caucasica and bv. xilingolensis of the nonmain subspecies *Y. pestis* subsp. *microtus*, may have been an element of the subsequent reductive intra-species microevolution during the adaptation of *Y. pestis* to circulation in the populations of certain vole species.

Meanwhile, a number of LPS features, such as the temperature-dependent variation of both core and lipid A structures, were inherited by *Y. pestis* from *Y. pseudotuberculosis* without any noticeable changes. Some of these variations, such as the phosphorylation with PEtN, the glycosylation with Ara4N, and the oxidation of Kdo into Ko, presumably do not possess any fundamental significance in the new lifestyle of the plague microbe and are required only for the normal functioning of the outer membrane, which is achieved by imparting a certain hydrophilicity and a certain charge to it. On the other hand, they may contribute to plague pathogenesis by facilitating the optimal adaptation of *Y. pestis* at different phases of its existence under the considerably different conditions in mammal hosts and insect vectors. The unique phenomenon of the plague microbe and plague can be a result of the synergic effect of the inherited and newly acquired pathogenicity factors, including those associated with LPS.

Thus, the recent data highlight LPS as a multifunctional pathogenicity factor of *Y. pestis* which plays a key role in the adaptive variability of the plague microbe. However, it should be noted that even if there is a correlation between the LPS structure and the properties of the bacterial culture, the exact biological significance of various LPS modifications cannot be considered completely ascertained, since the cultivation of bacteria in laboratory conditions cannot simulate exactly *in vivo* conditions. If detailed structures of the LPS synthesized by the bacteria in flea and a warm-blooded host were identified, this would enable selection of the *in vitro* conditions necessary for production of the LPS forms characteristic of a particular infected animal for further laboratory experiments.

The identification of the structure-to-function relationships in *Y. pestis* LPS opens up new prospects for the design of efficacious live vaccines against plague which are based on the attenuated strains with reduced adverse effects. The approach based on genetic-engineering modification of the *Y. pestis* LPS, which reduces the degree of lipid A acylation, is highly promising. The ability to avoid recognition by the host's defence system at the earliest stage of infection allows the attenuated strain with the mutant LPS to rapidly multiply. However, the absence of one of the major pathogenicity factors as a result of a mutation used for attenuation of a *Y. pestis* strain prevents the generalization of the infection. This subsequently ensures efficient production of antibodies against the major antigens of the plague microbe and the unset of acquired immunity. Development of efficacious and highly selective next-generation antimicrobial preparations based on high-affinity oligonucleotide ligands can become an alternative approach in plague treatment. Understanding the genetic control of the key stages of the biosynthesis of LPS, intervention in which disturbs the functioning of the outer membrane and attenuates the effect of other virulence factors, will enable to propose new molecular targets for these antibiotics. ●

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A Polygenic Approach to the Study of Polygenic Diseases

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ABSTRACT **Polygenic diseases are caused by the joint contribution of a number of independently acting or interacting polymorphic genes; the individual contribution of each gene may be small or even unnoticeable. The carriage of certain combinations of genes can determine the occurrence of clinically heterogeneous forms of the disease and treatment efficacy. This review describes the approaches used in a polygenic analysis of data in medical genomics, in particular, pharmacogenomics, aimed at identifying the cumulative effect of genes. This effect may result from the summation of gains of different genes or be caused by the epistatic interaction between the genes. Both cases are undoubtedly of great interest in investigating the nature of polygenic diseases. The means that allow one to discriminate between these two possibilities are discussed. The methods for searching for combinations of alleles of different genes associated with the polygenic phenotypic traits of the disease, as well as the methods for presenting and validating the results, are described and compared. An attempt is made to evaluate the applicability of the existing methods to an epistasis analysis. The results obtained by the authors using the APSampler software are described and summarized.**

KEYWORDS **medical genomics; pharmacogenomics; polygenic analysis; epistasis**

ABBREVIATIONS **CDCV – common disease / common variant; CDRV – common disease / rare variant; CI – confidence interval; CMC – combined multivariate and collapsing; FDR – false discovery rate; GWAS – genome-wide association study; MCMC – Markov Chain Monte Carlo; MDR – multifactor dimensionality reduction; MS – multiple sclerosis; IS – ischemic stroke; OR – odds ratio; ORR – odds ratios ratio; RR – relative risk; SF – synergy factor; TDT – transmission disequilibrium test.**

INTRODUCTION

The concepts of modern genetics subdivide hereditary diseases into Mendelian and complex disorders. The Mendelian disorders are determined by carriage of a mutant variant of a single gene, whereas complex diseases depend both on a genetic component determined by the joint contribution of a large number of independent or interacting polymorphic genes and on other factors. Meanwhile, the individual contribution of each gene to the development of a polygenic disease can be small or modest. The carriage of certain allelic combinations of genes can also determine the emergence of clinically heterogeneous forms of diseases and the therapeutic efficacy of certain pharmaceutical agents.

In humans, polygenic disorders occur much more frequently than monogenic ones; they have a great social and economic impact. However, their molecular genetic nature has not been elucidated thus far. The search for the genes that are involved in the development of polygenic diseases is carried out with the use of two major strategies, namely, establishing the role of a certain candidate gene selected relying on the tentative role of its protein product in the etiopathogenesis of the disease and whole genome sequencing using the panel of genetic markers that are more or less uniformly distributed across the genome. The experimental approaches to determine the role of certain genes or the function of particular genomic regions consist

in the analysis of their linkage or association with the disease.

Linkage analysis is carried out in families with several individuals affected; the role of the gene in the formation of the susceptibility to the disease can be considered to be confirmed if allelic variants that are shared between the affected individuals are revealed. Low sensitivity is a drawback of this method; therefore, methods with greater statistical power that are based on the association analysis have recently taken center stage.

An association study is an attempt to find new statistical relationships between different events or verify the already known ones. The actual causes of these relationships are often beyond the knowledge or the experimental facilities of a researcher. However, once one has collected the statistics of occurrence of combinations of different observed outcomes, a conclusion can be made regarding the significance (which is assessed based on the probability of randomly obtaining the result observed) and intensity of these relationships. The association between a certain polymorphic genome region and a phenotypic trait is analyzed by comparing the distributions of its alleles and genotypes in the representative samples of individuals, which are formed with respect to the presence/absence of this trait and need to match in terms of sex, age, and ethnicity. The allelic variants under analysis can be localized in any DNA region, including the coding sequences (exons), introns, and promoter regions of the genes, where the transcriptional regulatory regions are frequently located, as well as the other DNA regions. In exon analysis, not only the nonsynonymous substitutions determining the changes in the amino acid sequence of the protein molecule being encoded are of interest, but also the synonymous substitutions, since they can affect the mRNA structure and stability, as well as the translation kinetics due to the use of different isoacceptor tRNAs.. However, it should be remembered that in addition to the direct relation between the investigated locus and the hereditary trait, the association may be based on linkage disequilibrium between the marker locus and the true locus of the disease, if these loci are located sufficiently close to one another.

The aim of association studies is to link the phenotypic traits that are significant for medicine with such characteristics as allelic variations in the genome, epigenetic modifications, effects of environmental factors, lifestyle, etc. The phenotypic traits that are of significance for personalized medicine typically include the onset of a disease, its course (clinical presentation, extent of injury in the systems of the organism, etc.) or the efficacy of therapy with a certain drug (the area of interest of phamacogenomics). In this review, we will focus on the association between the individual traits and the carriage of allelic variants of the genome. Identification of these associations enables one to assess the risk of disease development (susceptibility), predict the character of its course, and give a preference to certain methods of prevention, diagnosis and therapy based on the features of the individual genome.

The analysis of the associations between polygenic diseases and the combined occurrence of alleles of different genes remains a relatively poorly developed research area. This can be mainly attributed to the fact that any increase in the number of genes being analyzed results in an exponential growth in the number of combinations of their allelic variants, which makes any analysis using conventional exhaustive search techniques almost infeasible.

The present review is devoted to bioinformatic methods that search for such allelic combinations of different genes that are associated with the phenotypic traits of a polygenic disease, as well as to the methods for presenting and validating the results obtained. These methods (for the sake of brevity, they will be referred to as the polygenic analysis methods) are used to understand the cumulative effect of the genes and the nature of this effect. The association with the combination may be caused by the interplay of the phenotypic effects of the alleles on the phenotype; i.e., by nonlinear (epistatic) interaction between the genes. Alternatively, an allelic combination with a significant impact on the development of the trait can occur due to the summation of small independent subthreshold contributions of the alleles composing the combination. Both these cases will be discussed in the review.

ASSOCIATION STUDIES

The two major types of association studies (namely, cohort studies and case–control studies) differ in terms of the time sequences in which data is collected; therefore, they also differ in terms of the parameters that can be assessed based on monitoring. In cohort studies, a selected group of individuals is divided into two subgroups; individuals who have and those who do not have a certain indicator trait (e.g., subgroups of carriers and noncarriers of a certain genotype; smoker and nonsmoker subgroups). These subgroups are monitored during a certain time interval for the development of a trait that is of interest in terms of its prediction (the target trait); e.g., a disorder. This approach enables one to numerically assess the intensity of the contribution of an indicator trait to the development of the target trait via the ratio of probabilities of disease occurrence in the carriers and noncarriers of an indicator trait. This value is assessed using the relative risk (RR).

The case–control studies are a more common type of association studies. The sample here is divided into two subgroups: the individuals who possess and those who do not possess a target trait at an instance of study (e.g., affected and healthy individuals). The presence of indicator traits that possibly affect the emergence of the disease is assessed in each group. Nothing is known about the individuals who died before the launch of the study, thus the higher the disease mortality, the less accurate the estimation of the level of association in terms of RR. The odds ratio (OR) is typically used as a criterion for the degree of difference between the carriers and noncarriers of an indicator trait in case–control studies [1]. If absolute risk of the disease in noncarriers is low, the OR and RR values are close. The higher the risk, the larger the difference between OR and RR. OR is always higher compared to RR.

The results obtained using the case–control method can be distorted because of the ethnic heterogeneity of the groups being compared or due to the environmental factors that have not been taken into account [2]. The family-based methods (e.g., comparison of the affected and healthy brothers and sisters [3]) are less susceptible to distortion. However, there are requirements for the input data (pairs of affected and healthy immediate relatives, preferably siblings, are needed) that limit their applicability for obtaining reliable dependences. The transmission disequilibrium test (TDT) [4] imposes less strict requirements on the input sample. TDT is based on the analysis of the transfer of a marker allele from heterozygous healthy parents to an affected child. The data obtained are compared with the ones expected upon Mendelian inheritance; in the case of disequilibrium of the transfer of an allele, association between the allele and the disease is inferred. The AFBAC (affected family-based control) is another family-based method of association analysis in which the control group consists of a combination of the alleles of healthy parents that have not been inherited by the affected child (one allele from each parent) [5].

In the association analysis, both the predicted (dependent) and predicting (independent) traits are the categories that divide the sample into two classes (e.g., "affected" and "healthy" or "carrier" and "noncarrier"). It is convenient to present the intersections of the classes as a 2×2 table (contingency table). Its values are used to characterize the strength of association (OR) and its significance (p-value). The p-value is calculated using the Fisher's exact test that was proposed in 1922 and is still widely applicable [6].

If a trait is represented by more than two classes that can be ranked (e.g., using the disease severity scale assigned by the medical community), $2n$-field contingency tables (where n is the number of gradations of a trait) are compiled; the Goodman-Kruskal gamma test is used to assess the strength and significance level of an association [7]. If ranking makes no sense, either the Freeman–Halton test that extends the Fisher's test to more than two categories [8] or the χ^2 test [9] can be used.

METHODS FOR POLYGENIC ANALYSIS

All the approaches to multivariate analysis and to polygenic association studies in particular can be divided into two fundamentally different types: 1) the use of a reduced amount of input variables based on some *a priori* data and 2) complete analysis of all available variables. The reduction of the amount of possible variables in polygenic studies involves selection of several candidate genes to carry out the association analysis [10]. This approach allows one to considerably reduce genotyping costs and the space of analysis, thus reducing its complexity and the time required for computations. On the other hand, if a gene effect manifests itself only in combination with other genes and is not observed upon its individual consideration (i.e., there is no marginal effect [11], [12]), the probability that this gene will be selected as a candidate gene is extremely low, although its role may be significant. Genome-wide association studies (GWAS) [13–16] are currently gaining popularity due to the development of both computation and genotyping technologies. GWAS belongs to the second type of polygenic analyses, i.e., the analysis of all available variables.

When analyzing genome-wide data, one inevitably encounters many extremely rare alleles. Individual consideration of these alleles does not allow one to arrive at a conclusion regarding the impact of each allele on the disease. However, when considering the effect of several alleles altogether, the observed data can be sufficient to validate the assumption that they have a combined effect. In other words, data on each of the rare alleles is insufficient; however, that data should not be neglected, since association can be reliably established when data on several rare alleles is accumulated. This effect is known as the additive effect; it can also be observed for objects other than rare alleles. However, in the case of rare alleles, additive effect detection is one of the most promising methods for an association study. Correspondingly, the theory attributing the emergence of a large number of common diseases to the carriage of rare alleles is named CDRV (common disease / rare variant). This theory, which is currently gaining common acceptance, is an alternative to the CDCV (common disease / common variant) theory. A set of methods have been specially designed for the assessment of the additive contribution of rare alleles, e.g., the combined multivariate and collapsing (CMC) method [19], weighted sum statistics [20], and the gene burden test [21].

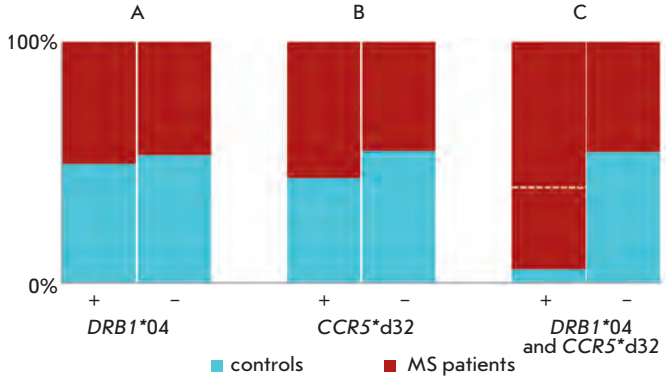

Fig. 1. Visualization of 2x2 tables of carriage by the MS patients and control group individuals of: alleles of the major histocompatibility complex HLA-*DRB1* (A), the chemokine receptor *CCR5* (B), and their combination (C) (based on data from [28] for ethnic Russians). Red areas correspond to the case; blue – to the control group. The ratio of the vertical fields reflects the distribution of carriers (+) and noncarriers (-) of *DRB1**04 (A), *CCR5**d32 (B) and a combination of *DRB1**04 and *CCR5**d32 (C). The horizontal dashed line in (C) corresponds to the expected ratio of the number of patients and controls among the carriers of the allele combination calculated under the assumption that the allele effects are independent.

The problem of correcting for multiple hypothesis testing becomes especially urgent upon polygenic analysis. This problem can be briefly formulated in the following way: an increasing number of tested hypotheses results in an increase in the probability of a random (including unlikely) outcome, which reduces the significance of the postulate that the statistical relationships observed represent specific non-random dependences.

If a number of comparisons used for studying the association of a phenotypic trait with several alleles of one highly polymorphic gene or upon simultaneous assessment of the role of several biallelic candidate genes is small (although not equal to 1), such an increase in significance is taken into account using the Bonferroni correction [22], which simply multiplies the corresponding *p*-values by the number of tests carried out. However, the Bonferroni correction turns out to be too conservative because of the underlying assumption that the tests are independent. A more accurate correction can be obtained using the Westfall–Young method [23], which does not imply independency and compares the best observation with the best results for the permuted samples. Another approach to this problem consists in assessing the false discovery rate (FDR) instead of the family-wise error rate (FWER) [24, 25].

Gene-gene interaction (epistasis) has recently turned into a widely discussed theme. This interest is to a significant extent due to the poor reproducibility of the results in assessment of the role of individual genes in the formation of susceptibility to polygenic diseases; in particular, in GWAS. There is a certain ambiguity in the terms "epistasis" and "epistatic interaction." They were originally used to denote complete masking of the effect of a polymorphism in one locus by the polymorphism of another locus; later, it was extended to refer to any other type of influence that certain polymorphisms have on the manifestation of other polymorphisms in the phenotype. The differences in interpretations of the term "epistasis," as well as the problems arising due to these discrepancies, have been thoroughly described in [26, 27].

The results of an analysis of the contribution of carriage of the HLA class II allele *DRB1**04 (*A*), an allele with a 32 nucleotide deletion in the chemokine receptor gene *CCR5* (*CCR5**d32) (*B*), and their combination (*C*) to the development of multiple sclerosis (MS), a typical polygenic disease, is shown in *Fig. 1* in the form of visualized 2x2 contingency tables (the experimental data were taken from [28]). In all the cases, MS patients and healthy individuals were divided into two classes based on carriage/noncarriage of the allele (homo- and heterozygotes with respect to this allele were not distinguished). The polymorphism of the *CCR5* gene was indeed biallelic (the deletion allele and the wild-type allele), whereas 18 groups of alleles of the *DRB1* gene were analyzed for this highly polymorphic gene. The group of noncarriers of the *DRB1**04 allele was made up of the carriers of the remaining alleles of this gene. It is clear from *Fig. 1C* that the carriage of the combination of *DRB1**04 and *CCR5**d32 is associated with the disease to a higher extent than might be expected based on the additive contribution of the constituting alleles. This fact can be construed as resulting from the epistatic interaction between the genes under consideration. This example is an illustration of the simplest type of polygenic analyses, when only the joint contribution of two alleles to phenotype formation is taken into account.

We have proposed the use of the odds ratios ratio (ORR) as a numeric measure of epistasis [29]. It is based on the concept that if at least two alleles within a combination do not interact with each other, the OR value for carriers of this combination will be made up of the product of the ORs of individual alleles within the combination. The product is regarded as the expected OR and compared with the observed OR. The more this ratio differs from unity, the stronger the predicted epistatic interaction between the genes.

The ORR value [29] can be used to analyze the interaction between two or more alleles. However, the

absence of a method to assess the confidence interval (CI) is a significant drawback here. The Synergy Factor (SF), a measure of epistasis described in [11], has contrasting advantages and drawbacks. The method for CI calculation has been designed for it; however, this value can be used for the analysis of the interaction between two alleles (or other binary indicator traits). Both values are the ratios between the OR observed for the allelic combination and the product of the OR observed individually for its components; however, the OR values are calculated using different methods. ORR compares the number of carriers and noncarriers of the indicator trait (whether this is an allelic combination or an individual allele) in patients and the control group, as is shown in *Fig. 1*. In the case of SF, the carriers of an allelic pair are compared with the carriers of neither allele, as well as the carriers of each allele constituting the combination that are also noncarriers of another allele. Identically to the situation with ORR, SF > 1 attests to a positive (mutually enhancing) interaction, whereas SF < 1 attests to a negative (compensatory) interaction. The SF value can actually be determined for more than two alleles; however, the result will depend on their order of combination to form complex traits. Thus, it is reasonable to use both of these assessments.

The available tools for an analysis of the cumulative effect of several genetic variables use various algorithms for data mining and are discussed below.

The conventional logistic regression, in which the coefficients of model terms at the second order and higher correspond to the interaction, is the most popular method [30]. Iterative simulation is required to use this method to search for the most closely interacting allelic combination, which weakens the statistical power of the method. The two-step variant implemented in GenABEL [31, 32] allows one to solve the problem of iterative testing by using the data on dispersion in individual loci to select the ones with a higher interaction probability. Various heuristic approaches, such as genetic programming [33], neural networks [34], pattern mining [35], dimensionality reduction techniques [36], and Markov Chain Monte Carlo (MCMC) methods (which include APSampler [37, 38], BEAM [39, 40], and logic regression LogicReg [41–43]) are used.

The association between carriage of any combination of alleles (or another indicator trait) with the phenotype can be assessed in the same manner as is done for one allele (trait). In other words, each combination can be regarded as a compound trait and can be characterized by the significance level of association and the RR or OR values. Numerous combinations are possible; therefore, the task of searching for the combinations characterized by the most significant association moves to the fore.

The multifactor association analysis can also be carried out using family-based data. There are multiallele and multilocus versions of TDT [4] (the method that is based on McNemar's test and was originally designed for biallelic single loci). Methods extending TDT to several allelic variants have been proposed by a number of authors. These methods include calculating the marginal homogeneity [44]; iterative grouping of alleles into two groups: the "allele under study" and "the remaining alleles," followed by the McNemar's test [45] and multiple testing correction; and calculation of the disequilibrium in the allele transfer using logistic regression [46], which is best suited for highly polymorphic loci. When carrying out the analysis simultaneously at several loci, methods involving the comparison of the actual child's genotype with all the theoretical genotypes that are possible for his parents are used [45, 47, 48]. Linkage disequilibrium between the loci under analysis is either calculated from the sample or taken from known data (e.g., from HAPMAP [49] in the FAMHAP [48, 50] software).

Some commonly used tools for polygenic association analysis are thoroughly discussed and compared below.

PLINK

PLINK freeware was developed at Harvard University [30, 51]; it is a large interrelated collection of various algorithms for the analysis of genotypic and phenotypic data, including the methods for polygenic analysis. PLINK has been used in a number of studies focused on genetic interaction (e.g., [52–55]).

One of the methods for the analysis of gene interaction in PLINK is based on the consideration of regression models [56]. The logistic regression model assuming that the probability of an event (in our case, disease) is described as a logistic function of a linear combination of independent variables (predictors) is used upon a binary outcome (e.g., "healthy–affected") [57]. The common linear regression of the same predictors is used for quantitative phenotypes (such as three degrees of arterial hypertension). In this case, independent variables are indicator functions that can assume either a 1 or 0 value, depending on whether a certain allele or genotype is present in the genome (or upon the presence of any other indicator trait). The analysis yields a set of regression coefficients for the indicator functions of the alleles and their combinations, and the levels of the statistical significance of the values by which these coefficients differ from zero. High significance of the difference of the coefficient corresponding to a certain combination of alleles from zero attests to their association. That is how the "PLINK -epistasis" test proceeds.

The "PLINK -case-only" is a simpler test on interaction; it verifies the correlation between carriage of several genotypes by patients. If the correlation between a genotype pair is high and their linkage can be excluded from consideration, it means that they interact. This test is based on an a priori assumption that the revealed correlation is typical only of the affected individuals. The two-step procedure verifying the presence of the correlation in the total sample does not include this assumption; however, the results provided by it may still be biased [58]. The key advantages of the PLINK software include its applicability for GWAS and a wide set of analysis tools, whereas its drawback consists in the limitations on the data format, since only biallelic markers can be used for work using this software.

MDR

The multifactor dimensionality reduction (MDR) algorithm has been widely used for mining polygenic associations in case-control studies [59–62].

At the first step, all data is randomly divided into two sets: the training set (e.g., 9/10 of the data) and the testing set (e.g., 1/10 of the data). A parameter characterizing the ratio between the number of affected and healthy individuals carrying the combination of alleles and genotypes is determined for each combination. The combinations are classified into categories (e.g., low-risk and high-risk combinations) based on the value of this parameter. Thus, a transition is made from the n-dimensional space of all single polymorphic loci and phenotypes to a two-dimensional space, where the risk level is one dimension, and the carriage of a certain allelic combination is another dimension. Among all combinations, there will be one having the lowest classification error in the training (Training Accuracy) and testing (Testing Accuracy) sets. The division into groups is repeated 10 times with the parameters of the random number generator varied. The cross-validation consistency is defined as the number of cross-validation replicates out of 10 in which that same model was chosen as the best model. The model is considered to be valid if its cross-validation consistency is at least 9/10.

In addition to a text representation of the results, the MDR software package includes dendrograms showing a pairwise interaction analysis, where the type of interlocus interaction is shown with different colors (from epistasis to independence); the bond length shows the interaction strength.

MCMC-BASED METHODS

The exhaustive search of all combinations (e.g., that used in MDR by default) loses its efficiency when the number of alleles under analysis increases because of the large number of possible combinations. The so-called combinatorial explosion occurs. Moreover, the statistical significance of the combinations obtained using this procedure becomes less obvious due to the multiple testing problem. On the other hand, simple gradient ("greedy") methods, which refine the intermediate result in a stepwise manner, frequently yield no adequate results at all, since they are prone to trapping in local optima rather than reaching the global ones.

There are different heuristic methods enabling one to mine the global optimum without using the exhaustive search procedure. The Markov Chain Monte Carlo (MCMC) algorithm is one such method [38, 40, 63–65].

The main idea in this method is that, as with the gradient search, it strives for a better solution than the already existing one. However, unlike the gradient search, it can also proceed to a worse solution with some probability; this probability decreases as the fit to the data for the proposed solution becomes poorer.

BEAM

In the search for associations, the BEAM (Bayesian Epistasis Association Mapping) algorithm [40, 66] is based on the fact that the distribution of genotypes in patients with disease-associated loci differs from that in the control group. The algorithm is aimed at classifying all the loci into loci that are not associated with the disease, loci individually associated with the disease, and the associated and epistatically interacting loci. The software uses the MCMC method to find the partition of the loci set into these three classes, which is the most probable one for the given genotypes and disease degrees. The loci are regarded as epistatically interacting if the joint distribution of their alleles/genotypes fits the data better than the distribution derived from the independent model (product of the allelic/genotypic distributions). BEAM can account for haplotype data in order to differentiate them from epistatic interactions.

Logic Regression

The Logic Regression algorithm uses MCMC to optimize the models of regression search for polygenic associations [43, 65]. The name of the method refers to the well-known logistic regression that solves a similar problem in a different way. The indicator functions of logic combinations (logic functions) of the presence of different alleles are used as predictors of logic regression; the combination of the optimal functions is determined using MCMC. The logic functions obtained show the type of allelic interaction.

APSampler

The logic of the analysis of polygenic data using the APSampler software [38] differs considerably from the previously described software packages, where

the predicted phenotypic trait can possess only two values (e.g., "affected" and "healthy"). The use of the nonparametric Wilcoxon test in APSampler software permits the analysis of data with more than two values of the target trait if ranking of the outcome is possible, allowing the use of a number of internationally recognized scales to define the groups for analysis. For example, in the case of stroke, such scales could be the degree of depression of consciousness, the initial severity of the disease, stroke outcome, which all have their own values and the number of values of at least three levels. The genetic pattern (i.e., the combinations of alleles and genotypes of different loci associated with a phenotypic trait) is the major object in the APSampler software for predicting an indicator trait. The pattern search is carried out using MCMC; several patterns being considered at each step simultaneously. The set of patterns is optimized from step to step in terms of the probability of all the patterns within the set being independently and simultaneously associated with a trait. The nonparametric Wilcoxon test is used to assess the probability of association of each pattern; the subsets being compared differ in carriage of only one pattern within the set. The algorithm includes two steps. The first step yields a list of patterns that have been encountered during the MCMC search and validates the findings by determining the significance of association for each pattern from the list using the Fisher's test (in the case of a dichotomous outcome) or the Goodman's and Kruskal's test [7], if there are more than two categories. At the second step, the software then repeatedly mixes the labels of the phenotypic trait and runs the search for associated patterns again. The reliabilities of association based on the results of these permuted runs provide the distribution of the reliabilities of the findings on the assumption of the null hypothesis of no association. This null distribution is used to validate the combinations obtained in the first step.

The *Table* summarizes data pertaining to the functional possibilities of the described software for polygenic association analysis. The data presented in the *Table* attest to the fact that the software proposed for polygenic analysis have considerably different functions. The software being compared can be used in different instances, depending on the available genetic and phenotypic data, on the content and format of the desired results, as well as the ability of a user to run the software at the level of the command line. One also needs to make allowance for the fact that the target result notably differs for different programs.

MDR is very convenient due to the presence of a user interface and graphical visualization of the results, including epistasis. It provides the obtained phenotype-associated loci and their combinations, whereas APSampler takes into account the direction of association, which is determined by carriage of alleles of the loci and their combinations. Both APSampler and MDR operate with polyvariant input traits, whereas the rest of the programs operate only with binary indicator traits. These two algorithms are also similar in the fact that they allow one to analyze epistatic interaction after association has been determined, whereas BEAM a priori divides all alleles into three groups: the ones with the marginal effect, the ones with epistasis, and those with no effects. The characteristics of combinations of loci, which are given by MDR, are statistically reasonable. However, their correlation with the association strength is not obvious. LogicReg provides no conventionally interpretable association values at all. APSampler and BEAM solve this problem by performing the Fisher's exact test for the association between the resulting indicator traits and the phenotype. In general, BEAM, PLINK, MDR, and LogicReg can be applied in basic research, including in studies devoted to gene interaction or for operation within a larger integrated software environment. However, they a priori do not have the necessary set of functions to solve such applied medical and genetic tasks as searching for the markers of susceptibility or searching for pharmacogenetic markers, for which the APSampler software can be used.

These five programs were used with the data taken from [29] in the user mode (i.e., with all default settings). BEAM found no associations with $p < 0.05$; LogicReg required additional data processing. The results of using APSampler, MDR, and PLINK are given in *Fig. 2*. It is clear that APSampler found both the combinations found by MDR and those found by PLINK; moreover, all the validated findings of APSampler have also been validated by at least one of these programs.

STUDIES PERFORMED USING APSAMPLER

A large number of studies using the APSampler software have been carried out since the first publication [38]; the authors participated in most studies due to the fact that at the initial stages of development, the software was relatively difficult to operate. This fact allowed them to upgrade the software according to the users' requests, supplement it with new features broadening the potential of validation [67], data management, visualization of the results and the help files elucidating the use and structure of the APSampler software. At the moment of writing, the software is open-source and can be used free of charge [37].

The authors used the APSampler software to analyze the cumulative effect of the alleles of a number of candidate genes on the development of multiple sclerosis (MS) [68], different forms of arterial hypertension

Table. Brief comparison of the potential of different software for polygenic association analysis

	APSampler [38]	BEAM [40]	LogicReg [43]	MDR [60]	PLINK [30]
Graphical user interface	-	-[1]	-[2]	+	+
Binary phenotype	+	+	+	+	+
Quantitative rank phenotype	+	-[3]	-	-	+
Working with missing data	+	+	+	-[4]	+
Statistical mining of combinations of particular alleles associated with phenotype	+	+	+	-[5]	+[6]
Assessment of the association for the established combinations using the Fisher's exact test	+	+	-	-	-
Validation procedure	+	+	+	+	-
Polyallelic loci	+	-[7]	-	+	-
Mining epistasis	+[8]	+	+	+	+
Graphical representation of epistasis	-[9]	-	-	+	-
Possibility of carrying out the association analysis for the allelic combination specified by the user	+	-	-	+	-[10]
Genome-wide analysis	-	+	-	-[11]	+
Possibility of using the command line to run software (e.g., on a server).	+	+	+	+	+
Available for UNIX	+	+	+	+	+
Available for Windows	+	+	+	+	+
Parallel computing	+	-[12]	-	-[11]	-

[1] There is a version of the BEAM software integrated into the GALAXY server application [83].
[2] The algorithm has been used in the software environment for statistical computing and graphics R [84].
[3] The software automatically divides the data into two categories using the mean value.
[4] The authors propose specialized software, MDR Data Tool [85], for filling in the missing values.
[5] The software finds the interacting and phenotype-associated loci rather than their alleles.
[6] Only pairwise mining is available.
[7] The number of alleles in each locus has to be equal.
[8] Despite the fact that mining of epistatically interacting alleles has not been claimed to be a specific function of the APSampler software, the experience of practical use of the software attests to the possibility of using it for mining epistasis.
[9] Perl software for graphical representation of epistasis has been designed [37].
[10] The haplotype-association analysis is proposed.
[11] A specialized software has been provided for this purpose [86].
[12] Specialized software PBEAM for parallel computing [87].

[69–71], myocardial infarction [72], ischemic stroke (IS) [73, 74], and hemorrhagic stroke [75]. The studies were carried out in compliance with the principle of ethnic homogeneity in Russians or Yakuts. The Yakut population is of particular interest in terms of ethnogenomics, since the founder effect and a certain geographic and cultural isolation are observed in it [76]. APSampler was also used in pharmacogenetic studies of MS for the investigation of the relationship between the genetic status in patients and the efficacy of treatment with immunomodulatory drugs, interferon beta (in Irish patients, [67]) and glatiramer acetate (in Russian patients, [29, 77]).

In most of the aforementioned studies, the group of nonrelative patients was compared pairwise with the control group of unaffected nonrelative individuals, which was similar to the affected sample in terms of their ethnicity, sex ratio, and the mean age. Two groups of patients with clinically heterogeneous forms of the same diseases (e.g., arterial hypertension with

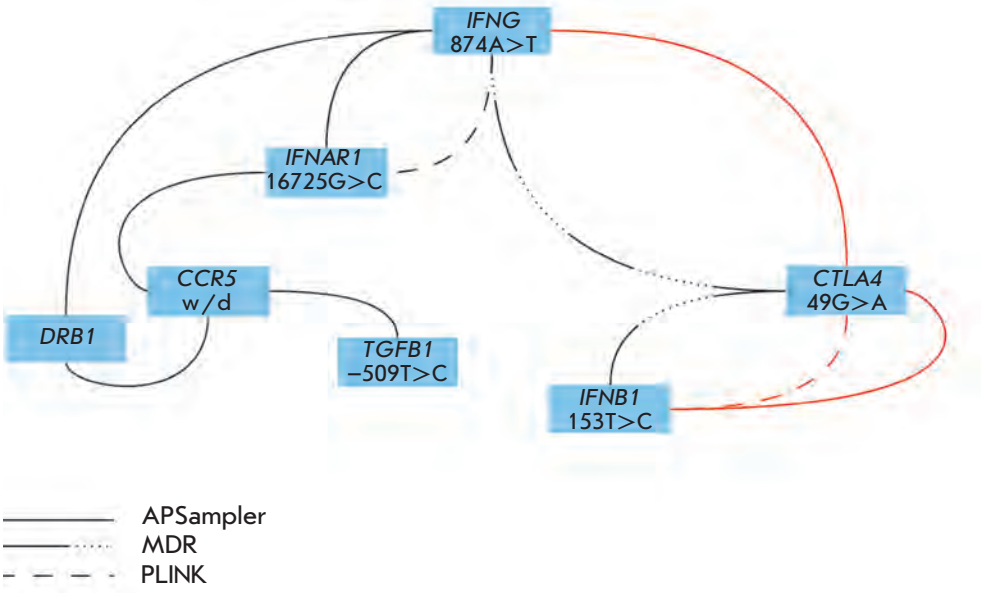

Fig. 2 Search for biallelic combinations of immune response genes associated with the response to treatment of MS with glatiramer acetate (based on data from [29] for the ethnic Russians) with APSampler, MDR, and PLINK. APSampler [38] finds all biallelic markers found by the other software as well as identifies additional combinations. The red color marks the findings that have been validated by permutations in APSampler ($p < 0.1$) or MDR cross-validation (CVC > 8/10).

and without hyperaldosteronism [69]) were compared in some cases. When studying the genetic susceptibility to arterial hypertension preceding the development of IS, the patients were at first divided into two subgroups in accordance with the hypertension level. The 2×4 contingency table was subsequently used to find such allelic combinations among identified ones carriage of which is characterized by monotonous increase from normotonics to third degree hypertensive patients [71]. In pharmacogenetic studies, the patients responding and not responding to treatment were also compared pairwise using the "comparison of extremes" approach.

The candidate genes were selected based on the existing conceptions of participation of their protein products in the processes involved in the disease pathogenesis. When analyzing the genetic susceptibility to cardiovascular diseases, the following genes were selected: the ones whose protein products participate in inflammation, the genes of hemostasis, transport, and lipid metabolism systems, the genes of the renin-angiotensin-aldosterone system, and some other genes. For MS, the candidate gene products participate in the development of the immune response and chronic inflammatory process. Polymorphic regions (mostly, single nucleotide polymorphisms, or SNPs, being of interest in terms of their function; i.e., knowingly affecting the amount or property of the encoded protein product) were usually typed in these genes. The joint contributions of 10 or more polymorphic markers have been analyzed in relatively small samples consisting of no more than 500 individuals. Although this sample size, which is typical of Russian studies, cannot be compared to the size of the groups formed by the international consortiums, we have found highly significant associations between allelic/genotype combinations and the phenotype under study using the APSampler software. This can be illustrated by the data pertaining to an association between the triallelic combination *FGB**−249C + *APOE**ε4 + *CMA**−1903A and the level of arterial hypertension preceding the development of IS in the Yakut population (*Fig. 3*). A monotonous rise in the carriage frequency of the named triallelic combination was observed in a sample consisting of 115 patients: from 0 in normotonics to 47% of the total number of individuals in the subgroup of third degree hypertensive patients; the *p*-value assessed based on the Fisher's test in the 2x4 contingency table was 0.0003. In this case, a vivid example of the effect of the joint contribution of the genes encoding components of three different key systems of the homeostasis, namely, hemostasis system (*FGB*), the lipid metabolism system (*APOE*), and the renin-angiotensin-aldosterone system (*CMA*), to the development of arterial hypertension has been observed. Thus, the disease is most likely to emerge with the summation of the independent contributions of individual genes.

The reason for such a high information value upon a rather modest amount of experimental data can be attributed to the advantages provided by the ethnic and clinical homogeneity of the groups used in the analysis, whereas groups consisting of tens of thousands of patients from different countries and patient care institutions, which are formed within the framework of consortiums, usually fulfil the homogeneity requirements in terms of neither ethnicity nor clinical presentation. This fact may smooth their genetic deviations from the control group. However, the major reason for the

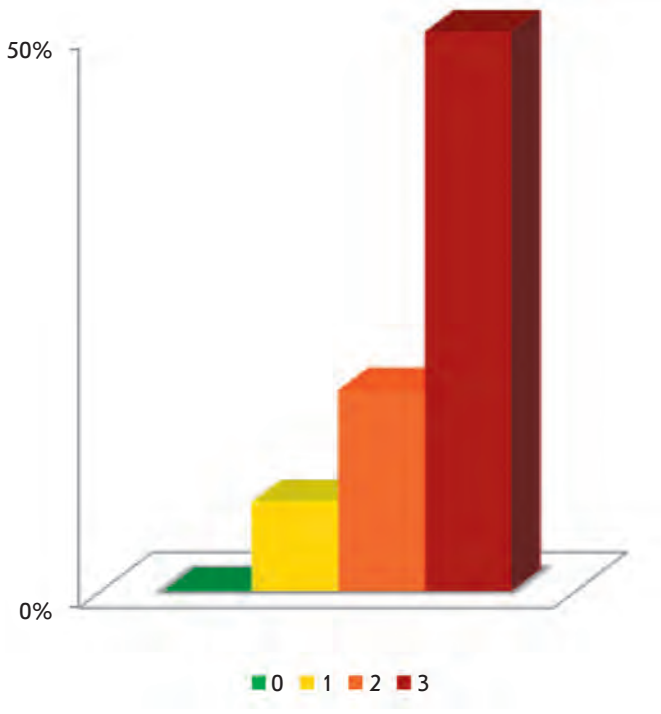

Fig. 3. Carriage of the triallelic combination *FGB**−249C + *APOE**ε4 + *CMA**−1903A, which was found with APSampler in Yakut ischemic stroke patients with different levels of the preceding blood pressure [71]. 0 – normotonics, 1–3 – first, second and third degree hypertensive patients, respectively, according to the criteria of 2003 ESH/ESC [82]. Carriage is represented as a percentage of the total number of patients in the subgroup.

high information value of the results obtained using the APSampler software can presumably be attributed to the high statistical power of the analysis. Without going into the details of what underlies this phenomenon, one can summarize by saying that identification of the association of the alleles/genotypes of individual genes during the analysis of any studied disease was a rare event, whereas phenotype-associated combinations of two-four alleles were found in almost all cases. It is appropriate to note here that both positive and negative associations could be observed; a differently directed effect of the alternative alleles has been successfully revealed for most, but not all, cases.

The association of MS with the *DRB1**15 allele of the major histocompatibility complex [78, 79], with the microsatellite marker TNFa9 [80] and with the biallelic combination of *DRB1**04 and *CCR5**d32 [28] (see *Fig. 1*) in the Russian population was previously demonstrated without the use of the APSampler algorithm and reproduced in an independent sample using the APSampler algorithm [68]. The replication of the data pertaining to the association of these genetic factors with the development of MS complies with the criteria widely accepted across the world's scientific community for validation of the results and attests to the software's efficiency.

Based on the aforementioned observations, the concept of the minimal set (combination) of alleles as a genetic risk factor that is revealed in a certain study has been formulated [68]. This means that any subset of this set is characterized by a lower significance of association. Thus, we have identified [68] two MS-associated triallelic combinations comprising alleles of the polymorphic regions of the *DRB1*, *TGFB1*, *CTLA4*, and *TNF* genes. The differences between the groups of affected and healthy individuals in the carriage frequencies of the biallelic combinations and of the individual alleles within the triallelic combinations 1 and 2 did not reach the significance level ($p < 0.01$). It is important to note that the subgroups of individuals carrying the MS-predisposing combinations 1 and 2 did not overlap and corresponded to approximately 5 and 9% of MS patients, respectively, whereas they were not present in the control group. Thus, identically to the case of classical monogenic dominant disorders, all the carriers of either combination in our sample turned out to be affected. Identical results were obtained in our other studies. In either case, the minimum set of alleles is a compound genetic marker of the polygenic disease or of another phenotype.

We attempted to solve the question pertaining to the type of interaction between the alleles within the gene combination (epistatic or additive) in a pharmacogenetic study where the association between the efficacy of treatment of MS patients with the immodulatory drug glatiramer acetate and the allelic polymorphisms in a number of the immune response genes was analyzed [29]. The carriage of allelic combinations of four genes (*DRB1**15 + *TGFB1**−509T + *CCR5**d + *IFNAR1**16725G) exhibited a 14-fold increase in the risk of ineffective response to glatiramer acetate therapy (OR = 0.072 [CI = 0.02−0.28]; $p = 0.00018$); the association withstood permutation testing ($p_{perm} = 0.0056$), which had been included into the software by the time the study was conducted. The triallelic combination (*DRB1**15 + *CCR5**d + *TGFB1**−509T) differed negligibly from the tetra-allelic combination as a marker of treatment inefficacy, whereas the association between all the other components of the tetra-allelic combination and treatment inefficacy was considerably weaker. Graphical visualization (the Venn diagram) of the character of the interaction between different components of the "unfavorable" allelic combination (*DRB1**15 + *TGFB1**−509T +

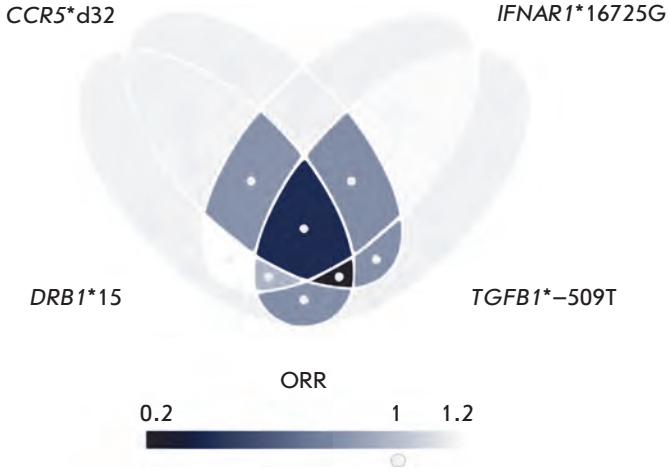

Fig. 4. Venn diagram describing the possible interaction between the components of the *DRB1**15 + *TGFB1**–509T + *CCR5**d + *IFNAR1**16725G combination, which is negatively associated with the efficiency of the treatment of MS with glatiramer acetate, as identified using the APSampler software [29]. Each of the four ellipses in the diagram corresponds to one of the four alleles in this combination. The intersections of the ellipses correspond to all possible combinations of the four alleles, color intensity reflects the ratio of the observed OR to the expected OR (ORR), in accordance with the gradient scale provided below. The gray areas corresponding to individual alleles, as well as the small circles, correspond to the reference ORR, which is equal to 1. The more the color of an area differs from gray, the stronger the epistatic interaction of the alleles represented by the area. The values of the expected OR are calculated for each combination as a product of the ORs of the individual alleles corresponding to the overlapping areas.

*CCR5**d + *IFNAR1**16725G) is given in *Fig. 4*. For the triallelic combination (*DRB1**15 + *TGFB1**–509T + *CCR5**d) ORR was 0.2 (i.e. it was fivefold lower than 1) and remained unchanged after the addition of the *IFNAR1**16725G allele. We regard these data as evidence of the epistatic interaction between the alleles of the *DRB1*, *CCR5*, and *TGFB1* genes.

Unexpected data on epistatic interactions upon formation of genetic susceptibility to IS in the Russian population were obtained in [73]. The analysis using the APSampler algorithm has revealed the protective biallelic combinations (*IL6**–174C/C + *FGA**4266A) and (*IL6**–174C/C + *FGB**–249C), which were associated with IS slightly more significantly than the protective genotype *IL6**–174C/C by itself and had practically the same OR value (0.32–0.35). Each of the alleles within these combinations (*FGA**4266A or *FGB**–249C) upon joint carriage of the *IL6* G allele, which is the alternative to the *IL6**–174C/C genotype, "neutralized" its significance as a risk allele by reducing both the significance levels and the OR values (from 2.9 to 1.9–2.1). In other words, association between IS and combinations of the alleles/genotypes of *IL6*, *FGA* and *FGB* has been observed; *IL6* played a key role, whereas the *FGA* and *FGB* genes had a modulating function. This observation presumably attests to the fact that the *FGA* and *FGB* genes contain interleukin-6-sensitive elements, which are capable of binding to STAT3 (the major transcription factor transmitting signals from the interleukin-6 receptor to the nucleus) [81].

CONCLUSIONS

Searching for polygenic combinations associated with a phenotypic trait (i.e., composite genetic markers) is an adequate analysis tool for studying polygenic diseases. The statistical methods enabling this type of analysis are currently rapidly being developed.

In accordance with all the aforementioned facts, composite genetic markers can result from epistatic interaction between components or be of additive nature. Taking into account the complexity of various cumulative effects and their direction, one can claim that identification of a reliable composite marker (even if it carries a small number of components) is an important step in understanding the etiopathogenesis of the disease. Indeed, such a marker may attest to the key link in a complex regulatory network of interactions between biological macromolecules. ●

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Cardiological Biopharmaceuticals in the Conception of Drug Targeting Delivery: Practical Results and Research Perspectives

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ABSTRACT **The results of the clinical use of thrombolytic and antithrombotic preparations developed on the basis of protein conjugates obtained within the framework of the conception of drug targeting delivery in the organism are considered. A decrease has been noted in the number of biomedical projects focused on these derivatives as a result of various factors: the significant depletion of financial and organizational funds, the saturation of the pharmaceutical market with preparations of this kind, and the appearance of original means for interventional procedures. Factors that actively facilitate the conspicuous potentiation of the efficacy of bioconjugates were revealed: the biomedical testing of protein domains and their selected combinations, the optimization of molecular sizes for the bioconjugates obtained, the density of target localization, the application of cell adhesion molecules as targets, and the application of connected enzyme activities. Enzyme antioxidants and the opportunity for further elaboration of the drug delivery conception via the elucidation and formation of therapeutic targets for effective drug reactions by means of pharmacological pre- and postconditioning of myocardium arouse significant interest.**

KEYWORDS **drug targeting delivery; protein bioconjugates; thrombolytics; antithrombotic agents; molecular size of bioconjugates; density of molecular targets; enzyme connected antioxidants; cell adhesion molecules; pharmacological pre- and post-conditioning of myocardium.**

ABBREVIATIONS **EC-SOD – extracellular superoxide dismutase; CAT – catalase; EMA – emergency medical aid; SOD – superoxide dismutase; CHS – chondroitin sulphate; SOD-CHS-CAT – covalent bienzyme superoxide dismutase chondroitin sulphat–catalase conjugate; ECG – electrocardiogram.**

INTRODUCTION

Popular belief held that drugs can be delivered to a focus of pathological lesion via Paul Ehrlich's 'magic bullets' [1]. This notion underpins the conception of drug-targeting delivery into the organism [2]; protein conjugates obtained via chemical and biological synthesis being among its objects [3, 4]. Thrombolysis became a significant area of the targeted extracellular application of these conjugates [5]. Successive decades have presented many opportunities for the results of the application of these agents (biopharmaceuticals) in thrombolytic and adjunctive therapy to be evaluated, as well as serving to outline the necessary directions for further biopharmacological innovations. This analytical review comprising data from PubMed, SCOPUS, Index Medicus/MEDLINE, and other databases, as well as the data of the Medical Research Library of the Russian Cardiology Research and Production Complex (Moscow), is devoted to the aforementioned issues.

NEW DRUGS FOR THROMBOLYTIC THERAPY

The high prevalence of cardiovascular diseases is a well-known fact. In the Russian Federation, deaths due to these diseases account for more than half of the number of deaths [6]. Serious and overwhelming symptoms of cardiovascular disorders may appear either gradually or rather suddenly. The emergence of retrosternal pain (ischemic discomfort) is cause to suspect the progression of an acute coronary syndrome (ACS) [7]. Recording an electrocardiogram (ECG) enables one to reveal a mural or occlusive (completely blocking the vascular lumen) thrombus, on the basis of the ST-segment level in the ECG. The clinical diagnosis can be refined by determining the blood levels of creatine kinase (the MB isoform) and/or troponine (T or I) [7, 8].

Urgent thrombolytic therapy is required for patients with acute myocardial infarction.

Streptokinase (1.5 million IU for intravenous infusion for 30–60 min), alteplase (recombinant tissue plasminogen activator, 15 mg of the drug is given in the form of intravenous bolus (injection) followed by a 0.75 mg/kg infusion for 30 min and an additional 0.5 mg/kg infusion for 60 min; the total amount of the drug administered being less than 100 mg), tenecteplase (a mutant form (mutein) of a tissue plasminogen activator; 30–50 mg of the drug is administered intravenously depending on the patient's body weight – 60 and over 90 kg), and purolase (prourokinase, 2 million IU of the drug is given intravenously followed by infusion of 4 million IU for 30–60 min) are used as thrombolytic agents in Russia. According to the standards of medical care in Russia, alteplase (trade name Actilyse), streptokinase and prourokinase (purolase) (i.e., the thrombolytics with a bolus-infusion scheme of administration) are prescribed to patients with acute myocardial infarction (by order of the Ministry of Healthcare and Social Development № 582 dated August 2, 2006). The use of such bolus agents as tenecteplase (trade name Metalyse) currently being promoted on the Russian pharmaceutical market has thus far been sporadic.

It should be noted that streptokinase (SK), the protein product of β-haemolytic streptococci, along with urokinase (UK), belongs to the first generation of plasminogen activators, whereas the tissue plasminogen activator (t-PA) and prourokinase (u-PA, pro-UK) belong to the second generation [9]. It is now possible to produce plasminogen activators in the form of nonglycosylated derivatives (Actilyse, purolase), owing to genetic engineering (*Fig. 1*). At present, tenecteplase (TNK-tPA, Metalyse) and reteplase (r-PA, Retavase) are third-generation clinically used plasminogen activators. The promotion of these pharmaceuticals toward clinical application emphasizes the specific features of modern biopharmacology and biotechnology, such as the significant amount of time required to design the pharmaceuticals and high cost of the resulting product (the price of an effective dose of the preparation is 2,000–3,000 USD). A number of new forms of plasminogen activators (anisoylated plasminogen/streptokinase activator complex – APSAC, lanoteplase – n-PA (mutant t-PA, mutein t-PA)) have not been widely used, because of a number of therapeutic indices; the alternatives (r-PA, TNK-tPA, purolase) are increasingly used.

Retavase (r-PA) is recommended for sequential double-bolus administration to patients with acute myocardial infarction. This pharmaceutical is a nonglycosylated t-PA with several domains (the finger-like domain, and a domain which is homologous to the epidermal growth factor, and the kringle domain 1) deleted from its molecule [10]. As a result of this modification, r-PA is capable of swift action, remaining in the bloodstream for a considerable time, and causing a lower depletion of the level of haemostatic blood proteins (systemic action) in comparison to the parent form of t-PA. Tenecteplase has a similar positive action (it is characterized by poorer suppression of the activity of plasminogen activator inhibitor type 1 and by a reduced contribution to fibrinogenolysis). The combination of mutations in the t-PA molecule (T103N, N117Q, KHRR(296–299) AAAA substitutions) was responsible for the emergence of the aforementioned properties and enabled the design of a pharmaceutical that is efficient after a single-bolus intravenous administration to patients with acute myocardial infarction [11, 12]. Targeting of the r-PA and TNK-tPA derivatives to thrombus (implementation of the targeting drug delivery concept) was successfully performed not via the use of an external vector (e.g., antifibrin monoclonal antibodies or their fragments) but by selection of the mutant forms of t-PA and the isolation of its domains. A normal t-PA molecule consists of several structural domains [9]: a fibronectin finger-like domain responsible for the high affinity binding to fibrin; the domain homologous to the epidermal growth factor which ensures the receptor binding to hepatic cells and accelerated clearance; and two kringle domains, one being essential for the binding of the domain 1 to endothelial cell receptors, and the second being responsible for low-affinity binding of domain 2 to fibrin. In addition, t-PA comprises the proteinase domain with plasminogen-specific activity. The proteinase domain contains the binding region of plasminogen activator inhibitor type 1. The molecular weight of this single-chain glycoprotein is ~ 64 kDa. Tenecteplase (Metalyse) and reteplase produced from it using genetic engineering techniques facilitate the further development of thrombolytic therapy (*Fig. 1*). Thus, emergency medical aid (EMA) teams staffed with medical or nursing personnel performed pre-admission bolus thrombolysis using tenecteplase according to the improved two-stage regimen (using ECG cardiotelemetry) [13]. The efficacy of the thrombolytic therapy was considerably determined by the symptom-to-needle time; its average value being 1 h 58 min. The door-to-needle time (from the time when the emergency medical aid team arrived to the injection) was 16 min. The noticeable reduction in time up to the beginning of therapy helped in the efficient treatment of 51.5% of the patients (one of the criteria was a decrease in the ST segment in ECG by more than 50% in the lead characterized by the greatest rise). The so-called interrupted myocardial infarction (when the ST segment decreases to the ECG isoline) was observed in 18.2% of

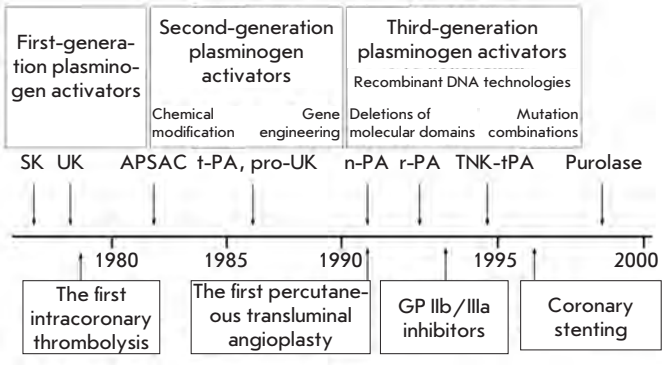

Fig. 1. Chronology of the emergence of plasminogen activators of different generations and angioplasty means (balloons, guidewires, stents) of reperfusion therapy in clinical practice.

the patients. In the presence of the EMA team, the lethality was 1.5%, and the lethality was 3.0% and 1.5% during the first 1 and 30th days, respectively. Thus, the lethality indicators did not increase and provided a significant decrease in the time taken to start the treatment due to the thrombolytic therapy performed by EMA teams, which can considerably improve the prognosis in patients with an acute myocardial infarction with a rise of the ST segment in ECG [13]. With allowance made for the necessity of settling the question regarding the price of tenecteplase pharmaceuticals, instrumentation of the EMA teams, organization of cardiac telemetry centres, and personnel training and education, this approach to providing the earliest thrombolytic treatment appears to be efficient and to undoubtedly help in the struggle against acute cardiovascular diseases.

FORMATION AND DEVELOPMENT OF REPERFUSION THERAPY

The formation of thrombolytic therapy has revolutionized the treatment of acute myocardial infarction. It is noteworthy that as recently as the middle of the XXth century, the level of lethality amongst hospitalized patients was 30–40 %, a figure that has been reduced by almost 50% (14–17%) due to the increased use of intensive therapy wards [14]. The development of thrombolytic therapy has contributed to a considerable reduction in the mortality rate (to the level of 6–8%). The demand for further reduction of lethality levels due to myocardial infarction has fuelled the need for the establishment and improvement of reperfusion therapy, which is based on the use of thrombolytic drugs, methods and tools of transluminal balloon angioplasty, as well as coronary stenting (*Fig. 1*). The efficiency of intervention methods for bloodstream recovery via mechanical action have appeared to be rather high; however, some limitations still exist. According to the European Society of Cardiology Guidelines, emergency medical aid is to be urgently rendered to patients with acute cardiovascular diseases. "Five doors" are to be quickly passed through: the house doors (1), consultation/examination by a general physician (2), emergency medical aid manager (3), rendering emergency medical aid and transportation of a patient by the EMA team (4), and admission to a hospital/vascular centre (5) in order to receive qualified treatment. The beginning of therapy is delayed because of slow requests for medical aid and by heavy traffic, which can determine different time intervals (from symptom manifestation to the beginning of therapy) for the selection of the treatment strategy [15]. Thrombolytic therapy can be performed by an EMA team during the pre-hospital stage [13]. In the future, it will be possible to provide self-aid thrombolytic therapy even at home. However, in spite of the fact that the vast majority of organizational problems are being solved slowly and irrespective of the current situation in the financial and medical spheres in Russia, thrombolysis and angioplasty are complementary rather than alternative methods [16]. This approach is determined by the existence of hospitals equipped with tools for vascular angioplasty and stenting, as well as the proximity of the patient to them; the actions of the EMA teams; and timely thrombolytic therapy (in particular, when percutaneous coronary intervention is infeasible). The combination of thrombolysis and angioplasty is used in a number of cases. The latter approach seems to have a higher potential at the current level of development of the Russian healthcare system. In general, the problems relating to patient education, the improvement of the organization of cardiology aid (the "five doors" approach) and its means (the design of new stents and thrombolytics) remain pressing. However, the diversity of reperfusion therapy procedures and the high costs associated with this type of therapy have reduced the attractiveness of the sector to investors, a point attested to by the results of current biomedical research, which is focused on thrombolytic pharmaceuticals.

CURRENT RESEARCH IN THE FIELD OF TARGETED THROMBOLYTICS

The investigation of new thrombolytic agents [5, 9], whose intensive research began as recently as 15–20 years ago, has now considerably narrowed. The construction of targeted bioconjugates is based on the vector (which determines the recognition and binding to the target) and drug (ensuring the therapeutic effect)

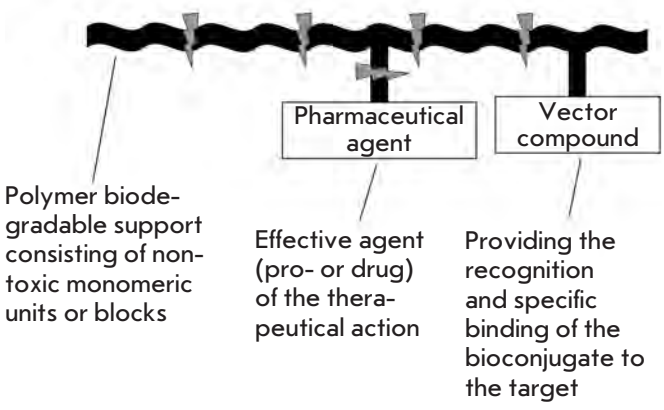

Fig. 2. Schematic representation of the bioconjugate model for drug targeting delivery in the organism. The vector and drug components of the conjugate are covalently linked to the biodegradable matrix of a polymeric carrier.

components bound to the biodegradable carrier matrix (*Fig. 2*). This model is currently being developed not as intensively as earlier. Antifibrin antibodies (or their fragments), fibrinogen (as a vector and carrier) or its components, as well as the complementary action of the combination of different t-PA and u-PA forms on the thrombus are no longer used. Vascular endothelial injury markers are now used as thrombotic leisure determinants [4]. Of course, their content in the blood and in other cell types that are available in the bloodstream should be low. Moreover, the density of their expression on endothelium should be sufficient for binding, which is required to achieve therapeutic effects and not result in negative side effects. Thus, the bioconjugate of urokinase with monoclonal antibodies (RE8F5) against the surface membrane protein of capillary pulmonary endothelium, which were bound via 4-succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene (SMPT) with retention of 85% of the initial urokinase activity was obtained for use in patients with pulmonary embolism [17]. With regards to the model for pulmonary embolism, this conjugate potentiated thrombolysis by 12–16 times, in comparison to urokinase and Retavase, without systemic activation of plasminogen and depletion of the fibrinogen level. Meanwhile, the covalent binding of the conjugate components via the disulphide bond (at its surface localization on the conjugate molecular structure) casts doubts on the conjugate's stability and the potential for its practical development. The approach in the prevention of cerebrovascular thromboses appears to be of considerable interest [18]. The association of biotinylated t-PA with biotinylated erythrocytes via streptavidin induced rapid and long-lasting reperfusion in mice with cerebral thrombosis, as opposed to the effect of t-PA administered alone even at a dose tenfold higher [19]. The resulting adduct was characterized by an increased bloodstream half-life, and it was capable of lysing fresh thrombi (but not the old haemostatic plugs). In addition, the adduct exhibited a weaker response to the action of plasminogen activator inhibitor type 1 [20]. The erythrocytes proved to be efficient carriers of t-PA for thrombosis prevention; however, *ex vivo* modification was required for binding to t-PA, prior to introduction into the organism. This complicated modification can be avoided via the use of antibodies against erythrocyte membrane proteins. Thus, glycophorin A occurs on the erythrocyte surface. The use of the anti-glycophorin A single-chain antibody (scFv) within the recombinant protein form with low molecular weight single-chain urokinase, selectively activated by thrombine (scu-PA-T) [21] or with t-PA mutein (the kringle domain 2 and the protease domain) [22], ensures their binding to erythrocytes (40–95%) and considerably enhances the circulation time in the bloodstream (~35% of the dose administered remains in the bloodstream after 48 h). According to the results of these studies, the preventive delivery of various forms of plasminogen activators to erythrocytes can be considered as a new approach to the clinical prevention of thromboses, when the risk of vascular occlusion is high.

The low molecular weight recombinant single-chain urokinase plasminogen activator (lmw-scu-PA), fused with a single-chain variable antibody fragment (scFv) against a platelet endothelial cell adhesion molecule (PECAM-1), was obtained [23]. It was demonstrated, using the fused protein as an example, that cell adhesion molecules located on endothelium can act as targets for drug delivery. The recombinant form of prodrug lmw-scu-PA-scFv was bound specifically to the cells expressing PECAM-1 [23] and became a fibrinolytically active tcu-PA form after the cleavage of the Lys158–Ile159 bond in the urokinase fragment (lmw-scu-PA) by plasmin (at the sites of thrombus formation). Following the intravenous administration, the drug accumulated in the lungs of wild-type mice (but not those of the PECAM-1 knockout-mice) and was vastly more efficient than that exhibited by lmw-scu-PA. The drug was capable of lysing pulmonary emboli, as well as rapid removal from the bloodstream. These facts attest to the high potential of using fused proteins based on cell adhesion molecules and plasminogen pro activators to prevent thrombosis [4, 23].

A research group from the University of Pennsylvania (United States) led by V.R. Muzykantov [4, 19–23] is focusing on the sequential study of bioconjugates with a targeted fibrinolytic effect. Other research groups have either changed the direction of their studies or

have released data sporadic ally[17, 18]. The questions relating to the immunogenicity of the recombinant forms, their applicability in acute lesions, and the development of adverse reactions remain open. The fact that tenecteplase (Metalyse) and reteplase (Retavase) have appeared as pharmaceuticals instills hope for successful developments.

TARGETED ANTITHROMBOTIC DRUGS IN CLINICAL PRACTICE

A large variety of antithrombotic drugs contribute to the stabilization of the effects of reperfusion therapy. These drugs include those with standard antithrombin effects (heparin, low molecular weight heparin (enoxaparin)), direct thrombin inhibitors (bivalirudin, dabigatran), factor Xa inhibitors – direct (apixaban, rivaroxaban, and otamixaban) and indirect ones (fondaparinux) [24], protease-activated receptor 1 (PAR-1) inhibitors, blockers inhibiting thromboxane A_2 (TXA_2) production (acetylsalicylic acid etc.), and $P2Y_{12}$ receptor antagonists (clopidogrel, prasugrel, ticagrelor, cangrelor, etc.) [25]. The application of glycoprotein IIb/IIIa antagonists [26] for the inhibition of platelet aggregation during angioplasty in patients with acute coronary syndrome [27] is of interest from the viewpoint of the conception of drug targeting delivery (targeted to protein derivatives). Clinically available drugs are shown in *Fig. 3*. It should be noted that tirofiban and eptifibatid, which are currently moving towards certification on the pharmaceutical market, are considerably cheaper compared to abciximab and monafram (ruciromab being its non-patented name in Russia). The peptidomimetic tirofiban is a low-molecular-weight compound of non-peptide nature; eptifibatide is a small peptide. In contrast, abciximab consists of the Fab fragment of the recombinant chimeric antibody from the variable domains of the mouse anti-glycoprotein IIb/IIIa monoclonal antibody 7E3 and the constant domains of human immunoglobulin G; monafram is an $F(ab')_2$ fragment of anti-glycoprotein IIb/IIIa monoclonal antibodies. At the time of writing, competition for the extended use of the aforementioned drugs in clinical practice still exists. It should be noted that the antibody nature of abciximab and monafram enables the efficient recognition of these drugs by glycoproteins IIb/IIIa and binding to thrombocytes, which inhibits their aggregation.

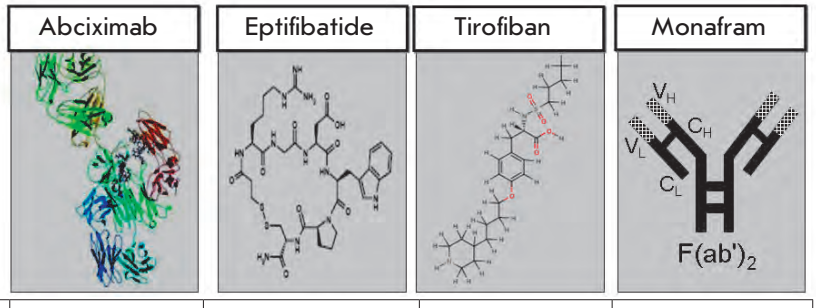

Fig. 3. Molecular form and basic parameters of glycoprotein IIb/IIIa receptor blockers.

	Abciximab (ReoPro) Elli Lilly	**Eptifibatide (integrilin) Cor Therapeutics/ Shering Plough**	**Tirofiban (aggrastat) Merck**	**Monafram (ruciromab) Russian Cardiology Research and Production Complex, Ministry of Healthcare and Social Development of the Russian Federation**
Type	Antibody	Peptide	Non-peptide	Antibody
Molecular weight, Da	~50 000	~800	~500	~100 000
Binding to thrombocytes	h	s	s	h
$t_{1/2}$ in plasma	min	2.5 h	3 h	< 3 h
Time of 50% platelet function recovery	12 h	2-4 h	~4 h	> 24 h
Relationship with the other integrins	α5β3 Mac-1	–	–	–

Among efficient antithrombotic pharmaceuticals, fragments of protein molecules rather than the full-size molecules (identical to the case of third-generation plasminogen activators) are of interest for clinical practice [28]. In terms of a number of pharmacological properties, compounds with a molecular weight lower than 400 Da appear to be better compared to the larger types. Moreover, the lipophilicity of a compound under study is typically increased for the purpose of increasing the efficiency of a derivative and the specificity of its interaction with cell receptors, or ease of penetration through the membrane. However, this makes the compound less soluble. The compound becomes metabolically stable, serious adverse effects manifest themselves abruptly, and the level of toxicity increases (as follows from the results of the comparison of the toxicity of the compounds investigated in 1991 and 2000). The investigation of an enormous number of potential drugs has been discontinued for this reason [28].

Four levels of organization are conventionally recognized in the protein structure: the primary, secondary, tertiary, and quaternary structures. However, other gradations also exist [29]. According to them, the primary (amino acid sequence), secondary (α-helix, β-structure, etc.), supersecondary (ensembles of secondary structures interacting with each other: e.g., supercoiling of α-helices, i.e., coiling of two α-helices around one another) structures, structural domains (in particular, those determined by analyzing the electron density maps and corresponding to a 2.5-nm diameter globule, which satisfies the principle of easy coiling of a protein chain), globular proteins, and aggregates can be distinguished in a protein molecule. Nowadays, the priority in the design of biopharmaceuticals for cardiological purposes is on protein domains and their various combinations. However, this fact does not eliminate the necessity for a thorough investigation of their immunogenicity and toxicity.

DEVELOPMENT OF DERIVATIVES FOR COMBINED ANTIOXIDANT THERAPY

The other approaches directed towards the retaining and enhancement of the effects of reperfusion therapy are to a larger extent associated with research studies as opposed to clinical ones. The antioxidants with trophicity to the lesion foci are being developed in order to block and reduce the adverse effect of oxidative stress, when excessive reactive oxygen species nonselectively damage molecules, tissues, and organs [30]. It is a newly forming area of antioxidant therapy, since the oxidative stress accompanies the development of cardiovascular disorders. Certain antioxidants (e.g., of vitamin or phenol nature) exhibit different clinical effects; meanwhile, oxidoreductases are notable for their high efficiency and specificity of their antioxidant action. Human superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase belong to the exhibiting antioxidant activity. SOD is represented by three isoforms: the cytosolic Cu,Zn-SOD (SOD-1), the mitochondrial Mn-SOD (SOD-2), and extracellular SOD (SOD-3, EC-SOD).

EXTRACELLULAR SUPEROXIDE DISMUTASE

An increased content of one of the types of reactive oxygen species, superoxide radical ($O_2^{\cdot-}$), was observed in the arteries of spontaneously hypertensive rats. The transfer of the EC-SOD gene to these rats improved the functioning of their endothelium and reduced arterial pressure [31]. It is assumed that the interaction between $O_2^{\cdot-}$ and NO initially occurs in the extracellular space [32]. Among all the antioxidant enzymes, only EC-SOD localizes on the vascular luminal surface where it interacts with heparan sulphate proteoglycan via its heparan-binding domain [30, 32]. EC-SOD can presumably be located along the entire depth of the vascular wall and also between the endothelium and the vascular muscle [33]. The introduction of heparin (at therapeutic concentrations) results in the release of the EC-SOD previously bound to endothelial and other cells into the bloodstream [32, 34]. The antioxidant effect of EC-SOD mainly manifests itself on the vascular wall rather than in the bloodstream volume [30, 32]. It was revealed that diseases of the coronary vessels in humans are associated with a reduced level of heparin-released EC-SOD [35, 36]. A positive correlation between the level of heparin-released EC-SOD, the content of high-density lipoprotein cholesterol, and age was noted [36]. The protective effect of EC-SOD was attributed to the protection of the NO vascular dilator, which diffuses from the endothelium to the guanylate cyclase of smooth muscle cells [30, 32, 37], which was confirmed with data obtained from a model of volume-dependent (high-volume) hypertension in mice (1 kidney, 1 clip) [38]. Meanwhile, the impairment of endothelium-dependent dilation, increased arterial pressure, and vascular oxidative stress are observed in wild-type and EC-SOD knockout mice. Recombinant EC-SOD reduced arterial pressure and enhanced NO biocompatibility in the aorta of wild-type and EC-SOD knockout mice; however, it did not reduce the arterial pressure in endothelial NO synthase knockout mice and in wild-type mice that had received a NO synthase inhibitor. These results provided an illustrative demonstration of the fact that the targeted vascular effects of the recombinant EC-SOD are NO-mediated [38] and, along with the other data [39–41], point to the significant role of this biocatalyst upon hypertension. In addition to atherosclerosis [30, 32] and hypertension, oxidative stress and enzymatic antioxidants play an important role in the development of diabetes mellitus

and heart failure [32]. The broad protective effect of enzymatic antioxidants emphasizes the topicality of using them to design new agents for combined therapy.

MODIFICATION OF SUPEROXIDE DISMUTASE

The low affinity of SOD-1 to membranes of the cells where reactive oxygen species are produced, its low stability in blood plasma, and the fact that it remained in the bloodstream for a short time is testament to the need for obtaining lecithinized SOD in which four phosphatidylcholine molecules would be covalently bound to the dimeric enzyme [42]. With the modification with lecithin, the SOD derivative exhibited an increased trophicity to the cell membrane; it reduced the lesion in mice with ulcerative colitis as early as 7 days after daily intravenous administration, whereas the native enzyme had to be introduced at 30-fold higher doses [42]. The considerably superior effect of using lecithinized SOD was also observed in mice with bleomycin-induced pulmonary fibrosis [43]. The targeting of protein agents to the lesion focus as a result of their modification is to a noticeable extent determined by the size of the resulting conjugates [44]. Thus, SOD conjugated with anti-PECAM-1 antibodies is characterized by optimal trophicity to lung endothelium when the conjugate is 300 nm in diameter. It is assumed that the targeting of the SOD conjugated with anti-PECAM-1 monoclonal antibodies to endothelial endosomes can have a pronounced anti-inflammatory effect [45].

COMBINATION OF SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES

The inactivation of the endogenous enzyme by hydrogen peroxide [38] was revealed in the course of the investigation of the feasibility of using superoxide dismutases for antioxidant protection [32, 34, 46, 47]. The *in vivo* use of CAT (an intravenous bolus injection of the catalase–polyethylene glycol derivative for 3 days) reduced arterial pressure in wild-type spontaneously hypertensive mice (but not in the EC-SOD knockout ones) and improved the *ex vivo* function of aortic endothelium. These data clearly attested to the key role of hydrogen peroxide in the inactivation of endogenous EC-SOD [38, 48]. The benefit of the reduction in the hydrogen peroxide level under oxidative stress conditions was demonstrated for the cell cultures. The super expression of CAT protected human aortic endothelium against apoptosis caused by the oxidized forms of low-density lipoproteins (oxLDL) [49]. These data attest to the fact that the simultaneous presence of SOD and CAT activity is reasonable to ensure protection against vascular oxidative stress. Different forms of these enzymes (both in the form of a mixture and in the form of conjugates) were used for this purpose.

LINKAGE OF SUPEROXIDE DISMUTASE AND CATALASE

The results of the combined application of native forms of SOD and CAT were rather inconsistent [30, 46]. The simultaneous functioning of SOD and CAT in the focus of lesion development is required for the manifestation of a therapeutic effect [50]. This condition was fulfilled using a bienzyme conjugate in which SOD-1 was covalently bound to CAT via chondroitin sulphate (CHS) – a glycosaminoglycan of the vascular wall – to obtain the SOD-CHS-CAT adduct [46]. The conjugation changed the properties of SOD-1 by converting it into the SOD-3 form, which is the most similar to the glycoprotein [30, 51, 52]. In the resulting SOD-CHS-CAT conjugate, SOD and CAT catalyze two sequential reactions in which hydrogen peroxide (the SOD product) acts as a substrate for the reaction catalyzed by CAT and is converted into safe compounds: water and molecular oxygen (the reaction scheme is shown below):

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$4O_2^- + 4H^+ \xrightarrow{COD/KAT} 2H_2O + 3O_2.$$

On the model of arterial thrombosis in rats induced via treatment of the vessel with a saturated solution of iron (II) chloride, the bienzyme conjugate SOD-CHS-

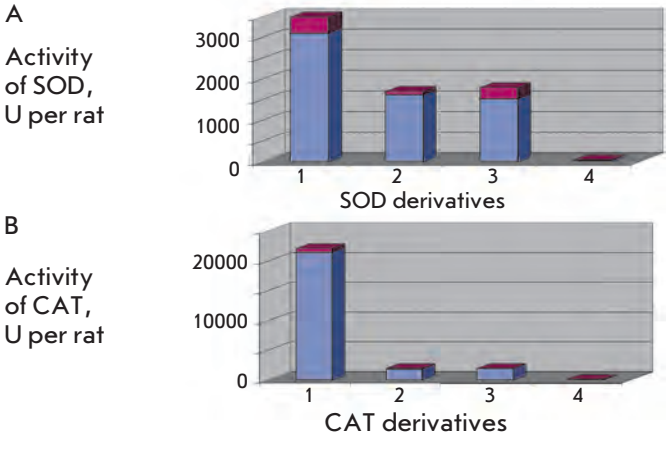

Fig. 4. The comparison of the optimal dose intervals for the antithrombotic action of SOD (A) and CAT (B) derivatives. Designation: 1 – native enzyme, 2 – covalent conjugate of the enzyme with chondroitin sulphate, 3 – mixture of SOD-CHS and CAT-CHS derivatives, 4 – bienzyme SOD-CHS-CAT conjugate.

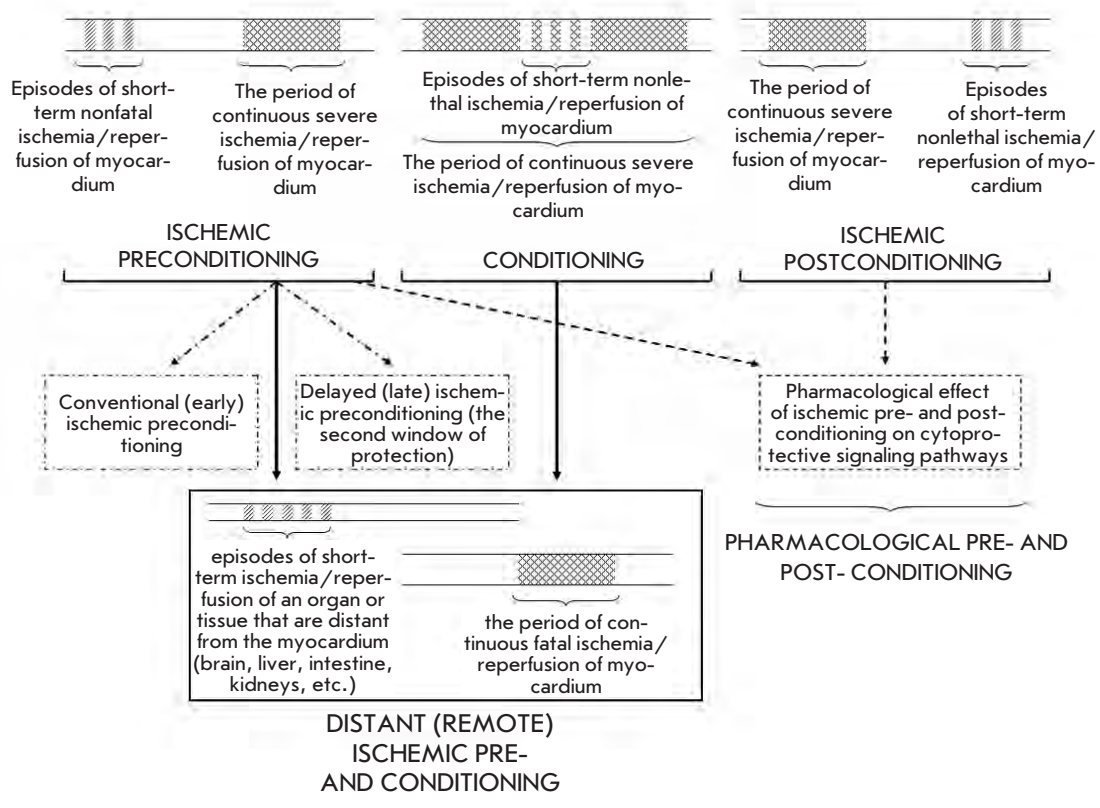

Fig. 5. Schematic representation of different forms of ischemic pre- and postconditioning of myocardium and its pharmacological conditioning.

CAT exhibited an antithrombotic effect when administered at doses lower by two orders of magnitude than those of the native SOD and CAT, and lower by an order of magnitudes than those of SOD and CAT (or their mixture) modified with chondroitin sulphate (*Fig. 4*) [50]. The linkage of proteins with CHS serves to target the bienzyme conjugate to the regions of the vascular lesion. It is known that the atherosclerotic lesion areas are characterized by an increased CHS content [30]. Early thickening of the intima of the vessel wall during atherogenesis is also associated with the accumulation of CHS [53]. After stents were mounted to New Zealand white rabbits with atherosclerosis, exposure of chondroitin sulphate proteoglycan was observed in the subendothelial arterial layer subjected to surgical intervention [54]. These data emphasize the feasibility and efficacy of using the components of vascular glycocalyx for drug-targeting delivery [53, 55].

PHARMACOLOGICAL CONDITIONING OF MYOCARDIUM

The efficacy of pharmaceutical correction of the disorders of cardiovascular metabolism is also associated with another approach that is based on the production of a model for pharmacological interaction. As a result of using intermittent short-term episodes of ischemia/reperfusion before or after the period of severe, relatively continuous ischemia, the consequences of the disease turned out to be considerably less severe as compared to those without this procedure (*Fig. 5*). If the metabolic targets that are suitable for the successful pharmaceutical correction are determined after the mechanical actions upon myocardium (pre- and postconditioning), it becomes possible to use the methods of pharmacological pre- and post-conditioning of myocardium (*Fig. 6*) [56]. Thus, in order to provide efficient interaction with a certain pharmaceutical, one needs to identify and prepare a target for cardivascular lesion that is sensitive to it.

CONCLUSIONS

It should be noted that the interest in the research performed within the framework of the conception of targeted drug delivery and research focused on the development of bioconjugates for cardiology has waned. Such "truncated" protein forms as tenecteplase, reteplase, abciximab, and monafram have achieved clinical application. It is becoming apparent that it is necessary to change the vector component of bioconjugates when the antibodies against the markers of the lesion are being developed (cell adhesion molecules, glycocalyx components, etc.) rather than when thrombus

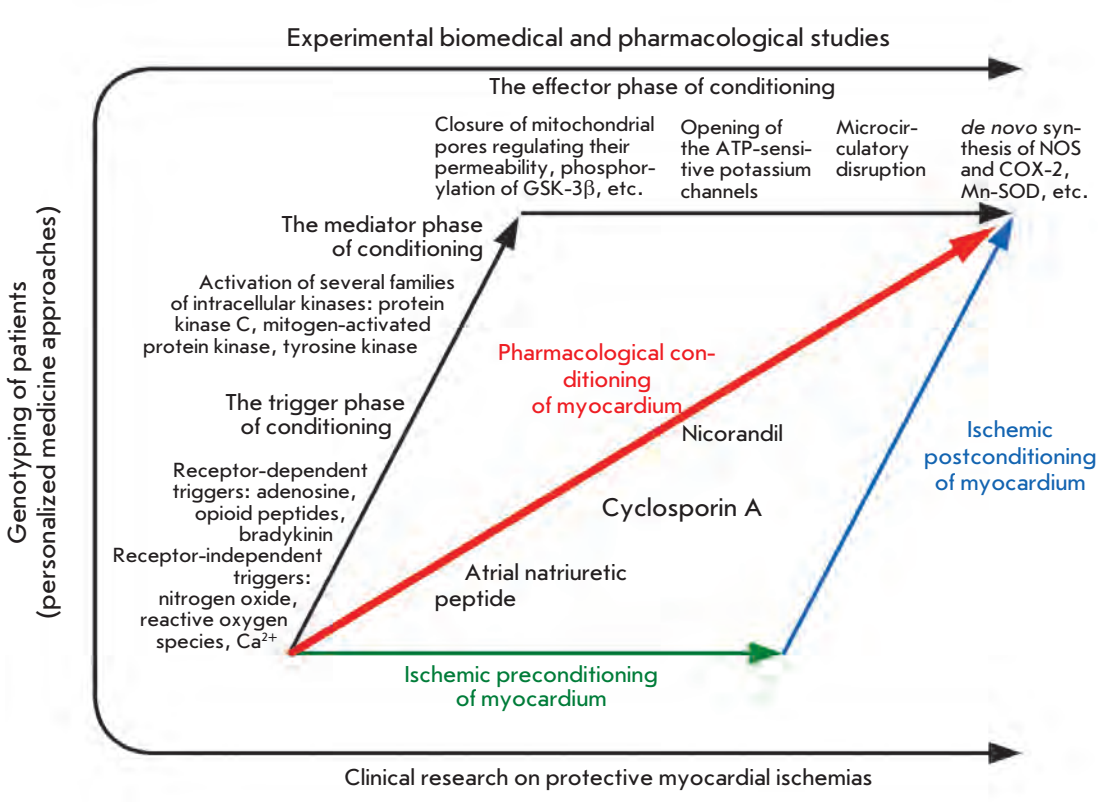

Fig. 6. Translation of ischemic pre- and postconditioning research related to biochemical and cell biology studies. Together, they constitute the resultant thrust of the pharmacological conditioning of myocardium in the frames of clinical and biomedical investigations integrated with increasing genotyping of patients.

components are applied at an increasing rate. The significance of the bioconjugate size, the density of the local accumulation of targeted markers in the focus of a developing lesion, and the use of a combined action of the catalysts of connected enzymatic reactions for efficient and specific drug targeting has now been revealed. The significance of the conception of targeted drug delivery, which is used to determine the strategy of bioconjugate construction, is decreasing. The modifications of the derivatives being designed acquire a significance; these modifications add useful properties (a lower effective dose, simplicity of use, and durable action) in addition to an appreciably high therapeutic effect and safety. New approaches to the conditioning of myocardium also emerge, facilitating the accurate identification and construction of significant targets for cardiovascular therapy. This will enable the modernisation of the conception of targeted drug transport during the development of cardiological biopharmaceuticals leading to the design of a new generation of targeting drugs.

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Non-thermal Plasma Causes p53-Dependent Apoptosis in Human Colon Carcinoma Cells

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ABSTRACT **Non-thermal plasma (NTP) consists of a huge amount of biologically active particles, whereas its temperature is close to ambient. This combination allows one to use NTP as a perspective tool for solving different biomedical tasks, including antitumor therapy. The treatment of tumor cells with NTP caused dose-dependent effects, such as growth arrest and apoptosis. However, while the outcome of NTP treatment has been established, the molecular mechanisms of the interaction between NTP and eukaryotic cells have not been thoroughly studied thus far. In this work, the mechanisms and the type of death of human colon carcinoma HCT 116 cells upon application of non-thermal argon plasma were studied. The effect of NTP on the major stress-activated protein p53 was investigated. The results demonstrate that the viability of HCT116 cells upon plasma treatment is dependent on the functional p53 protein. NTP treatment caused an increase in the intracellular concentration of p53 and the induction of the p53-controlled regulon. The p53-dependent accumulation of active proapoptotic caspase-3 was shown in NTP-treated cells. The study was the first to demonstrate that treatment of human colon carcinoma cells with NTP results in p53-dependent apoptosis. The results obtained contribute to our understanding of the applicability of NTP in antitumor therapy.**

KEYWORDS **non-thermal plasma; protein p53; apoptosis.**

ABBREVIATIONS **NTP – non-thermal plasma.**

INTRODUCTION

Non-thermal plasma (NTP) is a flow of partially ionized gas obtained under atmospheric pressure that has a macroscopic temperature that is close of the ambient temperature [1]. The potential of using NTP for medical purposes started to be intensively investigated about 10 years ago, although the first studies in the field (predominantly in Russia) began much earlier [2–4].

A non-thermal plasma torch consists of charged particles, neutral active particles (including free radicals and metastable particles), and ultraviolet radiation. The biological effects of NTP are attributed to the synergistic action of the aforementioned factors, whereas the subthreshold concentration of each component in most cases does not alter biological objects [5, 6].

There has been considerable interest in the potential use of NTP as an antibacterial agent, since NTP has been found to possess nonspecific bactericidal activity, which enables one to use NTP to sterilize thermosensitive surfaces and sanitize tissues, including wound surfaces [7–9]. Another potential field of application for NTP is antitumor therapy. Thus, the selectivity of the cytotoxic effect of plasma on various human cell types and the opportunity of selecting particular conditions that would provide selective death of a certain type of tumor cells have been reported [10, 11]. The exposure of tumor cells to NTP has been shown to result in cell cycle delay and induction of apoptosis [12–14].

Opposite to the final effects caused by NTP treatment of the cells, the molecular mechanisms underlying the interaction between NTP and eukaryotic cells remain poorly studied. These data are required to elucidate the nature of the selective effect of NTP with

respect to tumor cells and to determine the range of applicability of NTP. Therefore, our work aimed at studying the molecular mechanisms of the action of NTP on tumor cells and at determining the type of cell death in the cells subjected to NTP treatment.

METHODS

Cell lines and growth conditions

Two sublines of human colon cancer cells (HCT116) were used in this study: HCT116(p53+/+)-ConA-lacZ subline with an active *p53* gene and the β-galactosidase reporter gene under the control of a p53-dependent promoter, and HCT116(p53-/-)-ConA-lacZ subline that had deletions of both copies of the gene encoding the p53 protein. The HCT116(p53+/+) and HCT116(p53-/-) cell lines were kindly provided by A.V. Gudkov (Roswell Park Cancer Institute, USA). The cells were grown in a DMEM medium supplemented with a 10% fetal bovine serum (Hyclone, USA), 1 mg/ml glutamine (PanEco, Russia), 50 U/ml penicillin, and 50 μg/ml streptomycin (PanEco, Russia) at 37°C in a 5% CO_2 atmosphere. Cells were seeded at a 1 : 6 ratio on day 2 after the monolayer became confluent.

Counting the number of live cells

The cell survival rate was determined spectrophotometrically 25 h following the NTP treatment using the intensity of staining the live cells with a methylene blue dye. Optical density was measured at 540 and 620 nm. The relative number of cells that survived was calculated using the formula $x = OE_{620} - OE_{540}$.

Assessment of the activity of p53 under the control of a promoter based on the expression of the β-galactosidase reporter gene

After the culture medium was removed, a lysis buffer containing a β-galactosidase substrate (1 mM $MgCl_2$, 0.25 M Tris HCl, pH 7.4, 0.02% NP40, 2 g/l *o*-nitrophenyl-β-*D*-galactopyranoside) was added to the cells. Following incubation for 30 min at 37°C, the β-galactosidase activity level was determined spectrophotometrically by measuring the optical density of the solution at 414 nm.

NTP source

A source of non-thermal argon microwave plasma MicroPlaSter β was used for the experiments. The NTP source contained a 2.45 GHz current generator, a burner, and a gas (argon) supply system. The device uses two regimens, the argon plasma regimen and the regimen including the flow of a nonionized argon gas. The burner is capable of generating a highly stable low-power (60–150 W) plasma flow (torch) with a low rate of gas flow (4–8 l/min). The argon plasma torch has a length of about 5 cm and a diameter of 3.5 cm. The distance of the treated surfaces from the plasma source was equal to 2±0.2 cm. At this distance, the torch temperature was 36±2 °C.

NTP treatment of cells

A day prior to the experiment, the cells were seeded into 3-cm-diameter culture dishes (2×10^5 cells/dish). On the next day, just before the NTP treatment, the cultivation medium was removed and a 0.5-mm layer of the medium was left. The dishes were placed at a distance of 2 cm from the plasma torch and treated with NTP during the time specified below. Immediately after treatment, a fresh cultivation medium was added and the dishes were placed into a CO_2 incubator. The number of viable cells and activity of the β-galactosidase reporter gene were determined 24 h following the treatment.

Assessment of caspase-3 activity level

Caspase-3 activity was measured using antibodies specific to the active protein form conjugated to the fluorescent dye fluorescein isothiocyanate (FITC, BD Pharmingen, USA). Eighteen hours following the treatment with plasma, the cells were collected and precipitated via centrifugation at 1200 rpm for 10 min. A BD Cytofix/Cytoperm™ Fixation/Permeablization Kit (BD Pharmingen) was used for cell fixation and permeabilization. Intracellular staining of the active form of caspase-3 was carried out according to the manufacturer's (BD Pharmingen) protocol. Fluorescence was detected by flow cytofluorimetry using a BeckmanCoulter FC-500 instrument.

Assessment of p53 activity level

The p53 level was measured using anti-p53 antibodies conjugated to the fluorescent dye phycoerythrin (BD Pharmingen, USA). A day prior to the experiment, 2×10^5 HCT116(p53+/+)-ConA-lacZ and HCT116(p53-/-)-ConA-lacZ cells were seeded into each of two 3-cm culture dishes. The next day, when the cells reached a 60–80% confluence, the cultivation medium was removed from the dishes and a 0.3 mm layer of the medium was left to preserve the cells from desiccation during the treatment. The cells were treated with plasma for 2 min, and a fresh DMEM medium was added immediately after treatment. After incubation for 6 h, the cells were collected and precipitated by centrifugation (10 min, 1200 rpm). A BD Cytofix/Cytoperm™ Fixation/Permeablization Kit (BD Pharmingen) was used for cell fixation and permeabilization. Intracellular staining of p53 protein-3 was carried out according to the manufacturer's (BD Pharmingen) pro-

tocol. Fluorescence was detected by flow cytofluorimetry using a BeckmanCoulter FC-500 instrument.

Statistics

All experiments were performed using duplicate samples and repeated at least three times. The mean values and standard errors were calculated with the Excel software (Microsoft Office 2007).

RESULTS AND DISCUSSION

NTP treatment causes dose-dependent death of HCT116 cells

The ability of NTP to activate the transcription factor p53 and induce the development of p53-dependent programs leading to apoptotic death is presumably one of the possible reasons behind tumor cell death after treatment with NTP.

The human colon cell line HCT116(p53+/+) containing the functional *p53* gene was selected to verify this assumption. The ability of NTP to cause the death of these cells was determined at the first stage. HCT116(p53+/+) cells were treated with NTP with different exposure times. The number of cells alive was determined 24 h following the treatment. Nontreated cells and the cells treated with nonionized argon during the corresponding time period were used as controls. Treatment with NTP for 2 min resulted in no statistically significant decrease in the number of cells alive (*Fig. 1*). A twofold ($p < 0.01$) and 14.5-fold ($p < 0.005$) decrease in the number of cells alive was observed after a 5- and 7-min treatment, respectively, compared to the intact control cells. A decrease in the number of cells alive after plasma treatment for 7 min was statistically significantly different from the effect of nonionized argon ($p < 0.01$). Treatment with nonionized argon resulted in a decrease in the number of live cells compared to that of the control cells; however, this was statistically negligible at all exposure times. The reduction in the number of cells alive can presumably be attributed to the consequences of the gas flow, which could result in desiccation of the cultivation medium, or some other nonspecific effects.

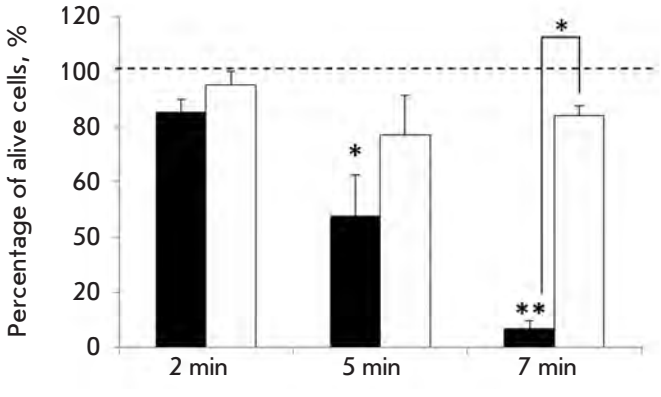

Fig. 1. Study of survival of HCT116(p53+/+) cells after NTP (black columns) and non-ionised argon (white columns) treatment as a function of exposure time. The percentage of living cells against the intact control (dashed line) is shown. Mean values ± SD are given. * – $p < 0.05$, ** – $p < 0.005$ (as compared to the intact cells).

Thus, we have demonstrated that treatment with NTP leads to a decrease in the number of live cells; the intensity of the cytotoxic effect depends on the duration of treatment with the plasma flow. The treatment with non-ionized argon caused a smaller decrease that was independent of the exposure time. The results allow to conclude that the cytotoxicity of NTP is due to the specific effect of ionized NTP particles on eukaryotic cells.

Protein p53 and p53-dependent elements are activated in HCT116 cells treated with NTP

Protein p53 is known to be one of the major stress-activated transcriptional regulators; its activation can initiate the development of a number of programs inducing cell death.

The effect of NTP on p53 activity was studied using HCT116 cell sublines (HCT116(p53+/+)-ConA-lacZ). The *lacZ* reporter gene encoding bacterial β-galactosidase was inserted into the genome of HCT116(p53+/+)-ConA-lacZ cells. The expression of the reporter gene was controlled by the p53-dependent promoter. The use of this reporter system allows one to determine the transcriptional activity of protein 53 based on the β-galactosidase activity level. Previously obtained data were used to determine the subtoxic time of treatment of the cells with NTP, which does not result in pronounced cell death (2 min). The β-galactosidase gene expression level was determined spectrophotometrically 24 h following the treatment with NTP. Cells treated with a nonionized argon flow were used a controls. The 2-min treatment of cells with NTP caused a statistically significant increase in the β-galactosidase activity level, attesting to the enhancement of p53 transcriptional activity in HCT116 cells (*Fig. 2*).

The amount of p53 was additionally determined via flow cytofluorimetry using fluorescently labelled monoclonal anti-p53 antibodies. The HCT116(p53-/-) cells that had deletions of both copies of the *p53* gene were used as the control cell line. The 2-min treatment with NTP was shown to result in a statistically significant increase in the amount of p53 in HCT116(p53+/+) cells compared to that in the intact cells (*Fig. 3A*). As

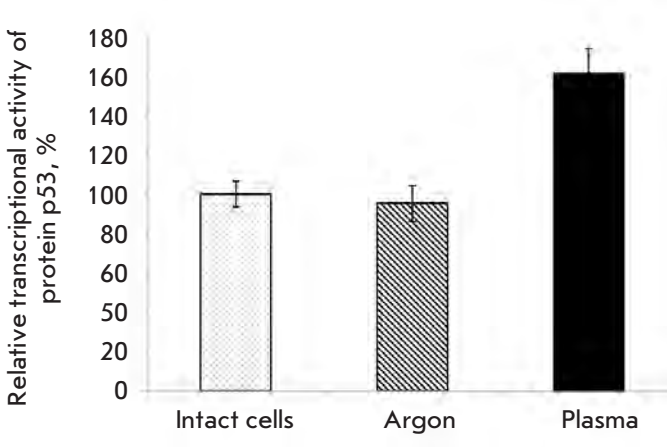

Fig. 2. The relative transcriptional activity of protein p53 in HCT116(p53+/+) cells treated with NTP. Mean values ± SD are shown.

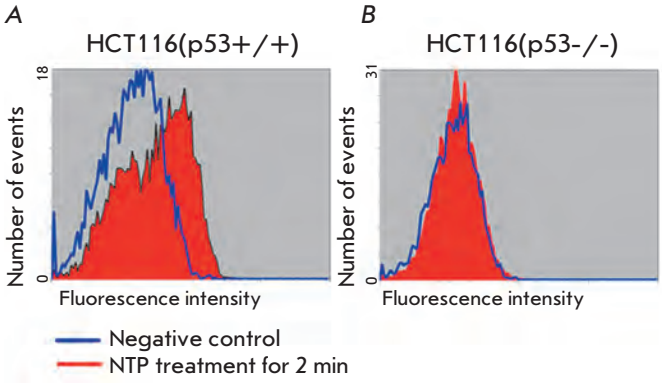

Fig. 3. Intracellular concentration of protein p53 in HCT116(p53+/+) (A) and HCT116(p53-/-) (B) cells, intact or treated with NTP for 3 min.

could have been be expected, NTP treatment did not alter the nonspecific signal in HCT116(p53-/-) cells (*Fig. 3B*).

Thus, it was ascertained that treatment of eukaryotic cells with NTP at sub-toxic exposure time results in a statistically significant ($p < 0.05$) increase in the amount of p53 and enhancement of its transcriptional activity.

Treatment with NTP induces apoptotic death of HCT116 cells

The type of cell death induced by NTP treatment had to be identified at the final stage. Apoptosis initiation through the activated p53-dependent pathway is one of the major cell death mechanisms that are known thus far [15]. Effector caspase 3 is one of the key enzymes activated upon apoptosis [16]. The activation of this protein is an integral feature of the final stages of apoptotic cell death. The level of activated caspase 3 in HCT116(p53+/+) and HCT116(p53-/-) cells treated with NTP was determined to reveal the association between p53 activation and cell death in HCT116.

A significant (up to 20%) increase in the percentage of cells containing active caspase 3 was observed in the HCT116(p53+/+) cell population treated with NTP for 2 min (*Fig. 4A*), whereas the NTP treatment in HCT116(p53-/-) cells had no such effect (*Fig. 4B*). It can be thus concluded that NTP treatment of cells results in p53-dependent activation of the main effector proapoptotic caspase 3.

Summarizing the results, one can arrive at the conclusion that NTP treatment of human cells induces activation of protein p53, the main regulator of the cellular stress response, and induces the expression of p53-dependent genes (including caspase 3), thus initi-

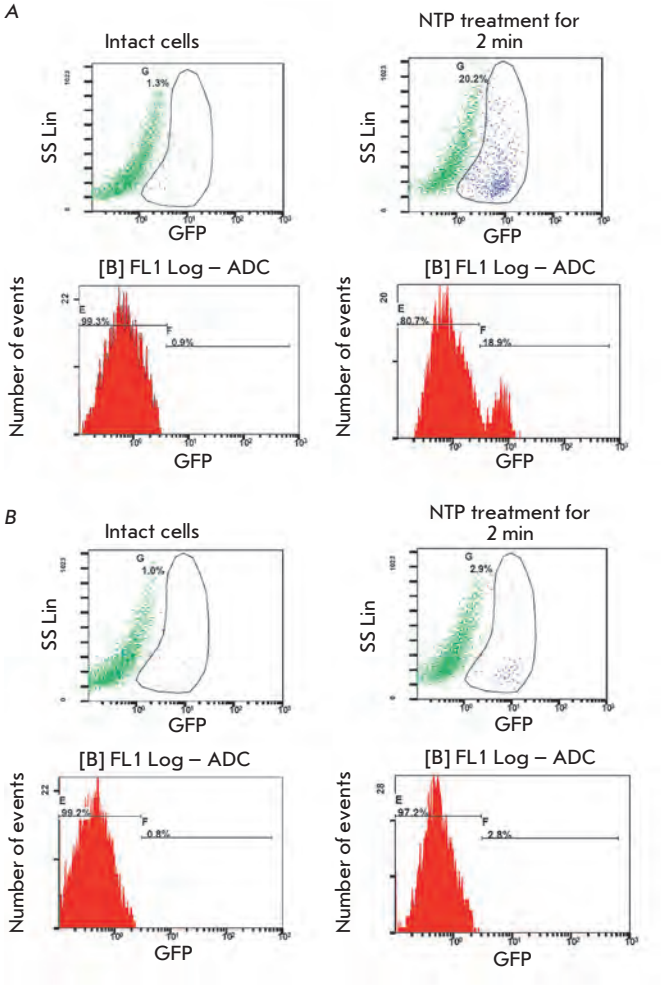

Fig. 4. Amounts of active caspase-3 in HCT116(p53+/+) (A) and HCT116(p53-/-) (B) cells, intact or treated with NTP for 3 min.

ating cell death via the apoptotic pathway. Apoptosis induced by NTP was first demonstrated to occur via the p53-dependent pathway. Based on data pertaining to the enhancement of the transcription of the *p53* gene and p53-regulated *p21* gene in human hepatoma (Hep2G cells), it has been assumed that p53 participates in the cellular response to NTP treatment [17]. However, no direct evidence to support the existence of any association between the cell survival rate and presence of functional protein p53 has been obtained. Our results correspond to the induction of the β-catenin signalling pathway in human colon cancer cells treated with NTP, since this pathway is associated with the p53-dependent signalling cascade [18]. Generation of reactive oxygen species (ROS) is another signalling system participating in the cellular response to NTP treatment [14]. Intracellular ROS that interact with the components of the signalling pathways (such as protein kinases, phosphatases, and transcription factors) in a direct or mediated manner act as secondary signalling molecules, which participate in cell cycle regulation and affect the final outcome of the events induced by p53 activation [19].

However, the sequence of signalling events occurring in a cell in response to NTP treatment has not been elucidated thus far. First of all, the type of damage resulting in p53 activation has not been thoroughly ascertained. A number of studies attest to the possibility of DNA injuries as a factor inducing apoptosis in cells treated with NTP. Thus, the action of a dielectric barrier discharge as an air plasma source on MCF10A breast cancer cells results in phosphorylation of histone H2A, which is a marker of the emergence of DNA double-strand breaks [14]. However, these results are inconsistent with data that were obtained on prokaryotes and purified DNA samples and attest to the fact that the amount of double-strand breaks caused by NTP treatment is minimal [20–23]. The authors interpreted this inconsistency by assuming that DNA double-strand breaks may be caused by the NTP-induced formation of intracellular ROS [14]. Damages to the cytoplasmic membrane may be another potential signal for apoptosis development. For instance, activation of acid sphingomyelinase caused by membrane damage and an increased ceramide production may result in the development of both p53-dependent and independent apoptosis [24]. Experimental data demonstrating that it is the surface cell structures (in particular, membrane) that are the major target of active NTP particles support this mechanism of apoptosis initiation [20, 25–27]. However, no evidence in favor of this mechanism of apoptosis initiation has been obtained thus far; the details of the events occurring in the cell immediately after treatment with NTP remain to be elucidated. Meanwhile, it is obvious that for a successful application of NTP for medical purposes, a thorough understanding of what signalling events are induced by NTP depending on the dose and type of plasma radiation is required, since it is this knowledge that would allow one to optimize the treatment parameters and achieve the desired effect.

CONCLUSIONS

It has been demonstrated that the survival rate of HCT116 colon cancer cells treated with NTP depends on the presence of the functional protein p53. NTP treatment increases the intracellular concentration of p53 and induces expression of p53-regulated genes, in particular, the major proapoptotic caspase 3. It has thus for the first time been shown that the treatment of colon cancer cells with argon NTP induces p53-dependent apoptosis. These results are of significance for better insight into the potential of using NTP as an antitumor agent. ●

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Mitochondrial Pathway of α-Tocopheryl Succinate-Induced Apoptosis in Human Epidermoid Carcinoma A431 Cells

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ABSTRACT Vitamin E derivatives are known to act as agents exhibiting cytotoxity against tumor cells. The effect of vitamin E succinate on human epidermoid carcinoma cell line A431 was investigated in this study using live imaging, immunocytochemistry, and transmission electron microscopy. α-Tocopheryl succinate-induced apoptotic cell death in A431 cells was shown to be both dose- and time-dependent. The hyperproduction of reactive oxygen species, changes in size, shape and ultrastructural characteristics of mitochondria followed by the release of cytochrome *c* from mitochondria to cytosol were observed. These results suggest that α-tocopheryl succinate induces apoptosis that occurs via the mitochondrial pathway. Mitochondria are shown to be crucial targets in α-tocopheryl succinate-induced caspase-dependent cell death in human carcinoma A431 cells.
KEYWORDS α-tocopheryl succinate; apoptosis; mitochondrial pathway; ROS; cytochrome *c*
ABBREVIATIONS α-TS – α-tocopheryl succinate; ROS – reactive oxygen species; AI – apoptotic index

INTRODUCTION

Many researchers are now focusing on searching for anti-tumor agents that would selectively affect malignant cells while being nontoxic to normal cells and tissues. Vitamin E derivatives are among such compounds.

The term "vitamin E" is now used to refer to a large group of both the natural and synthetic compounds known as tocopherols and tocotrienols, as well as to the acetyl and succinyl derivatives of tocopherol. The biological effects of vitamin E are diverse and remain understudied. Certain forms of vitamin E can be considered as potential anti-tumor agents, since they can scavenge free radicals, suppress growth and induce differentiation in transformed cells, affect the course of the cell cycle, induce apoptosis, and activate the immune system [1–3].

It has been shown that α-tocopherol exhibits virtually no anti-tumor activity, whereas a number of its derivatives, including vitamin E succinate (α-tocopheryl succinate, α-TS), exhibit antitumor properties. Unhydrolized α-TS is a redox-silent compound ; however, as opposed to free α-tocopherol, it exhibits unique antiproliferative and proapoptotic properties [4]. α-TS can affect tumor cells in culture [5, 6], as well as human tumor xenografts in animal models and tumors induced by chemical cancerogenes [7–11]. α-TS can induce cell death and cell cycle arrest [12, 13], inhibit angiogenesis [14], and protect the organism against ionizing radiation [15].

Intracellular targets of α-TS in cell lines of different origins have been subjected to intense investigation in recent years. α-TS has been demonstrated to induce the apoptotic death of a number of tumor cells (breast cancer, malignant mesothelioma, neuroblastoma cells) via the mitochondrial pathway [16–21]. Nevertheless, the mechanisms of α-TS -induced apoptosis remain rather poorly understood. The effect of α-TS has been investigated on tumor cell lines of different origins. Skin neoplastic diseases are characterized by high malignancy and belong to poorly treatable tumors; however, there are almost no studies devoted to the effect of vitamin E derivatives on transformed keratinocytes thus far. Malignant skin diseases often have an unfavorable prognosis.

Hence, the effect of α-tocopheryl succinate on human epidermoid carcinoma A431 cells was investigated in this study.

EXPERIMENTAL

Cell culture and experimental procedure

A431 cells (human epidermoid carcinoma) (Institute of Cytology of the Russian Academy of Sciences, Russia) were cultured in a DMEM medium (PanEco, Russia) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria) and 80 mg/ml gentamycin (PanEco) at 37°C with 5% CO_2.

96% ethanol (Sigma) was used as a vehicle for α-TS. Cells cultured in the unsupplemented medium were used

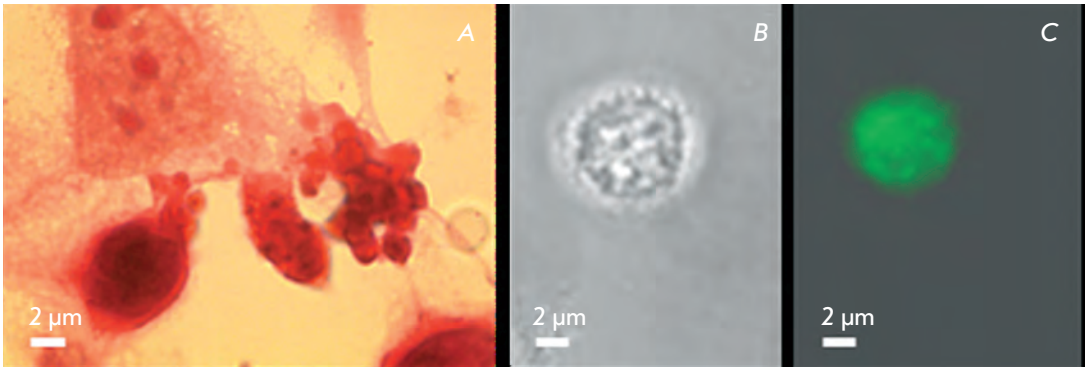

Fig. 1. Apoptosis in human epidermoid carcinoma A431cells: a – on hematoxilin and eosin-stained slides, b – phase contrast, c – immunocytochemical revealing of caspase-3 with anti-caspase-3 antibodies in the cell shown in "b".

as the first control. Cells cultured in the medium with vehicle were the second control. Cells were treated with the agent and ethanol used as the second control on day 2 following cell seeding and incubated for 24, 48, and 72 h.

Assessment of the level of cell death

The percentage of apoptotic cells in the population was counted on specimens stained with hematoxylin and eosin according to the standard procedure. Such morphological indicators as chromatin condensation, as well as cytoplasmic shrinkage and blabbing, were used as the criteria to identify the cells that went into apoptosis. The effect of α-TS at concentrations of 20, 40, 60, and 100 μM was assessed. The specimens were analyzed on a Leica DM 1000 microscope with a N PLAN 100x/1.25 Oil objective. The results were processed using the Microsoft Office Excel 2007 software.

Cytochemistry and immunocytochemistry

The cells for the immunocytochemical studies were fixed with 4% formaldehyde (MP Biochemicals, France) prepared using a 0.1 M PBS buffer (Sigma), pH 7.2. The specimens were stained with monoclonal antibodies against the active form of caspase-3 (Sigma) and with anti-cytochrom *c* sheep antibodies (Sigma). Anti-mouse IgG antibodies conjugated to Alexa Fluor-488 (Sigma) and anti-sheep IgG antibodies conjugated to Alexa Fluor-488 (Invitrogen, USA), respectively, were used as secondary antibodies. Cell nuclei were stained with DAPI (100 nM, Sigma). The specimens were embedded in a 1:1 PBS–glycerol solution and analyzed on an Axiovert 200M luminescent microscope (Carl Zeiss Inc., Germany) using a Plan-NEOFLUAR 100x/1.30 objective. The images were processed using the Adobe Photoshop and ImageJ software.

Mitochondria were stained with the potential-dependent dye Mitotracker Orange CMTMRos (100 nM, Invitrogen Molecular Probes). Cells were fixed with 4% formaldehyde (MP Biochemicals, France) prepared in a 0.1 M PBS buffer, pH 7.2, and embedded in a 1:1 PBS–glycerol mixture.

Live imaging

In order to detect ROS, the medium was supplemented with 10 μM 2',4'-dichlorofluorescein diacetate (DCFH-DA, BioChemika, USA) for 20 min. An oxidized fluorescent product, dichlorofluorescein, is formed in the presence of ROS (oxygen peroxide, peroxide anion, peroxide radical). DCFH-DA was added 48 h after α-TS had been added into the culture medium. Imaging was performed on an Axiovert 200M luminescent microscope (Carl Zeiss Inc.) using a Plan-NEOFLUAR 20x/0.50 objective. The percentage of ROS-positive cells in the images was counted. The data were statistically processed using the Microsoft Excel software. The images were processed using the Adobe Photoshop CS3 software.

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde (Sigma) with 2% formalin in 0.1 M PBS, pH 7.2 (Sigma) and postfixed with a 1% OsO_4 solution (Sigma) in PBS for 1 h in dark conditions. The standard procedures for transmission electron microscopy were then used. Ultrathin sections (60–80 nm) were cut with an Ultratom-5 ultramicrotome (LKB, Sweden). The sections were contrasted with a 1.5% aqueous solution of uranyl acetate (Serva, USA) and Reynolds' lead citrate (Serva). The specimens were examined in a JEM-1011 electron microscope (JEOL) equipped with a GATAN ES500W digital camera with the Digital Micrograph software (GATAN) and a JEM-100B transmission electron microscope (JEOL).

RESULTS AND DISCUSSION

α-TS induces apoptotic death of A431 cells in time- and dose-depended manner

The only type of cell death (apoptosis) was detected in specimens stained with hematoxylin and eosin. Apoptotic cells could be identified by using a number of criteria, such as cytoplasmic shrinkage, acquiring a round shape, chromatin condensation, blabbing, and disintegration into apoptotic bodies. No necrotic cells were detected (*Fig. 1A*). The presence of the active form of caspase-3 (*Fig. 1B,C*) confirms an apoptotic pathway of cell death.

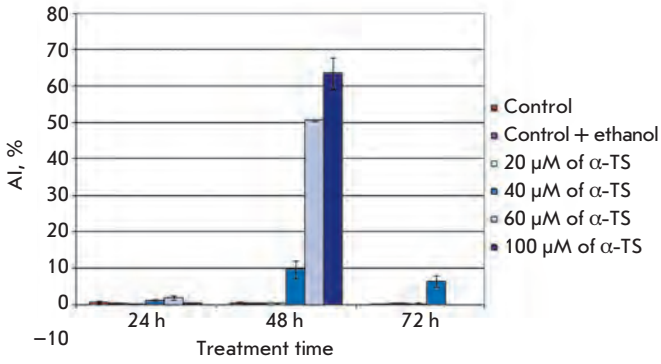

Fig. 2. α-TS induces apoptosis in A431 cells in a dose- and time-depending manner.

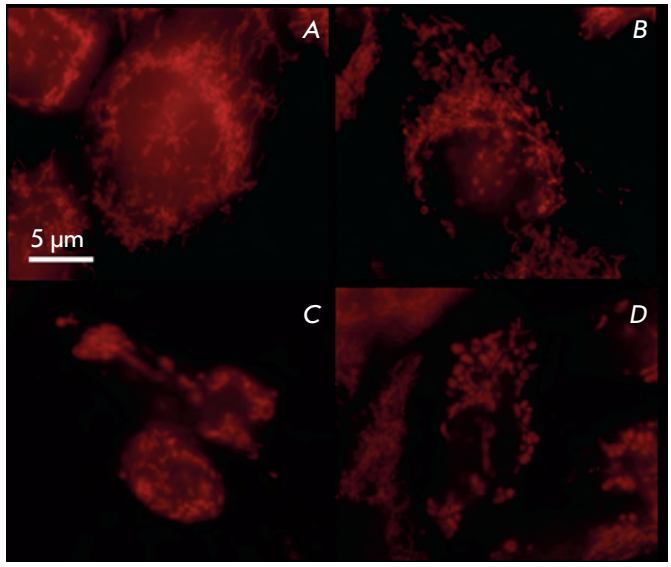

Fig. 3. Mitochondria in A431 cells stained with MitoTracker Orange: a – control; b – control with vehicle; c, d – 40 μM α-TS, 48 h.

The apoptotic index (AI) value in the control specimens of the A431 cell culture was 0.4–0.9%; the supplementation with 96% ethanol had virtually no effect on the AI value. AI increases significantly (9.67%) on day 2 of incubation of the cells with α-TS at a concentration of 40 μM; on day 3, it remains at this level. Treating the cells with 60 μM α-TS on day 1 virtually does not alter the AI; on day 2, it increases abruptly (by over 60%), and on day 3 no cells are detected on glass slides. A similar result was obtained upon the addition of 100 μM α-TS (63.5%); however, the AI values were the highest in this case (*Fig. 2*).

Thus, the statistical analysis demonstrated that treatment with α-TS results in an increase in the level of A431 cell death in a time- and dose-dependent manner.

40 μM α-TS after incubation during 48 h causes a significant increase in the AI value; however, mass cell death has yet to occur. This dose was, therefore, selected for the investigation of the mechanism of apoptosis induction.

α-TS alters the mitochondrial structure and induces the release of cytochrome *c* from mitochondria into the cytosol

According to the available data, α-TS triggers apoptosis in a number of cell lines via the mitochondrial pathway. To understand the role of the mitochondrial mechanism in apoptosis induction, the general structure of chondriome, the mitochondrial ultrastructure, localization of cytochrome *c*, and the level of ROS were analyzed. .

In order to analyze the state of chondriome in control and treated cells , the cells were stained with the potential-dependent dye Mitotracker Orange CMTMRos, which accumulates only in functional mitochondria. *Figure 3A,B* shows A431 cells in which the chondriome is formed by numerous mitochondria (small oval, round-shaped, filamentary, curved mitochondria, etc.). Mitochondria are typically uniformly distributed over the cytoplasm; they cluster around the nucleus very rarely and sometimes are localized in the peripheral cytoplasm. Large oval and round-shaped mitochondria occur in certain cases (*Fig. 3A*). Ethanol treatment does not alter the distribution and shape of mitochondria (*Fig. 3B*).

α-TS treatment of A431 cells results in changes in the shape of mitochondria; numerous large round-shaped and oval mitochondria that are significantly larger than those in the control cells emerge. The number of these mitochondria per cell may vary; however, in general, their number is usually noticeably smaller than that in the control cells. Localization of cytochrome *c* was determined immunocytochemically. The release of cytochrome *c* from mitochondria into the cytosol is the crucial stage in the apoptosis occurring via the mitochondrial ("internal") pathway.

In the control and control with vehicle, anti-cytochrome *c* antibodies detect this protein within mitochondria. It is clear from *Fig. 4A,B* that there are numerous small mitochondria that are frequently filamentary shaped. Small oval and round-shaped mitochondria occur in some cases.

Different degrees of cytoplasmic staining and different amounts of stained mitochondria can be traced in cells cultured in the presence of α-TS (*Fig. 4C–F*), which attests to the fact that cytochrome *c* is released

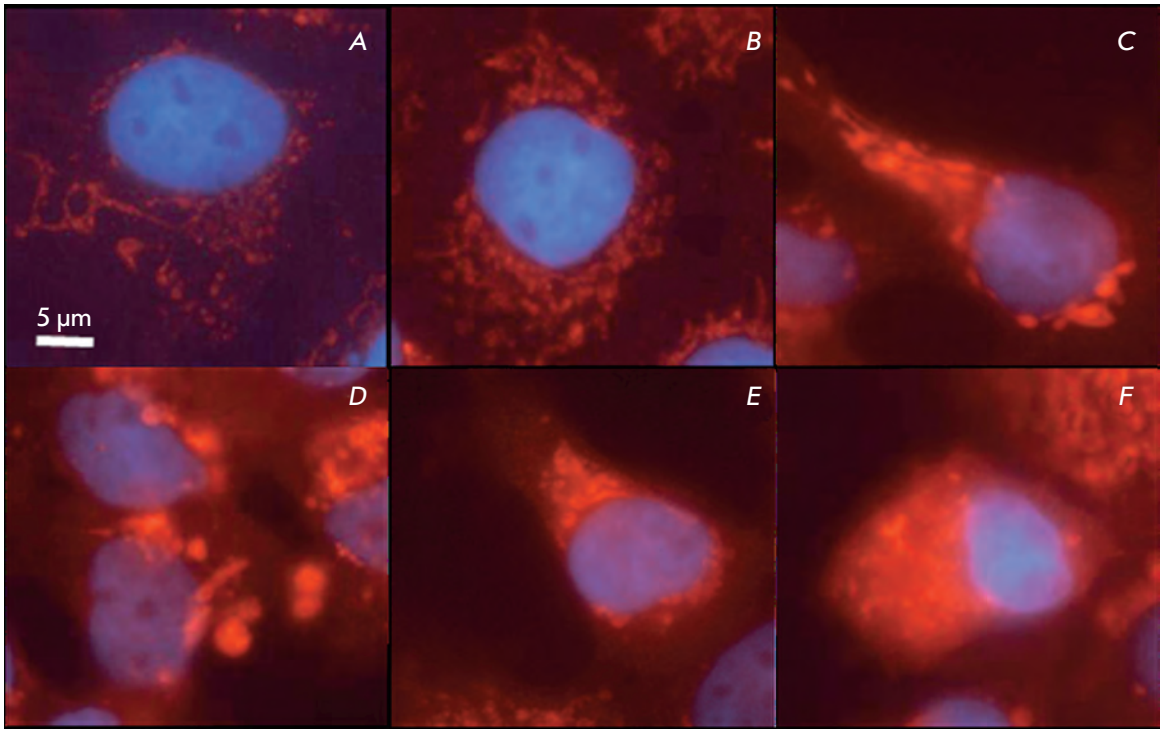

Fig. 4. Immunocytochemical staining of A431 cells with anti-cytochrome *c* antibodies: a, b – contol; c–f – 40 μM α-TS, 48 h.

from some mitochondria into the cytosol. Thus, *Fig. 4D* shows a cell with mitochondria of increased size and round and oval shapes. Meanwhile, the cytoplasm remains virtually unstained. *Figure 4D* shows a cell with mitochondria of a size similar to that of mitochondria in the control cells. However, unlike them, mitochondria shown in *Fig. 4D* are short oval bodies. The release of cytochrome *c* from mitochondria into the cytosol can be seen in *Fig. 4F*. It should be mentioned that some mitochondria contain cytochrome *c*.

An ultrastructural investigation of control A431 cells revealed small mitochondria with a light matrix and relatively sparse thin cristae (*Fig. 5A,B*). Treatment with α-TS results in significant alterations in mitochondria (*Fig. 5C–E*). Sections may contain giant mitochondria with a large number of cristae or mitochondria with invaginations, certain areas of which are filled with numerous cristae (*Fig. 5D*). Certain mitochondria contain a dense matrix and dilated cristae (*Fig. 5E*). There are also mitochondria with an ultrastructure virtually identical to that of mitochondria in the control cells. It is interesting to note that mitochondria with different ultrastructures can occur in the cytoplasm of one cell. The heterogeneity of the mitochondrial population presumably represents different stages of alterations occurring under the action of α-TS.

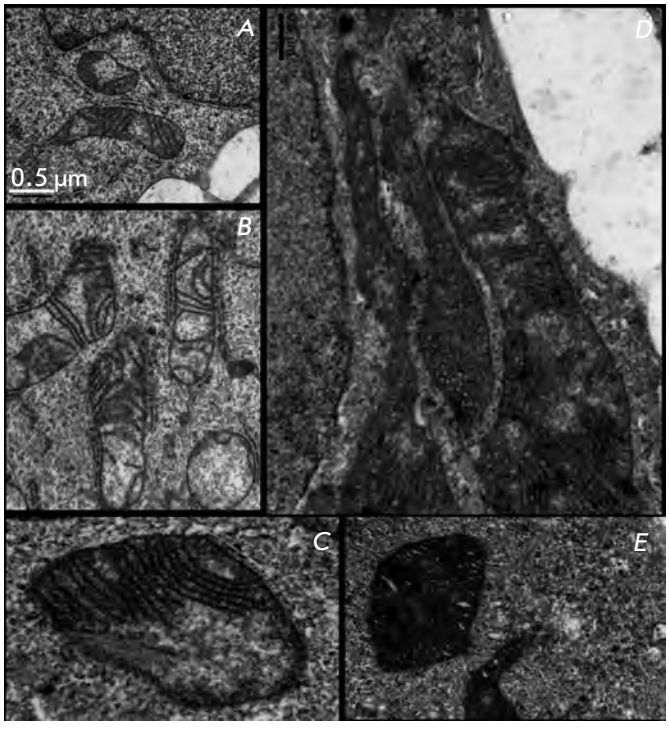

Fig. 5. Ultrastructure of mitochondria in A431 cells: a – control; b – control with vehicle; c–e – 40 μM α-TS, 48 h.

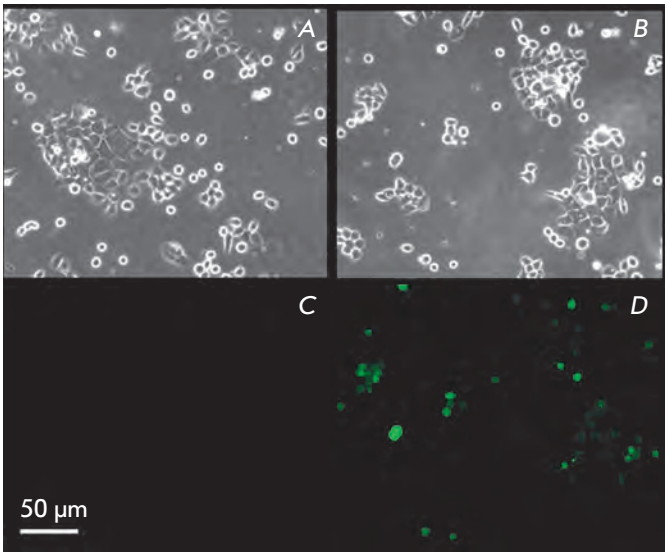

Fig. 6. Live-imaging of A431 cells stained with DCFH-DA: a, c – control; b, d – 40 μM α-TS.

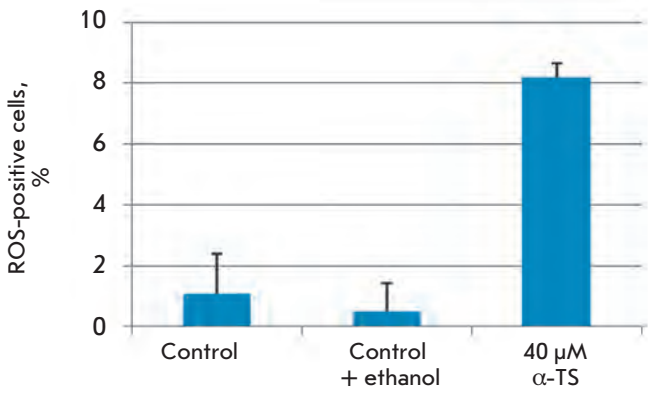

Fig. 7. Live-imaging of A431 cells stained with DCFH-DA: percentage of peroxide-positive cells in the control and after the incubation with 40 μM α-TS.

α-TS increases the ROS level in A431 cells

Cell death can be mediated by an increase in ROS production. In this regard, live imaging of cells with DCFH-DA enables one to detect hydrogen peroxide. Individual fluorescent cells (*Fig. 6A,B*) were detected in the control; ethanol caused no noticeable changes.

Treatment with 40 μM α-TS for 48 h significantly enhances ROS production in cells. The percentages of stained cells (*Fig. 7*) are relatively small both in the control and vehicle-treated cells (0.08 and 0.49%, respectively). Meanwhile, treatment with α-TS considerably increases the percentage of cells with excessive content of ROS (8.18%).

Thus, this study demonstrates that α-TS induces apoptotic cell death in human epidermoid carcinoma cell line A431 in a dose-dependent manner. In morphological terms, morphological characteristics of apoptosis are revealed, such as blabbing, chromatin condensation, nuclear fragmentation, and disintegration of a cell into apoptotic bodies. Furthermore, the apoptotic pathway of cell death was also confirmed by the fact that the cells were stained with antibodies against the active form of caspase-3. No cells with necrotic appearance, such as cell swelling, were observed. α-TS has been reported to induce apoptotic death of the following cell lines: gastric [22], colon [10], breast [23, 16], prostate [17], lung [24], cervical, ovary [25] cancer cells; hepatoma cells [26], osteosarcoma cells [12, 13], Jurkat T cell lymphoma cell line [8, 18, 29], and the other malignant hematopoietic cell lines [8, 19]; murine melanoma and glioma cell lines, and rat and human neuroblastoma [30, 31]. Apoptosis has been shown to be induced by α-TS in micromolar concentrations; the effect is time- and dose-dependent [8, 18, 32–35]. We have demonstrated that apoptosis induction in human epidermoid carcinoma cells is also time- and dose-dependent. Unlike α-tocopherol, which is known for its antioxidative properties, α-TS is a redox-silent compound and does not exhibit antioxidant properties [35]. Contrariwise, we and other authors [21] have shown that α-TS can act as a pro-oxidant in tumor cells and enhance generation of oxygen radicals.

It has been suggested in a large number of studies that mitochondria are the major targets in α-TS action on tumor cells [18, 19, 35, 36–38]. We have revealed a significant change in the mitochondrial ultrastructure and release of cytochrome *c* from mitochondria into the cytosol of A431 cells. We can assume that enhanced production of ROS and release of cytochrome *c* are related processes. Abundant data confirm the fact that α-TS can considerably enhance ROS generation in various cell lines, such as human and mouse breast tumor cells, a Jurkat T cell lymphoma cell line, Chinese hamster lung fibroblast cells, malignant mesothelioma cells, and human head and neck carcinoma cells [27, 36–40]. It has been mentioned in most studies that the superoxide anion radical plays a central role in apoptosis, which follows hyperproduction of ROS; however, Gu *et al.* [40] have demonstrated that hydrogen peroxide is dominant in epidermoid carcinoma cells, whereas the amount of superoxide is negligible. A significant increase in the percentage of cells exhibiting hyperproduction of ROS has also been revealed in this study. A DCFH-DA dye can interact with hydrogen peroxide; therefore, we have revealed the formation of hydrogen

peroxide. Since peroxide is generated in cells from a superoxide anion radical, it is most likely that O_2^- acts as the primary form of oxygen radicals.

Within the cell, the major source of ROS formation is mitochondria, where free radicals are generated due to the function of the electron transfer chain. α-TS has been shown to be capable of inhibiting the activity of complexes I [41] and II of the mitochondrial respiratory chain. The inhibition of complex II by α-TS has been observed in breast cancer cells, Jurkat cells, and rat thymocytes [36, 42, 43]. The activity of complex II has been reported to decrease due to the fact that α-TS acts as a pseudosubstrate for succinate dehydrogenase by binding to the Q_P and Q_D sites of the enzyme complex. Thus, the inhibition is competitive. When ubiquinone is replaced with α-TS , the electrons in the binding site of ubiquinone are not transported to FAD, [Fe-S] sites, haem, and ubiquinone via the hydrophilic part of succinate dehydrogenase. Instead, they recombine with molecular oxygen, yielding a superoxide anion radical; its accumulation may eventually result in the apoptosis of tumor cells [38].

It is a known fact that enhanced generation of ROS may trigger apoptosis through the mitochondrial pathway. ROS can mediate the formation of disulfide bonds between the Bax monomers in the cytosol, which results in the formation of channels in the outer mitochondrial membrane [42] and disturbs the binding of cytochrome *c* to cardiolipin, the mitochondrial membrane phospholipid, thus causing its hydroperoxidation [43, 44]. ROS generated in α-TS-treated cells can induce cytochrome *c* dissociation from cardiolipin and release of the protein into the cytosol, where cytochrome *c* induces caspase activation.

It is remarkable that the release of cytochrome *c* from all mitochondria is not a simultaneous process. Several mitochondria containing cytochrome *c* can be retained even in those cells where cytoplasm has been considerably strongly stained with anti-cytochrome *c* antibodies. These mitochondria are usually of increased size and oval or rounded shape. Large mitochondria are also detected by staining cells with a Mitotracker Orange potential-dependent dye. Since the release of cytochrome *c* requires disturbing the permeability of the mitochondrial membrane, the release must be accompanied by a decrease in the mitochondrial inner membrane potential. Thus, we have demonstrated that mitochondria containing cytochrome *c* and having the membrane potential (i.e., the mitochondria participating in the synthesis of ATP, whose production is required even at the late stages of apoptosis, the energy-dependent process) can be retained in cells upon treatment with α-TS .

CONCLUSIONS

We have demonstrated that mitochondria are the crucial target of α-TS action in epidermoid carcinoma A431 cells. α-TS was found to alter the shape and ultrastructure of mitochondria and enhance ROS production and release of cytochrome *c* from mitochondria into the cytosol, which induces caspase-dependent apoptosis. These findings enable us to propose the following mechanism of α-TS-induced cell death. α-Tocopheryl succinate inhibits the function of the respiratory chain complex II, which results in the disturbance of electron transport and acceleration of ROS formation. In turn, ROS accumulate in a cell and cause mitochondrial damage, which leads to the release of cytochrome *c* into the cytosol and triggers the caspase-dependent apoptotic cell death program. ●

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Gene Expression upon Proliferation and Differentiation of Hematopoietic Cells with Ph Chromosome *ex vivo*

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ABSTRACT **The genes *p53*, *mdm2*, *p21*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, and *gapdh* participate in the regulation of cell proliferation and differentiation, apoptosis and cell distribution for the cell cycle *ex vivo* in the Ph$^+$cells of chronic myeloid leukemia containing the Ph chromosome and *bcr/abl* oncogene. Expression of these genes correlates with regulation of cell proliferation and differentiation by alternating proliferation and maturation stages for three main Ph+cell types that occur under chronic myeloid leukemia. The *p53*, *p21*, *mdm2*, and *gapdh* genes overexpress in active proliferating myeloid cells in the cell cycle S+ G2/M phases and when the phases are coincident with the proliferation stage. Expression of these genes decreases to a considerable level under alternation of the Ph$^+$cell proliferation and maturation stages and whenever the expression is greatly diminished under significant neutrophil accumulation and especially under repeated alternation of the stages. In the course of neutrophil maturation, gene expression levels decrease in the range of *gapdh* > *actin* > *c-myc*, *bcr/abl*,*p21* > *p53* > *bcl2* > *bax*. The expression levels of these genes in neutrophils are lower than those in myelocytes and lower by an order of magnitude than that in the cells with a prolonged proliferation stage. The *Bcr/abl* expression gene under prolonged maturation and neutrophil accumulation is inhibited; however it is enhanced by 2–3 times for the proliferation stage with myelocyte accumulation. Minimal *bcr/abl* expression is observed under overexpression of *p53*, *mdm2*, *p21*, *c-myc*, as well as under cell maximum at the S and G2/M phases. *Bcr/abl* overexpression is observed under low expression of the *p53*, *p21*, *mdm2* genes. In the Ph$^+$ cells with a high P/D efficiency index (5–20), overexpression of the genes in the range of *bcr*> *gapdh*>*bcr/abl*, as well as a decreased expression of the *p53*, *bcl2*, *mdm2*, *p21*<< *gapdh* genes is observed for Ph$^+$ cells from the CML blast crisis and CML acceleration phase. Low control of cell proliferation and cell cycle by gene-regulators presumably promotes *bcr/abl* overexpression and activates the production of *bcr/abl*$^+$ cells. Apoptosis in the Ph$^+$ cells is induced by expression of the *bax* > *bcl2*, *p53*, *p21*, *c-myc and gapdh* genes. The blocking of Ph$^+$ cell apoptosis, neutrophil accumulation, and decrease in the expression of the *p53*, *mdm2* and *p21*, *c-myc*, *bcr/abl* genes occur at the maturation stage.**

KEYWORDS **gene expression; regulation of cell proliferation and differentiation; cells containing Ph chromosome; chronic myeloid leukemia; RT-PCR, cell cycle; apoptosis.**

ABBREVIATIONS **GEL – gene expression level; CML – chronic myeloid leukemia; Ph$^+$cells – hematopoietic cells containing Ph chromosome; PB – peripheral blood; BM – bone marrow; FBS – fetal bovine serum; RT-PCR – reverse transcription polymerase chain reaction; CPD – cell proliferation and differentiation.**

INTRODUCTION

Anomalies, translocations, inversions, deletions, and multiple mutations of chromosomes lead to the development of most leukemias ([1–5], and references therein). The Philadelphia chromosome (Ph) appears as a result of the chromosomal translocation t(9;22)(q34;q11) in a hematopoietic polypotent stem cell; this chromosome leads to the development of chronic myeloid leukemia (CML), as well as acute and chronic lympholeukemias. A chimeric oncogene *bcr/abl* encoding active tyrosine kinase p210/p185 that participates in CML pathogenesis is formed in cells containing the Ph chromosome (Ph$^+$ cells) due to reciprocal translocation of the 5' fragment of the *bcr* gene and the 3' fragment of the *abl* gene. Translocation results in the replacement of normal hematopoietic cells with Ph$^+$ cells. Numerous genes (*bcl2*, a number of *stat* genes, and the genes regulating the cell cycle and apoptosis) participate in

the cellular and molecular mechanisms of CML pathogenesis [1–57].

The ability of the *bcr/abl* oncogene to determine tumorgenic properties, enhance the viability, activate proliferation, and block apoptosis in Ph^+ cell lines has been thoroughly studied [9–7, 42–57]. The bcr/abl tyrosine kinase p210 was found to be capable of both suppressing apoptosis and making no contribution to it. The data relating to apoptosis blockage upon CML remain controversial [1–5, 42, 44, 45, 47 and our unpublished data]. The contribution of apoptosis to the proliferation and differentiation of Ph^+ cells had not been studied earlier. Our recent research demonstrates that apoptosis is dependent on the proliferation and maturation stages, as well on the type of Ph^+ cells derived from bone marrow (BM) and the peripheral blood (PB) of CML patients [Grineva *et al.*; unpublished data].

Ex vivo proliferation and differentiation of three main types of Ph^+ cells is regulated by alternating the cell proliferation stage (stage 1) and neutrophil maturation (stage 2). The proliferation rate is higher than the maturation rate at stage 1, whereas the maturation rate is higher at stage 2. The alternation of the stages and their rates maintains the optimal level of proliferation and differentiation efficiency in Ph^+ cells [1–4] and determines the wave-like regulation of these processes.

This study was aimed at putting the spotlight on the contribution of the expression of the genes that usually regulate proliferation and differentiation, apoptosis, and the cell cycle of normal hematopoietic cells to the regulation of these processes in Ph^+ cells. The kinetics of the expression of the *p53, c-myc, bcr/abl, mdm2, p21, bcl2, bax,* and *bcr* genes, as well as that of the control genes *gapdh* and *actin,* was studied. The ranges of gene expression kinetic curves and regularities of *ex vivo* proliferation, differentiation, apoptosis, and distribution in the phases of the cell cycle of Ph^+ cells isolated from CML patients were obtained.

CML Ph^+ cells consisting of 90% granulocytes are notable for their capacity to perform a complete proliferation and differentiation cycle, similar to that in the normal myeloid cells whose content is lower by an order of magnitude in the hematopoietic cell pool. This fact allows one to investigate the regularities of the regulation of proliferation and differentiation and their extrapolation onto normal haematopoietic cells.

MATERIALS AND METHODS

Heparin (Flow, UK); Limphoprep, α-MEM medium (MP Biomedical, USA); DEPC, HEPES, Tris, fetal bovine serum (FBS), sodium citrate, lauryl sarkosyl (ICN, USA); trypan blue stain, *L*-glutamine and 2-mercaptoethanol (Serva, Germany); TRI reagent, guanidine thiocyanate (Sigma, USA); RQ1 RNase-free DNAse, RNasin, dNTP, bovine serum albumin (BSA), Taq polymerase, RT buffer, MuMLV reverse transcriptase (Promega, USA); penicillin and streptomycin (OAO Biochimik, Saransk, Russia); tabletted PBS (10 mM phosphate buffer + 0.13 M NaCl + 2.7 mM KCl, pH 7.4) (NPO EKO-servis, Russia) were used in this study.

Oligonucleotide primers (*Table*) were synthesized and purified by PAGE gel electrophoresis or HPLC by Sintol company (Moscow).

The Ph^+ mononuclear cells used for the study were prepared from the PB and BM of CML patients in the chronic phase, acceleration phase, and blast crisis phase before and under treatment. In CML patients, mononuclears are mostly represented by leukocytes and granulocytes; hence, we researched these cells. The characteristics of the Ph^+ cells and CML patients from whose PB and BM the mononuclears were isolated are given in [2–5]. The types of *bcr/abl* mRNA (b3a2, b2a2 or e1a2) in the Ph^+ cells were determined by RT-PCR [2, 5].

The methods for isolating mononuclear cells and analyzing the proliferation and differentiation of Ph^+ cells were previously described [1–6]. Suspension $(0.8–1.2) \times 10^6$ cells/ml was incubated with an α-MEM medium containing 10–20% FBS, 2 mM *L*-glutamine, 10^{-4} M 2-mercaptoethanol, 100 U/ml penicillin, and 50 U/ml streptomycin, and 25 mM HEPES-NaOH pH 7.2–7.4 were cultured under strictly identical conditions; samples were collected for further analysis.

The degree of apoptosis and distribution of cultured Ph^+ cells over the phases of the cell cycle were analyzed cytofluorometrically [1–4] in the granulocyte gate on an EPICS-XL flow fluorimeter. Ph^+ cell samples (5,000 cells each) isolated from BM and PB in a Ficoll density gradient and the samples collected during the cultivation were centrifuged for 7 min at 600 g and 4°C, washed with PBS, and fixed dropwise adding cooled 70% ethanol during 30 min at 4°C. Prior to measurements, the cell suspension was washed with PBS and centrifuged; the precipitate was incubated in 0.5 ml PBS supplemented with propidium iodide (5 μg/ml) and RNase A (50 μg/ml) for 30 min at room temperature in the dark. The measurements were carried out in an EPICS-XL flow fluorimeter. The cells in the granulocyte gate were analyzed using forward-scattered light (FSC) and side-scattered light (SSC) with simultaneous registration of the FL2 fluorescence based on pulse amplitude and area (this allowed eliminating aggregated cells, conglomerates, and debris) in the linear and logarithmic scales. Apoptotic cells were detected simultaneously. FL2-H particles with hypodiploid DNA located as a separate peak leftward of the peak of diploid cells (a decrease in cell

Table. Oligonucleotide primers for RT-PCR

mRNA, target	Primers Sequence 5' → 3', Gene localization GenBank Acc.no		PCR fragment, bp
	Outer primers, 56oC annealing, 1st round	Inner primers, 60oC annealing, 2nd round	
bcr/abl b3a2, b2a2	TGGATGAACTGGAGGCAG NM_005157 (342–361 bp, 20b) TCA CAG GCG TGA TGT AGT T NM_007313 (835–854 bp, 20b) NM_004327 (2896–2913 bp, 22b) (90% гомология)	GGAGCTGCAGATGCTGACCAAC NM_004327 (3227–3248 bp, 22b) GCTTCACACCATTCCCCATT NM_007313 (3477–3496 bp, 20b) NM_005157 (289–308 bp, 20b)	378 b3a2, 303 b2a2
bcr	TGGATGAACTGGAGGCAG NM_004327 (2896–2913 bp, 22b) CAGTTTGGCTCAGCTGTGTCCC NM_004327 (3448–3469 bp, 22b)	GGAGCTGCAGATGCTGACCAAC. NM_004327 (3227–3248 bp, 22b) CAGTGGCTGAGTGGACGATGA NM_004327 (3340–3360 bp, 21b)	134
mdm2	ATGTGCAATACCAACATGTC NM_002392 (297–317 bp, 20b) TAGGGGAAATAAGTTAGCAC NM_002392 (1470–1492 bp, 20b)	CAAGAACTCTCAGATGAAGATG NM_002392 (1092–1114 bp, 22b) TTGATGGCTGAGAATAGTCTTC NM_002392 (1470–1492 bp, 22b)	401
p53	ATTGGCAGCCAGACTGCCTT NM_000546 (219–238 bp, 20b) GGAACAAGAAGTGGAGAATG NM_000546 (1434–1453 bp, 20b)	AGCTACTCCCCTGCCCTCAA NM_000546 (624–643 bp, 20b) GTCTTCCAGTGTGATGATGG NM_000546 (1009–1028 bp, 20b)	405
gapdh	GCTTGTCATCAATGGAAATC NM_002046 (300–319bp, 20b) CACGATACCAAAGTTGTCATG NM_002046 (595–615 bp, 21b)		316
bcl2		TGTGGAACTGTACGGCCCCAGCATGC NM_000633 (1087–1113 bp, 27b) GCCTGCAGCTTTGTTTCATGGTACATC NM_000633 (1286–1312 bp, 27b)	226
bax		CATCAGGGACTCAGTTGT NC_000019 (522–540 bp, 19b) CACTCCTCAAATCTGTGCCA NC_000019 (764–783 bp, 20b)	262
p21		GCCGGAGCTGGGCGCGGATT NM_07846(42–61 bp, 20b) GGCTTCCTCTTGGAGAAGAT NM_07846 (707–726 bp, 20b)	685
actin, beta (ACTB)		GCGGGAAATCGTGCGTGACATT M10277complete CDS (2280–2301 bp, 22b) GATGGAGGTTGAAGGTAGTTTCGTG M10277 complete CDS (2583–2606 bp, 24b)	327
c-myc	GAGGCTATTCTGCCCATTTG NM_002467 (440–459 bp, 20b) GGCAGCAGCTCGAATTTCTT NM_002467 (721–740 bp, 20b)		301

size not higher than 2 orders of magnitude) were considered to be apoptotic. The percentage of apoptotic granulocytes was estimated within the granulocytic gate containing no cell debris. The DNA histograms from the same cell samples were analyzed for cell cycle phase distribution (S, G2/M) using specialized software (SFIT method) [7, 10]. Samples containing 10^6 cells were used to isolate cellular RNA. Each sample underwent lysis by guanidine isocyanate according to [11], with small modifications [5].

The samples were treated with DNase according to [5]. RNA isolated from the sample (10^6 cells) was annealed with 50 ng of a hexamer mixture in 8 μl of water (70°C, 10 min). cDNA was synthesized during 1 h at 37°C in 25 μl of a RT buffer (Promega) containing 2.5 μM of each dNTP, 20 AU RNasin (Promega), and 20 AU MuMLV reverse transcriptase (Promega). The cDNA solution was stored at −70°C and immediately used to carry out PCR.

Transcription of the *p53*, *c-myc*, *bcr/abl*, *mdm2*, *p21*, *bcl2*, *bax*, and *bcr* genes and the control genes *gapdh* and *actin* was analyzed by RT PCR. RT PCR was carried out using specific primers on RNA isolated from each probe (*Table*) using one or two rounds.

PCR was carried out in 25 μl of a solution containing the PCR buffer (50 mM Tris HCl pH 8.9, 16 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 50 μl EDTA, 0.14 μg/ml BSA), 2–5 μl of a cDNA solution, 200 μM of each dNTP, 2.5 AU Taq polymerase (Promega), and 75 ng of each primer (*Table*). PCR (30 cycles) was carried out in accordance with the following scheme: denaturation – 1 min, 94°C; annealing – 1 min, 56°C for the 1st round and 60°C for the 2nd round; and synthesis – 3 min, 72°C. cDNA probes from the *bcr, p53, mdm2* and *bcr/abl* genes were annealed at 56 and 60°C for the outer and inner primers, respectively (*Table*). PCR products were analyzed by electrophoresis in 6% PAGE. Gels were stained with ethidium bromide (1 μg/ml). The current fluorescence intensity of the amplified fragments (*Jt*) was determined via computer densitometry using the Scion Image software with allowance for the volume of the RT PCR and electrophoresis probes.

Gene expression was judged based on the results of the RT-PCR carried out using the total RNA of Ph^+ cells with the primers specified in *Table*. The mRNA expression level was assessed based on the fluorescence intensity (*Jt*) of the bands corresponding to the cDNA amplification products. The level of expression of *gapdh* and/or *actin* mRNA in the same probe was used as an internal reference.

Expression of *bax* mRNA isoforms [9] was analyzed using primers for the amplification products of the *bax* RNA alternative splicing of intron I (*Table*); the accumulation of its PCR fragment correlates with the expected expression of the *bax*, *bcl2* and other genes, as well as with apoptosis kinetics (*Figs. 1–9*).

The kinetic plots of the gene expression, proliferation, differentiation, apoptosis, and distribution of the Ph^+ cells over the phases of the cell cycle were presented in a polynomial approximation. The alteration of the fluorescence intensity (*Jt*) was used to determine the positions of the peaks of RNA expression and their maximum; *Jt/Jgapdh* was used to assess the relative levels of mRNA expression. Hence, these results can be compared to the data that were obtained by measuring of the expression levels in separate probe, e.g. by a method widely used in other studies.

A polynomial approximation to the 6th power was used to process the curves of gene expression, cell proliferation and differentiation on the grounds that the curves are of a wave-like character with several maxima and minima and obey neither the logarithmic nor exponential law. The following advantages and limitations of the polynomial approximation were taken into account. The optimal number of generalized data is equal to the approximation power minus one. Approximation was considered reliable based on the accuracy of the experimental data ± 10% given in [1–6] ($R^2 \geq 0.81–1$). The number of approximated points could be higher than the approximation power index by one or two. The points belonging to the first growth period (five to eight points in our experiments for a time interval of 8–10 days) are of special importance for characterization of the kinetic curves. Probing after 24 h upon *ex vivo* CPD corresponds to the expected time of development of the cell cycle in animal cells *in vivo*, which is close to 24 h. One or two points were missing at the peak vertex if the kinetics was known (calculated and predicted by software based on the peak start) to allow one to plot the whole kinetic curve.

A morphological analysis was used to plot the kinetic curves of the proliferation and differentiation of Ph^+ granulocytes and their subpopulations, the myeloid cells (blasts, promyelocytes, myelocytes, metamyelocytes, segment and band neutrophils). Cell composition was analyzed using smears (three areas for each smear, each area containing 100 cells). The concentration of cell subpopulations in the probes was determined based on their content in the smears recalculated for 10^6 cells/ml of the original suspension [1–6].

The kinetic curves of the P/D efficiency index (the ratio between the neutrophil proliferation and maturation rates) were obtained as ratios between the accumulation of immature proliferating cells, P (blasts, promyelocytes, myelocytes), and the accumulation of neutrophils maturing without dividing, mature cells, D (metamyelocytes, bands and segments) according to [1–4].

RESULTS

The kinetic curves of the gene expression levels (GEL) of *p53, p21, c-myc, bcr/abl, mdm2, bcl2, bax, bcr*, which participate in the regulation of the cell cycle [14, 24, 28, 45–48, 52, 58, 59], apoptosis [3, 14, 16–22, 28, 42, 47, 49, 50, 56, 58, 60], proliferation and differentiation, were obtained by cultivation of Ph^+ mononuclear cells consisting of 95% myeloid Ph^+ cells; i.e., upon CML-affected myelopoiesis [1–3, 24, 26–28, 42, 43, 46, 48, 51–54, 57–68].

The kinetic curves of the expression of the *c-myc, p53, bcr/abl, mdm2, p21, bcl2, bax, gapdh, actin, bcr* genes were compared to those of the regulation of the proliferation and differentiation of three main types of myeloid Ph^+ cells and their apoptosis and distribution in the phases of the cell cycle. The GEL and CPD curves were obtained using the same probe for each assay.

mRNA expression levels were assessed based on the fluorescence intensity (*Jt*) of the corresponding RT-PCR products of the genes under study. The *gapdh* and *actin* genes were used as the control. The *Jt* value was used to estimate changes in gene expression and peak positions. The values of *Jt/Jgapdh* allow one to

estimate the ratio between gene expressions; however, the positions of the peaks, as well as their maxima and minima, are noticeably altered due to the changes in the *gapdh* expression. Early changes in the expression level of *gapdh* were also observed in other studies [55, 56].

The kinetic curves of gene expression were compared to the regularities of the proliferation and differentiation of the granulocyte populations, the apoptosis and distribution of Ph^+ cells in the phases of the cell cycle, with alternating the proliferation and maturation stages, which regulate the P/D index efficiency of these processes [1–4] and are given in a polynomial approximation. The regularities of the proliferation and differentiation of Ph^+ cells have already been studied [1–6]; the polynomial approximation of these curves is considered here, since they fail to obey either the logarithmic or exponential law, and the kinetic curves corresponding to these dependences have several maxima and minima (*Figs. 1–9*).

According to [1–4], the regularities of the cell distribution over phases of the cell cycle, apoptosis level, and P/D efficiency index for three types of Ph^+ cells obtained from CML patients vary. The cell types, their proliferation, and differentiation differ by the sequence of stage alternations, as well as the number and duration of the stages. This study provides evidence to support the assumption that gene expression shows features of the regulation of the proliferation and differentiation of three types of Ph^+ cells, as well as their apoptosis and distribution over the phases of the cell cycle.

Gene expression upon proliferation and differentiation of type 1 Ph^+ cells

Type 1 Ph^+ cells are characterized by a prolonged proliferation stage (stage 1) at a rate higher than the maturation rate; the concentration of immature cells is higher than that of mature cells for an appreciably long time; P/D^1 index ≥ 1–20. These cells are notable for their enhanced accumulation of myelocytes, promyelocytes, and blasts with a small accumulation of neutrophils maturating without dividing, and active apoptosis of neutrophils [1–3].

Figures *1A–H* show the kinetic plots of the gene expression, proliferation and differentiation of type 1 Ph^+ cells obtained from the BM and PB of CML patients with a moderate proliferative potential and a P/D index = 1–5. It is clear that the peaks with maximum and minimum gene expression in BM cells are clustered in three zones. Based on the peak area, gene expression in these zones can be divided into active and moderately active. Active expression of *bcl2* and *bax* genes can be seen in zone 1 (on days 1–2). The second zone within the range of days 2–7 is characterized by a wide peak of overexpression of the *p53, mdm2*, and *p21* genes with the maximum on days 3–5 (*Figs. 1A–D*, BM cells).

The maximum expression of the *p21, mdm2, p53, actin, gapdh, and c-myc* genes decreases to a different extent within the same range to attain its minimum on days 8–9. The *c-myc, bcr/abl, gapdh, actin, and bcr* genes are expressed less actively in the second zone. All the genes, except for *bcr/abl,* have two expression minima: on days 1–2 and 8–9. In Ph^+ cells obtained from PB, the *p53, p21, mdm2, c-myc, bax,* and *bcl2* genes are overexpressed in a similar manner; however, the peaks of expression of *p21, mdm2, c-myc* and *gapdh* are noticeably narrower (*Figs. 1E,F*).

Overexpression of *p21, mdm2* and *p53* attains a maximum under cell proliferation and differentiation in accordance with cell distribution in the S and G2/M phases; i.e., it occurs in actively proliferating myeloid precursor cells. Expression of these genes decreases to some level to the end of the proliferation and differentiation cycle, with cell death on days 6–7 (*Fig. 1C,G*); it increases again on days 7–8. Meanwhile, expression of *c-myc, bcr/abl* and *gapdh* is moderate. The concentration of proliferating (immature) cells is considerably higher than that of neutrophils (mature cells). Throughout the processes of proliferation and differentiation, the accumulation rate of proliferating cells is higher than that of maturating neutrophils; all cells have a common gene expression maximum corresponding to a high content of immature cells and a rather low content of neutrophils.

Active expression of *p53, mdm2, p21* (*c-myc,* to a lower extent) correlates with changes in cell concentration, cycle regulation, and cell apoptosis on days 3–4 and 7–10 (*Figs. 1A–C, E–G*). Overexpression of *p21, p53, mdm2* and moderate and low expression of the other genes (*actin, c-myc, gapdh, bcr,* and *bcr/abl*) in Ph^+ cells derived from BM should be regarded as gene expression in proliferative pool cells, which actively accumulate in the G1 and S phases of the cell cycle on days 3–4. The G1 phase including the synthesis of cyclins and kinases, formation of their ensembles, and phosphorylation of the Rb protein with the participation of p21 and the proteins responsible for passing the control points of the G1/S transition presumably occurs during this period [23, 24, 58, 64, 67–71]. This stage is accompanied by *p53* overexpression, which means that p53 fully performs its functions; i.e., it regulates transcription, cell cycle and its control points, differentiation, and apoptosis [10–16].

Maximum apoptosis of the cells prepared from bone marrow (~30%) is observed on day 4 and further slightly decreases. Minimum apoptosis is revealed 24 h after a rapid decrease in the beginning. Apoptosis intensity

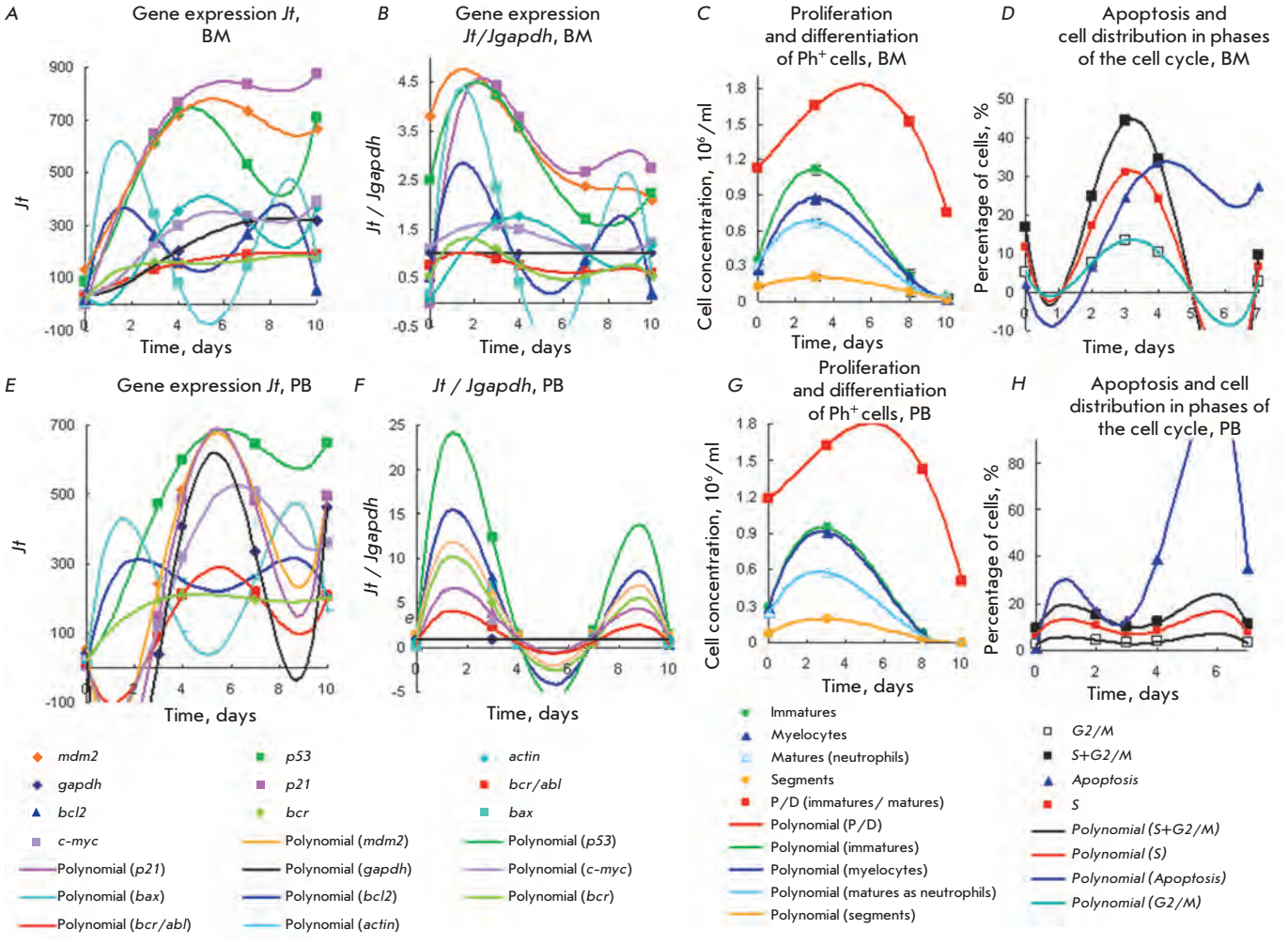

Fig. 1. Expression of *p53*, *mdm2*, *p21*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, *gapdh* , *actin* genes (a, *b*, *e*, *f*) for CML Ph+cells of type 1 represented by a prolonged proliferation stage with moderate proliferation efficiency. Comparison of kinetic plots for the expression levels of these genes with the kinetic plots of proliferation and differentiation (*c*,*g*), apoptosis and cell distribution in the cell cycle (*d*, *h*). Kinetic plots are assayed in the same probes for every process of Ph+ cells from BM (*a*—*d*) and PB (*e*—*h*). Gene expression levels (GEL) are given as fluorescence units *Jt* (*a*, *e*) of total RNA from10⁶cells estimated by RT-PCR and as the *Jt* /*Jgapdh* ratio (*b*, *f*). PD of Ph+ cells (*c*, *g*), apoptosis and cell distribution in cell cycle (*d*, *h*). There are [immature] > [mature] cells and P/D index 1.2–1.8 on days 0–10. Polynomial approximation to the 6th power.

is higher in cells obtained from peripheral blood; it has two GEL maxima on days 1 and 5–6 and a minimum on days 2–3 (*Figs. 1,3*). This does not correlate with GEL of *bcl2* and *bax*, which are responsible for apoptosis [13, 14, 16–22].

Expression of *bcl2* and *bax* in BM cells is characterized by two peaks with maxima on day 2 and a minimum on days 4–5, which is inconsistent with the apoptosis maxima in cells derived from BM and PB (*Figs. 1D,H*). It is a known fact that apoptosis can also be stimulated by actively expressed genes *p21*, *p53*, *gapdh*, *c-myc* [10–28, 32, 34, 49–51, 55, 56, 67–69]. The p21 protein inhibits cyclin-dependent kinases and mediates a number of p53 functions. Expression of *p21* is responsible for cell growth delay during the G1 phase, regulation of the cell cycle, and apoptosis [23–28, 64, 67, 68, 71]. If *p21* overexpression does not cause cell growth delay during the G1 phase, additional p21 molecules induce apoptosis and/or differentiation termination [24, 64, 68]. Apoptosis activation in response to *p21* expression occurs during this phase on day 4, provided that *bcl2* and *bax* are not expressed (*Figs. 1A–D*).

The apoptosis level in PB-derived Ph+ cells at the second peak with a maximum on days 5–6 is significantly

higher than that in BM-derived cells (*Figs. 1D,H*). Comparison of the GEL (*Fig. 1*) reveals a similarity in the expression of the *p53, bcl2,* and *bax* genes in BM and PB cells and a narrower expression peak in PB cells. However, activation of gene expression *bax* > *bcl2* with maxima on days 5–6 is absent in BM cells and does not match the second apoptosis peak observed on day 4. It is assumed that *p21* (which regulates apoptosis, according to [28, 57, 60]) participates in the regulation of this peak in BM cells. *gapdh* is simultaneously overexpressed in PB cells (the expression maximum is observed on days 4–6). Expression of *gapdh* and apoptosis in BM-derived Ph^+ cells increase several-fold (*Figs. 1D,H*).

Overexpression of *mdm2* is associated with the functions of this transcription factor, which modulates the properties of a number of genes and interacts with various growth factors and transcription factors. The mdm2 and p53 proteins mutually interact and negatively regulate the expression of each other [29–36]. Overexpression of *mdm2* presumably modulates the functions of p53 and p21, regulates the duration of the S and G2/M phases of the cell cycle, and enhances the proliferative potential of Ph^+ cells at a weak level of *bcr/abl* expression.

The tumor suppressor p53, which is activated by genotoxic and cellular stress, protects instable cells via the expression of the genes that trigger the cell cycle and inhibit proliferation, blocking apoptosis, and repairing DNA. Meanwhile, p53 and mdm2 activate each other and are simultaneously either stabilized or degraded. Stress-induced activation via a feedback mechanism results in the activation of *p53* and *mdm2* [31–36] and protects cells against death. The interaction between *p53* and *mdm2* is attested by the coincidence of their kinetic plots (*Figs. 1A,D,E,H*) with the maximum of cell accumulation during the S and G2/M phases. *Mdm2* overexpression can be attributed to the activation of the delayed cell transition to the S and G2/M phases of the cell cycle on day 6 (*Fig. 1H*). Mdm2 is known to stimulate uncontrolled cell transition to the S phase [29]. Furthermore, overexpression of mdm2, which directly interacts with the *p53* and *p21* promoters, results in uncontrolled cell transition to the S phase and their transformation [24, 29–31, 67, 68, 71].

The *bcr/abl* expression is known to activate the proliferation of Ph^+ cells [43–48]. In this study, Ph^+ cells derived from BM and PB were characterized by a very low level of *bcr/abl* expression, which was significantly lower than that of *p53, mdm2, p21*, and *c-myc*. Rather low levels of *bcr/abl* expression lie within the zone of the maximum expression of *p53, mdm2, p21*, and even *c-myc* on days 3–10, which is in agreement with proliferation and differentiation efficiency values that are low for type 1 Ph^+ cells (P/D indices = 1.2–1.8–0.8). Expression of *bcr/abl* in PB cells is higher to some extent compared to that in BM cells. A maximum (day 5) and two minima (days 1 and 9) were observed in PB cells. In BM cells, *bcr/abl* expression slowly increases by days 4–10. These differences do not affect the P/D indices, attesting to the fact that the proliferation and maturation rates in cells derived from BM and PB are comparable. Low *bcr/abl* expression with high cell content during the S and G2/M phases can presumably be attributed to the suppression of *bcr/abl* upon overexpression of *p53, p21, mdm2, c-myc*, the main regulators of the cell cycle [10–16, 23–28, 31–36, 51–54, 67, 68]. Expression of these genes is also required for the proliferation of myeloid cells and termination of their differentiation. A decrease in the expression level can be in agreement with the decreasing concentration of immature dividing cells.

It is clear that the peak representing gene expression in PB-derived Ph^+ cells is narrower than that for BM-derived Ph^+ cells (*Figs. 1D–H*). Expression of *p53, bcl2,* and *bax* in PB and BM cells begins immediately and occurs in a similar manner, attaining its maximum on days 2 and 9 (*bcl2*, *bax*) and on day 5 (*p53*). In PB-derived cells, the *p21, mdm2,* and *c-myc* genes are expressed with a 3-day delay; the maximum level of expression corresponds to days 5–6. A rapid decrease in expression with a higher apoptosis peak is subsequently observed in these cells compared to that in BM cells (*Figs. 1D,H*). It is clear that maximum expression of each individual gene (*p21, p53, mdm2* and *c-myc*) corresponds to the maximum content of cells derived from BM and PB during the S and G2/M phases (*Figs. 1D,H*). PB-derived cells are presumably synchronized to a larger extent compared to BM-derived cells.

Based on the *Jt/Jgapdh* ratio (*Fig. 1B*), one can assume that expression of the genes associated with the proliferation of BM-derived cells decreases for the range *mdm2* ~ *p21* ~ *p53* > *actin* ~ *c-myc* > *gapdh* ~ *bcr/abl* ~ *bcr*. A 4.5-fold decrease in GEL corresponding to the peak maxima in BM-derived cells compared to that for *gapdh* is observed. In PB-derived cells, *gapdh* overexpression is combined with an abrupt decrease in the expression levels of other genes; thus, it makes no sense to use the *Jt /Jgapdh* coordinates for comparison.

It is clear from *Fig. 1* that the expression of a number of genes (including *bcr/abl*) correlates with the regularities of the proliferation and differentiation, apoptosis, and distribution of Ph^+ cells in the cell cycle phases. The correlation between the maximum accumulation of proliferating and differentiating cells and gene expression means that the *p21, mdm2, p53, c-myc, bcr, bcl2,* and *bax* genes participate in the regulation of proliferation, differentiation, and apoptosis of type 1 Ph^+ cells. However, expression of these genes cannot be linked to

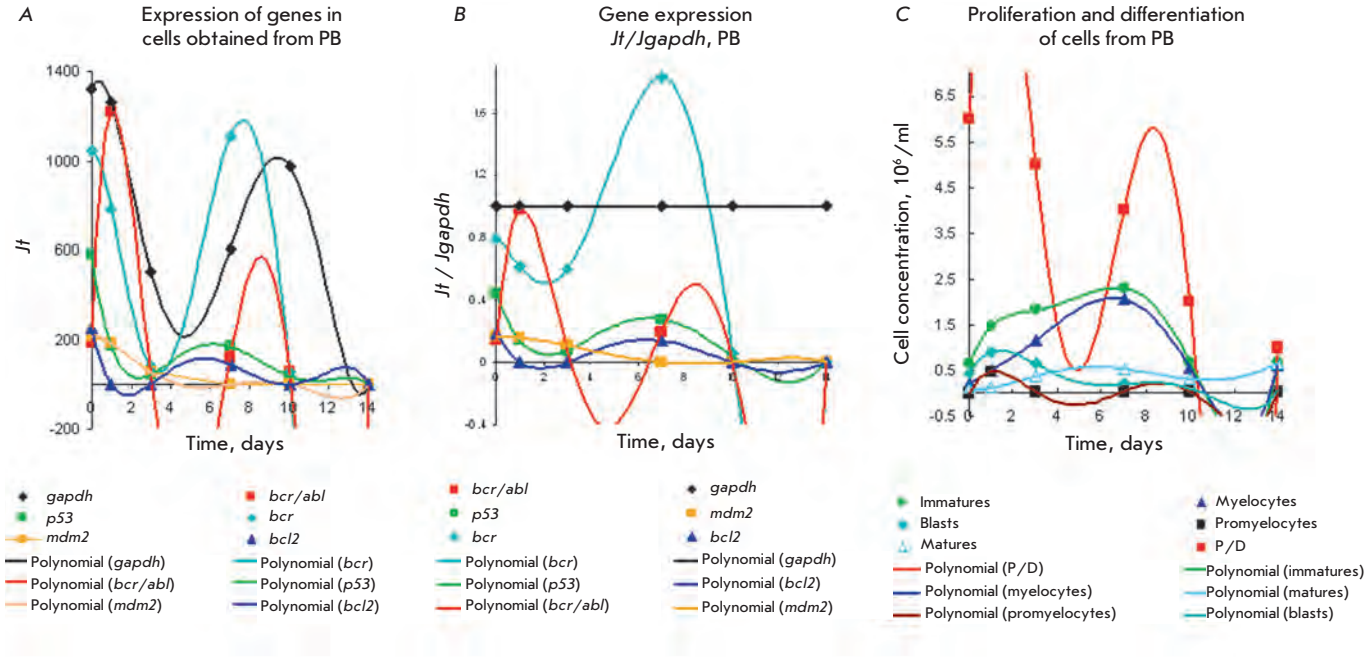

Fig. 2. Gene expression levels of *p53*, *mdm2*, *bcr/abl*, *bcr*, *bcl2*, *gapdh* (*a*, *b*) for type 1 Ph$^+$cells from PB with prolonged proliferation and a high efficiency P/D index =5–12 in comparison with the kinetic plots for proliferation and differentiation (*c*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*). Duration of the proliferation stage with [immature] > [mature] cells is 14 days.

various subpopulations derived from type 1 Ph$^+$ cells, since they are produced by a single peak with the same time maximum.

Overexpression of genes *bcr* > *gapdh* > *bcr/abl* with two maxima on day 1 and days 7–10 and a minimum on days 4–5 can be observed for a sample of type 1 Ph$^+$ cells derived from the PB of a CML patient in blast crisis. These cells possess a high proliferative potential (the efficiency index P/D = 2–12) and a significant content of CD34$^+$ cells [6]. A moderate level of expression of *p53*, *mdm2*, and *bcl2* with maxima on days 0.5, 6, and 9 and minima on days 2–4 and 11 corresponds to a wide proliferation and differentiation peak with the maximum peak of blast cells on days 1–3. Meanwhile, the concentration of immature cells is considerably higher than that of myelocytes. The peak of immature cells increases by days 5–8; however, by this time it mostly consists of myelocytes. The level of *bcr* expression rises, while *bcr/abl* expression decreases (*Figs. 2A–C*).

High levels of *bcr/abl* expression (*Figs. 2A,B*) with two maxima correspond to the profile of the P/D indices, as well as to the accumulation of blasts and myelocytes under proliferation and differentiation (*Fig. 2C*). They also represent the beginning of cycles 1 and 2 of proliferation and differentiation with gene expression in early myeloid precursor cells [6].

Thus, the peak of the P/D index on day 1 and the distribution of gene expression in the range of *gapdh* ~ *bcr/abl* > *bcr* >> *p53* ~ *mdm2* > *bcl2* are typical mostly of blast cells (myeloid precursor cells consisting of ~75% blasts and promyelocytes). It can be seen that the expression level of *p53*, *mdm2*, and *bcl2* is fivefold lower than that of *bcr/abl* and *gapdh*. It is possible that either overexpression of *bcr/abl* and *gapdh* results in the inhibition of *p53*, *mdm2*, and *bcl2;* or a decrease in the expression level of *p53* and *mdm2* causes uncontrolled division of Ph$^+$ cells.

The peak of the proliferation and differentiation of immature proliferating cells on day 7 includes mostly myelocytes; gene expression in the range *bcr* >> *gapdh* >> *p53* > *bcl2* ~ *mdm2* > *bcr/abl* on days 4–6 is also determined by myelocytes. Gene expression in myelocytes and neutrophils subsequently decreases, which is in agreement with the low expression of a number of proteins and growth factors in neutrophils [51, 57, 64, 65, 68, 69].

On the other hand, it is known that protein BCR$_{(64-413)}$, overexpressed in Ph$^+$ cells in CML mice, is phosphorylated by the bcr/abl protein at the tyrosine residue, thus reducing the kinase activity of the bcr/abl oncoprotein by 80% [37–40]. Overexpression of *bcr* (*Fig. 2*) results in significant (but not complete) *bcr/abl* inhibi-

tion. The maximum of the expression peak of *bcr* is observed two days earlier than the maximum of the expression peak of *bcr/abl* and corresponds to high P/D indices = 6–12 and rapid development of a CML blast crisis in the patient [2].

A low level of p53 expression was also observed in the other Ph^+ cells during the acceleration phases and CML blast crisis phases with a high proliferative potential and a P/D index = 3–23. Thus, the level of *p53* expression on day 3 is no higher than that of *gapdh*. In these cells, the expression levels of *bcr/abl*, *mdm2* and *bcl2* are comparable to that of *gapdh*, whereas the *bcr* expression level is twice as high. Ph^+ cells with a high P/D index (obtained from another CML patient) are characterized by a similar gene expression profile. These cells of the CML blast crisis may contain a defective *p53* gene, although mutations in this gene are atypical of CML.

Thus, the composition and level of gene expression are different for type 1 Ph^+ cells with prolonged proliferation, the concentration of immature cells being higher than that of mature cells, and P/D index = 2–20. The cells with P/D index ~ 5–20 are typically characterized by an increased content of blast cells (from $CD34^+$ to promyelocytes) with overexpression of *bcr* > *gapdh* > *bcr/abl* and reduced expression of *p53*, *bcl2* and *mdm*, *p21*< *gapdh*. Activation of *bcr/abl* in myeloid precursor cells is accompanied by a low level of *p53*, *p21*, and *mdm2* expression. The absence of a control performed by the genes regulating proliferation and the cell cycle presumably provides propitious conditions for the active proliferation of bcr/abl^+ cells. These Ph^+ cells may also contain the mutant gene *p53*.

Type 1 Ph^+ cells with a low proliferative potential, P/D ~ 1.2–4, and content of immature cells higher than that of mature cells are characterized by a moderate *bcr/abl* expression with simultaneous overexpression of *p21*, *mdm2*, *p53*, *bcl2*, and *bax*, as well as proliferation and differentiation preferable for this Ph^+ clone. These genes participate in the regulation of the cell cycle; a wide peak on days 2–5 with a maximum on day 3 representing cell distribution over the S and G2/M phases of the cell cycle. This period is characterized by expression of the *p21*, *p53*, and *mdm2* genes and interaction between p53 and mdm2, which mutually regulate each other's expression.

Efficient proliferation with accumulation of immature cells and overexpression of *p21*, *p53* and *mdm2* takes place in type 1 Ph^+ cells. Mature cells (neutrophils) formed during the period from day 3 to day 7 quickly enter apoptosis. The concentration of mature cells diminishes by almost an order of magnitude, which is an additional reason for the decrease in gene expression in the range *p21*> *mdm2*> *p53*. The aforementioned data is evidence of the fact that gene expression of *p21* > *mdm2*> *p53* in the first zone of proliferation and differentiation (days 1–4) of type 1 Ph^+ cells (*Figs.1* and *2*) is 4–4.5 times higher than *gapdh* expression. On days 4–10, when the cell content in the S and G2/M phases is diminised significantly, the expression levels of these genes decrease by 3, 2.5, and 1.5 times as compared to those of *gapdh*, respectively. On days 8–9, the expression levels of these genes on the kinetic plot have a close minimum.

Gene expression upon proliferation and differentiation of type 2 Ph^+ cells

Significant accumulation of neutrophils (in particular, segments, which block apoptosis to a significant extent and inhibit the proliferation of Ph^+ cells) is typical of type 2 Ph^+ cells under the maturation stage. Proliferation and differentiation last for a long time and are characterized by low efficiency ($P/D^2 \leq 1$), a higher maturation rate compared to the proliferation rate, and higher concentration of mature cells (neutrophils) compared to immature ones [1–4].

Type 2 Ph^+ cells *(Figs.3A–D)* were characterized by active expression of the *mdm2* > *p53* gene, a significantly weaker level of expression of *actin* ~ *gapdh*> *p21*> *bcr* > *c-myc* ~ *bcr/abl* > *bax* > *bcl2* (a wide peak with a maximum on day 2), its duration and position of the maxima corresponding to increased (30–40%) cell accumulation in the S and G2/M phases for 3–4 days at a low apoptosis level (2–5%, *Fig. 3D*). The expression levels of the *p21* > *c-myc* ~ *bcr/abl* > *bax*> *bcl2* genes are lower than that of *gapdh*. By the time myelocyte production attains its maximum (days 4–5), expression of the *mdm2* > *p53* genes reaches its minimum (day 4). Meanwhile, neutrophil concentration was twice as high as myelocyte one during the entire observation time (5 days); according to [1, 3], this noticeably slows down the accumulation of immature cells and inhibits proliferation during days 1–5. Despite a higher level of neutrophil accumulation compared to the accumulation of immature cells with an identical time corresponding to their maxima and high cell content in the G2/M and S phases (~40%), the expression level of *mdm2* > *p53* > *gapdh* remains significant.

Under these conditions, the expression levels of *gapdh*, *actin*, *p21*, *bcr*, *c-myc*, and *bax* change negligibly; the level of *bcl2* expression being no higher than half that of the *gapdh* level; this indicator being even lower for the other genes. Thus, despite the fact that the content of neutrophils and myelocytes is high, they have little impact on the expression of these genes. The expression levels of the *p21*, *bcr*, *c-myc*, *bcl2*, and *bax* genes in type 2 cells are 2 to 5-fold lower than those in type 1 cells. This allows to attribute overexpression of the *p53* and *mdm2* genes in type 2 Ph^+ cells to prolifer-

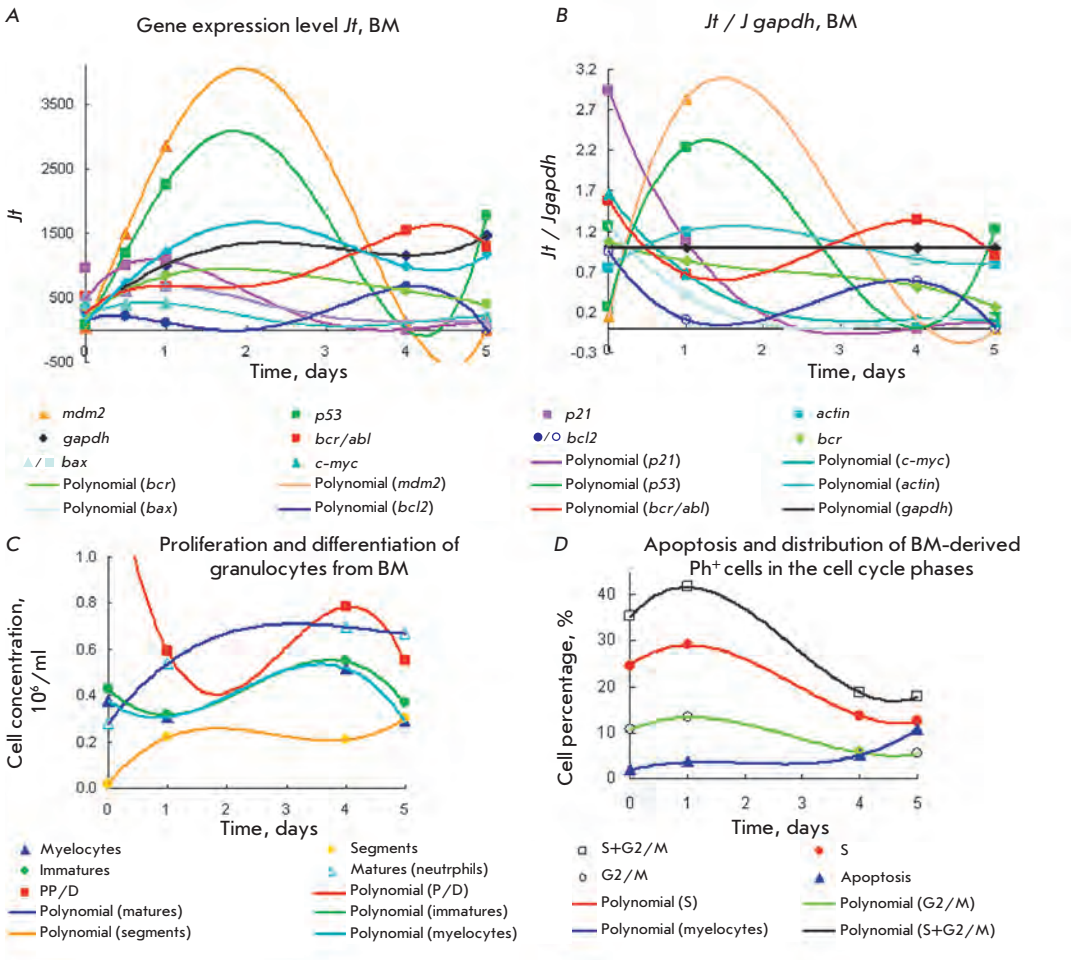

Fig. 3. Expression of the *p53*, *mdm2* and *p21*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, *gapdh*, and *actin* genes (*a*, *b*) for CML Ph^+ cells of type 2 from BM with prolonged maturation stage and a low P/D efficiency index ≤ 1 and [matures] >[immatures]. Comparison of the kinetic plots for the gene expression level (*a*,*b*) with those for proliferation and differentiation (*c*), apoptosis, as well as with cell distribution in cell cycle (*d*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*).

ating cells under the S and G2/M phases rather than to myelocytes and neutrophils under the maturation stage. The expression levels of *mdm2* and *p53* under the S and G2/M phases in both cell types are similar and equal to 4.5 and 2–3 compared to those of *gapdh*.

The maximum levels of *bcr/abl* and *bcl2* expression (appreciably low) correspond to the maximum of the myelocyte peak. In the case of *bcr/abl*, the maximum corresponds to the highest myelocyte accumulation, an increase in the P/D index on day 4, and the maximum level of *bcr/abl* > *gapdh* expression on days 4–5. The *bcr/abl* expression decreases simultaneously with neutrophil accumulation and increases approximately twofold, along with myelocyte production. The low levels of *bax* and *bcl2* correspond to a low apoptosis percentage, in particular for *bcl2* > *bax*, when apoptosis is blocked by *bcl2*. In other words, myelocytes and neutrophils are characterized by a low expression level of the *gapdh* ~ *actin* > *bcr*, *p21*, *bax*, *mdm2*, *p53* and *c-myc* genes, whereas the gene expression level of *bcr/abl* reaches its maximum in myelocytes (*Figs. 3A–C*).

Gene expression upon proliferation and differentiation of type 3 Ph^+ cells

Regulation of the proliferation and differentiation of type 3 Ph^+ cells depends on the order of alternation stages and the alternation scheme (1/2/1 or 2/1/2); i.e., what stage, proliferation (1) or maturation (2), is the first stage in the alternation. According to [1–4], proliferation and maturation are simultaneous processes; however, the rate of the preceding alternating stage is higher compared to the following one. The maximum proliferation rate corresponds to the minimum maturation rate, and vice versa. At the points where the accumulation plots of immature cells and neutrophils intersect, the rates of the stages are identical and their P/D is equal to 1. Thus, stage alternation determines the wave-like process of cell proliferation and differentiation. The alternation of stages according to schemes 1/2/1 or 2/1/2 differs not only by alternating rate decrease (either proliferation or maturation), but also in proliferation inhibition by high neutrophil concentrations under condition [mature] >> [immature] [1–4].

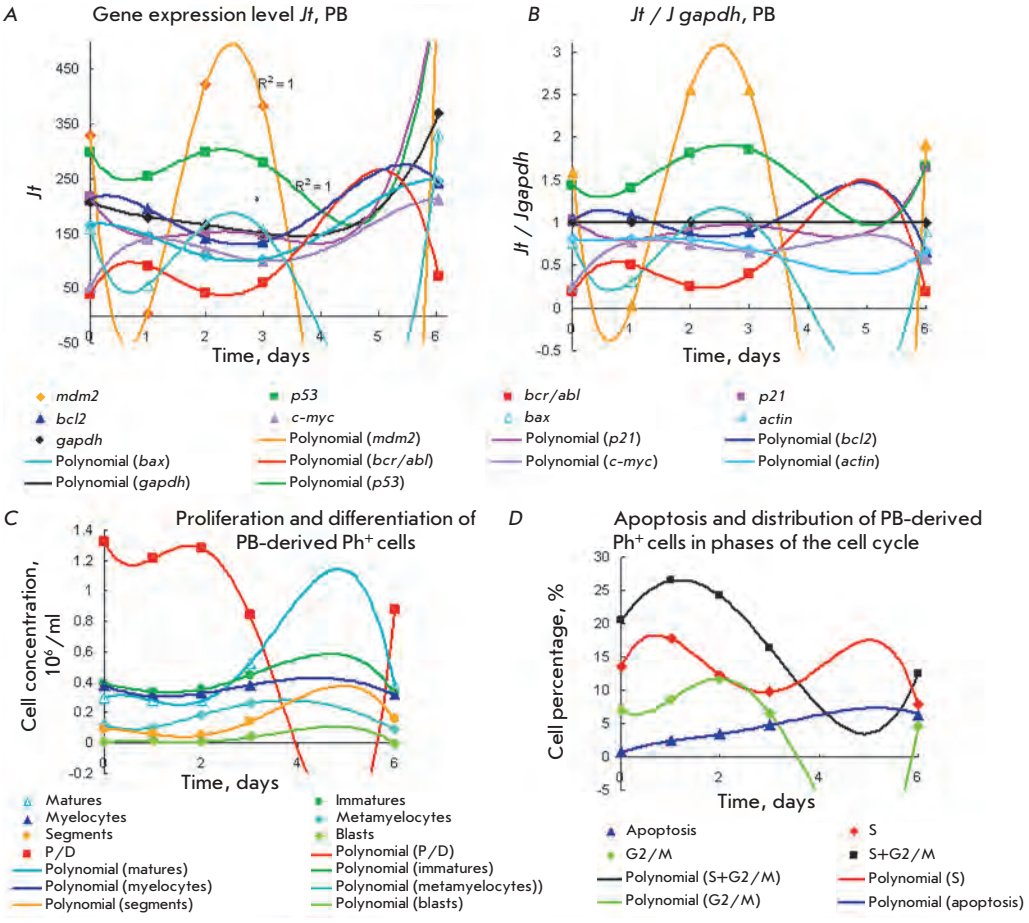

Fig. 4. Kinetic plots for the gene expression levels of the *p53*, *mdm2*, *p21*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, *gapdh*, and *actin* genes (*a* , *b*) for CML Ph+ cells of type 3 from PB with stage alternating according to scheme 1/2. Comparison with the plots for proliferation and differentiation (*c*), apoptosis and cell distribution in the cell cycle (*d*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*). Proliferation stage with [immatures] > [matures] on days 0–3. Maturation stage with [matures] > [immatures] cells occurred on days 3–6.

The character of gene expression in Ph+ cells, as well as their proliferation and differentiation, depends on the order of the alternating stages and on the initial stage.

Gene expression upon alteration of proliferation and maturation according to scheme 1/2/1

Figures. 4A–D show that active gene expression coincides with the maxima of cell distribution during the G2/M + S phases and the maxima of the P/D indices (the maximum on days 2–3, *Figs. 4C,D*). At the first stage (days 0–3), the proliferation and maturation rates differ negligibly (in terms of accumulation of immature cells and neutrophils) without a pronounced maximum (*Fig. 4C*). Approximately on day 3 (after intersecting the accumulation curves of immature and mature cells), the proliferation stage (P/D = 1.4–1.1 and with a concentration of immature cells higher than that of mature cells) proceeds to the maturation stage (days 3–6) with maximum accumulation of neutrophils and their components (metamyelocytes, segments and bands) and decreasing efficiency index ($P/D^2 < 1$). Meanwhile, stage 2 is characterized by a significant (4-fold) increase in neutrophil concentration, attaining its maximum on day 5. The concentration of immature cells and myelocytes increases by only ~20%, also attaining its maximum on day 5. The amount of mature cells is three times higher than that of immature ones (low apoptosis level – 3–7%, *Figs. 4C,D*). It can be seen that cell accumulation during the S phase on day 5 is accompanied by an insignificant increase in their apoptosis, which results in no increase in cell content in the G2/M phase (*Fig. 4D*). It is also clear that a 4-fold increase in the neutrophil content noticeably inhibits proliferation under the maturation stage.

It can be seen in *Figs. 4A–D* that the proliferation stage (1) on days 2–3 corresponds to the expression maxima of *mdm2* > *p53* > *bax* > *p21*, expression minima of *bcl2* > *c-myc* >> *bcr/abl*, the first maximum of the S phase, and the maxima of G2/M, S+G2/M, and the P/D indices. The first maximum of *bcr/abl* expression, minima of *p53*, *mdm2*, and *bax* expression (maxima of mature >> immature > metamyelocytes > segments >> blasts, and rather low apoptosis maximum) corresponds to the expression of minima of *p53* > *p21* >> *bax*, *mdm2* on days 4–5; cell minima in the G2/M,

G2+S phases; P/D index on days 5–6; as well as the expression maxima of *bcr/abl* and *bcl2*. Meanwhile, the maxima of *c-myc* and G2+S and the second minimum of P/D on days 5–6, as well as the expression minima of *p53 ~ p21>> bax*, and G2/M minimum on days 4–5, correspond to peak 2 of the nonproductive S phase (not leading to the G2/M phase) on days 4–6.

Gene expression levels (*Figs. 4A,B*) at the first stage (days 2–3) decrease in the range *mdm2 >> p53 > bax ~ gapdh ~ p21 ~ bcl2 > bcr/abl*, whereas expression of the genes *bcl2, c-myc > bcr/abl* attains its minimum. Stage 2 (day 5) is characterized by expression maxima of *bcr/abl ~ bcl2 > gapdh* and an increase in the level of *actin, p53 ~ p21,* and *c-myc* at the minimum *bax* level. Overexpression of *mdm2 >> p53 >> bax > gapdh* (its maximum being observed on day 2) corresponds to the maximum cell content in the S and G2/M phases. After the end of proliferation and proceeding to the maturation stage, the expression of *p53* and *mdm2* decreases abruptly, whereas the *bcr/abl* and *bcl2* expression increases. At the maturation stage, the maximum expression level (days 4–6) of the genes disposes into the following range: *bcr/abl ~ bcl2 > gapdh ~ actin ~ p21 ~ c-myc*. The maximum levels of *bcr/abl* and *bcl2* expression are observed under insignificant accumulation of immature cells and myelocytes on day 5. Apoptosis is blocked (2–4% and no higher than 7% on days 5–6) upon expression of *bcl2 >> bax*. This emphasizes the role of *bcl2* overexpression compared to low *bax* expression in such a significant suppression of apoptosis (*Figs. 4A,D*). In the case of *bcl2 > bax* or asynchronous maxima and minima of their expression, apoptosis inhibition was also observed in types 1 and 2 Ph^+ cells. An increase in the expression levels of a number of genes by days 5–6 can be regarded as a precursor of the proliferation stage, which follows the maturation stage.

During the proliferation stage, when the content of proliferating cells is just about higher than the neutrophil content, overexpression in the range *mdm2 >> p53> bax > gapdh* corresponds to the maximum of proliferating cells in the S and G2/M phases; small maxima of *bcr/abl ~ bcl2 > gapdh* expression emerge during the maturation stage. The expression levels of the remaining genes are lower than that of *gapdh* during both the proliferation and maturation stages. The expression level of the *mdm2* and *p53* genes increases abruptly under the proliferation stage and rapidly decreases under the maturation stage in accordance with cell percentage in the G2/M phase. This means that *mdm2* expression is significant in proliferating cells and low or completely absent in neutrophils. Active *mdm2* expression can presumably act as a marker of the proliferation stage and cell activation of the cell cycle G2/M phase. The same character of change of the expression maxima of *mdm2, p53,* and *p21* coinciding with the cell maxima in the G2/M phase (*Figs. 5*) has also been observed under the maturation stage with the alternating scheme 2/1.

The *bcr/abl* expression is characterized by two maxima (*Figs. 4A,B*): the maximum of $bcr/abl^1 < gapdh$ under the proliferation stage with the number of immature cells being insignificantly higher than that of mature cells. However, the maturation stage (upon high concentration of mature cells, their content being significantly higher than that of immature cells) is characterized by a maximum expression level in the range $bcr/abl^2 > gapdh$ and $bcr/abl^1 < bcr/abl^2$ (*Figs. 4A–C*). Let us note that *bcr/abl* expression also increases with decreasing GEL of *p53, mdm2*, and *p21* upon proliferation and maturation of Ph^+ cells according to the alternating scheme 1/2. In types 2 and 3 Ph^+ cells, the expression level is $bcr/abl^1 < bcr/abl^2$ (*Figs. 3* and *4*). However, the range of $bcr/abl^1 < bcr/abl^2$, and $bcr/abl^1 > bcr/abl^2$ can also occur in the type 1 Ph^+ cells.

Thus, gene expression correlates with regulation of the proliferation and differentiation of type 3 Ph^+ cells with alternation of the proliferation and maturation stages according to scheme 1/2. In this case, the increased expression level of *p53, mdm2,* and *p21* coincides with the maximum of the S+G2/M phases and corresponds to a low level of *bcr/abl* expression.

Gene expression in Ph^+ cells with stage alternation according to scheme 2/1/2

When the proliferation and maturation stages were alternated according to schemes 2/1–2/1/2/1, sequential changes in the concentration of type 3 Ph^+ cells were observed in the following range: [mature] > [immature] → [immature] > [mature] → [mature] > [immature] (*Figs. 5–9*).

It is clear from *Figs. 5* and *6* that gene expression levels upon maturation and proliferation correspond to a low content of proliferating cells in the phases of the cell cycle (10–20%), whereas apoptosis induction is significant (40–80%). Meanwhile, a high content of neutrophils that are incapable of dividing results in a decrease in the proliferative cell pool in the S+G2/M phases, which is particularly noticeable in *Fig. 6*. This pool does not increase upon proliferation on days 2–6. The cell maximum in these phases does not presumably coincide with a significant accumulation of immature cells during the proliferation stage. However, neutrophils maturating without division naturally decreases the accumulation of proliferating cells in the S and G2/M phases. Meanwhile, gene expression in neutrophils is significantly diminished, and gene expression with increased activity occurs only in the proliferating cell pool of the S + G2/M phases. Thus, neutrophil (mature cells) accumulation resulted in decreasing gene expression.

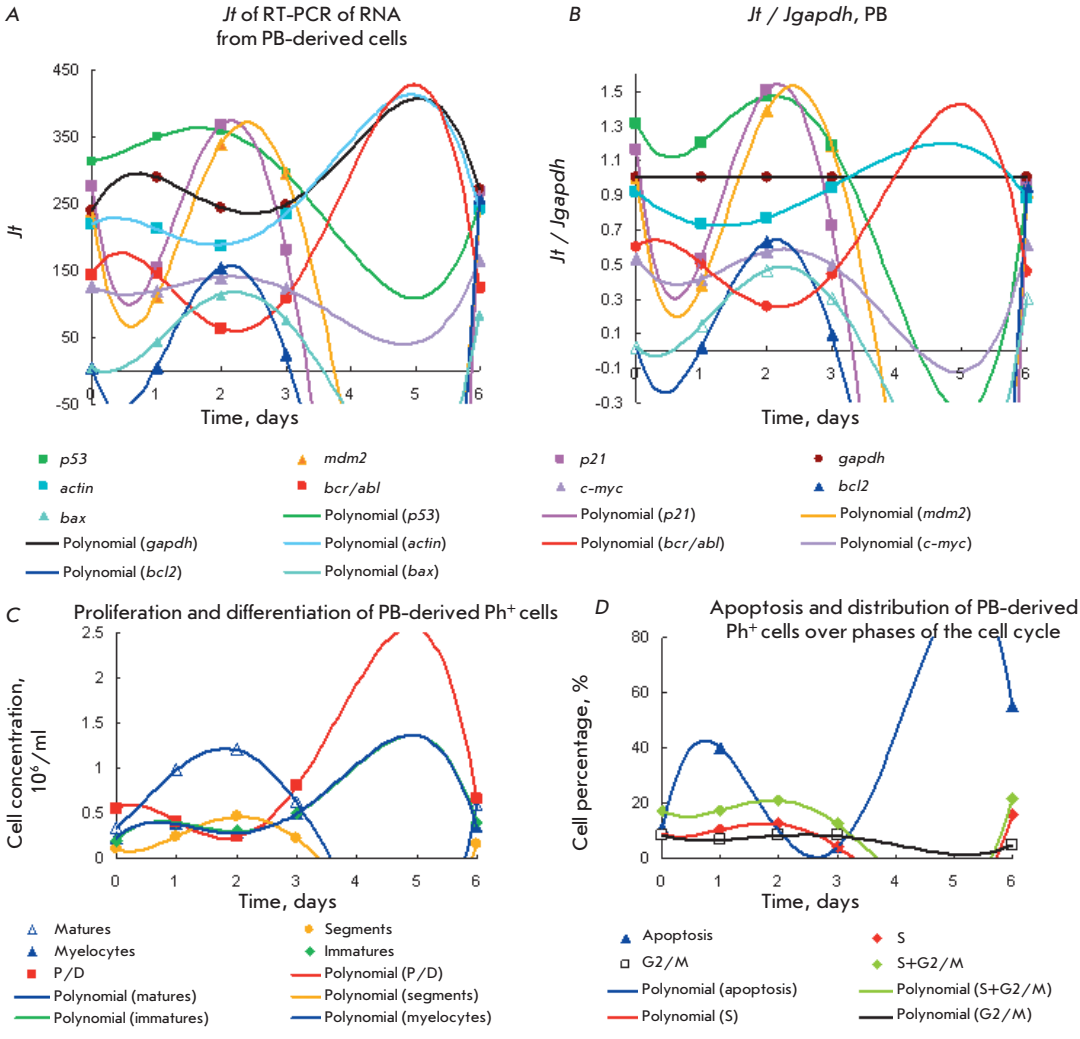

Fig. 5. Gene expression levels of *p53*, *p21*, *mdm2*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, *gapdh*, *actin* (*a*, *b*) for CML type 3 Ph^+ cells from BM with stage alternating according to scheme 2/1/2. Comparison with the kinetic plots for proliferation and differentiation (*c*), apoptosis and cell distribution in the cell cycle (*d*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*). Maturation stage with [matures] > [immatures] occurred on days 0–3 and 6. Proliferation stage with [immature] > [mature] cells occurred on days 3–6.

The expression levels of the genes under investigation are considerably lower here compared to the aforementioned examples, including the expression level compared to *gapdh*.

Upon proliferation and differentiation of Ph^+ cells starting with the maturation stage, along with a significant level of neutrophil accumulation and proliferation inhibition on days 0–3 (*Figs. 5* and *6*), the maximum of neutrophil accumulation corresponds to the minima of the efficiency index P/D and accumulation of immature cells and myelocytes. When proceeding to the proliferation stage on days 3–5, the minima of accumulation of neutrophils (mature cells), a decrease in P/D, and minima of neutrophil accumulation become clear. The concentrations of mature and immature cells in their maxima differ by 4–5 times, which allows one to attribute gene expression to the neutrophils or myelocytes that are incapable of dividing.

Under the maturation stage (*Figs. 5A–D*), the maximum expression of *p21, mdm2, p53* > *bcl2,* >*bax* on day 2 characterizes proliferating cells in the S and G2/M phases (20%) rather than neutrophils, since a 5-fold increase in myelocyte accumulation during the proliferation stage on day 5 results in a decrease in the expression level of these genes to the minimal values.

The maximum expression level of *bcr/abl*, *actin*, *gapdh c-myc* observed on day 5 characterizes myelocytes ($P/D^2 = 2.5$). Two peaks of *bcr/abl* expression (compared to *gapdh*, *Figs. 5A,B*) upon myelocyte proliferation are twice as high as those upon neutrophil maturation (days 5 and 0.5). The minima of gene expression *gapdh* > *actin* > *bcr/abl* can also be seen during the maturation stage (on day 2). This means that expression of the genes regulating the cell cycle in proliferating immature cells is also activated during the maturation stage in accordance with the cell maximum in the S and G2/M phases; however, the expression level is 2- to 3-fold lower than that in types 1 and 2 Ph^+ cells.

The expression levels of the genes *p21* ~ *mdm2* ~ *p53* > *gapdh* in *Fig. 5* are higher than those in *Fig. 6*. *Fig-*

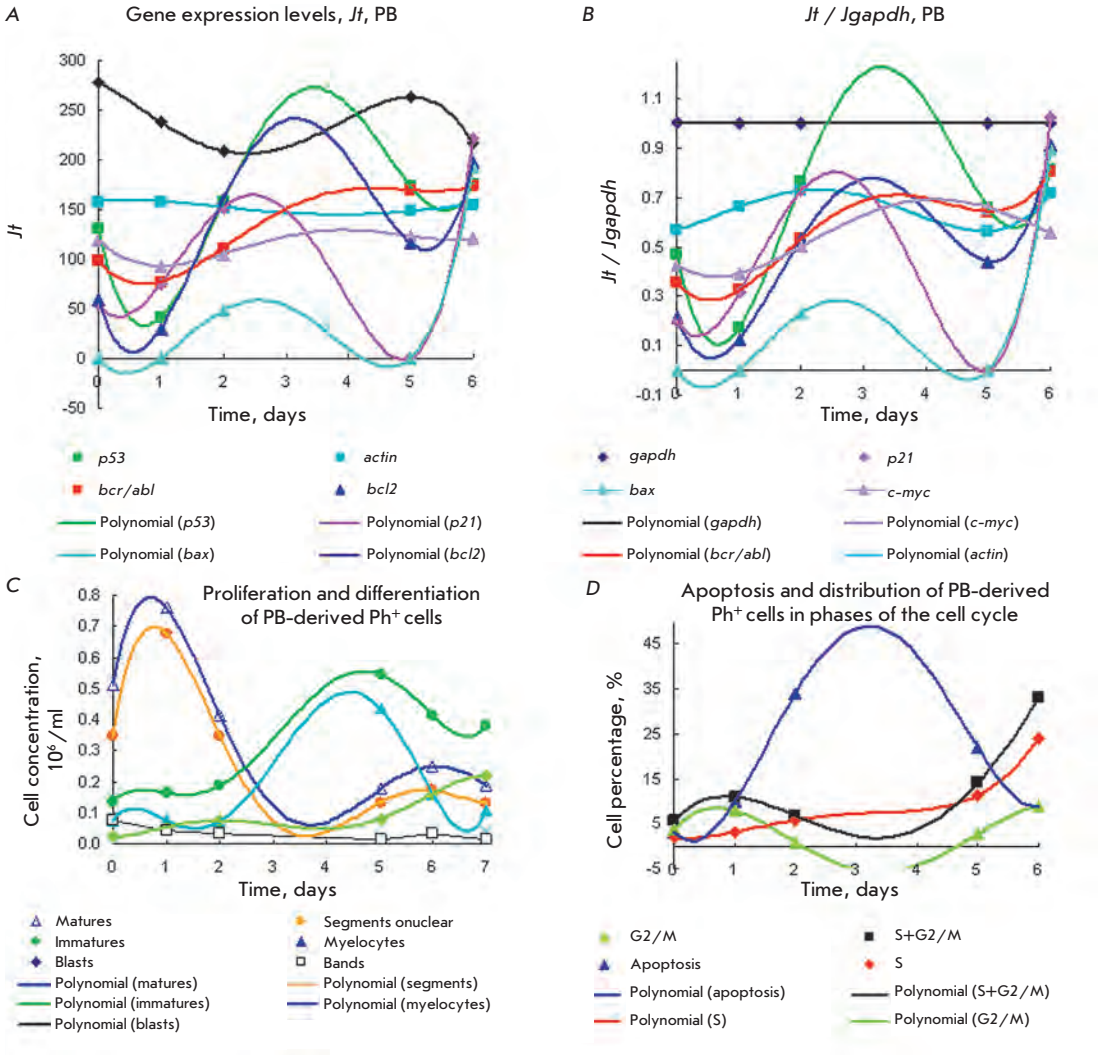

Fig. 6. Gene expression levels of *p53*, *p21*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *bax*, *gapdh*, *actin* (*a* , *b*) for CML type 3 Ph^+ cells from BM with stage alternating according to scheme 2/1/2 in comparison with the kinetic plots for cell proliferation and differentiation (*c*), apoptosis and cell distribution in the cell cycle (*d*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*). Maturation stage with [mature] > [immature] on days 0–3. Proliferation stage with [immature] > [mature] cells occurred on days 3–6.

ure 6 demonstrates that at the maturation stage, the cell content in the S + G2/M phases is twice as low, and that neutrophil content is five times higher than that of immature cells, whereas the content of segments is considerably higher. In other words, increasing neutrophil content results in low content of cells accumulated in the S and G2/M phases and a decrease in the relative levels of expression of the *p21*, *mdm2*, *p53*, and *gapdh* genes (*Figs. 5* and *6*).

Under the proliferation stage with a maximum peak of myelocytes (on day 5) only for the *bcr/abl* and *actin* genes, the expression levels are higher than those of *gapdh*, whereas the expression levels for *p53* > *c-myc* > *bax* > *mdm2* > *p21* are lower. Two maxima of *gapdh* expression correlate with the apoptosis maxima (*Figs. 5A,B,D*). *Figures 6A,B,D* demonstrate that only *p53* and *bcl2* are characterized by a more active expression on days 2–4 compared to *gapdh*. The maximum level of expression of genes *p53* > *gapdh* >> *mdm2* > *p21* on days 2–4 also corresponds to the maximum of the wide apoptosis peak (on days 2–5). This differs from the moderate *gapdh* expression in previously discussed examples of proliferation and differentiation without stage alternation and can be presumably attributed to the participation of *gapdh* in apoptosis induction with the maxima on days 1 and 5. Let us also note that expression of *p53*, *c-myc* and *bcl2*, which is minimal at the proliferation stage on days 3–6, is equal to 0.5–0.7 of the maximum level of *gapdh* expression (*Fig. 6*). It is clear from Fig. 6 that the expression maxima of the *p53* > *mdm2* > *p21* genes on days 2–4 also correspond to the maximum of a wide apoptosis peak (on days 2–5).

It is known that the expression of *p21*, *p53*, *gapdh*, and *c-myc* can be responsible for apoptosis induction [13–16, 20, 21, 28, 55, 56]. At the proliferation stage on days 3–6 in the absence of *bax* and *bcl2* expression, apoptosis is apparently induced by the *gapdh*, *p21*, and *p53* genes (*Figs. 5* and *6*). Let us note that the level of

bcr/abl expression on day 0.5–1 corresponds to a maximum short-term accumulation of myelocytes and immature myelocyte precursor cells. Gene expression, attaining its maximum by day 0.5, changes in the range *p53* > *gapdh* > *actin* > *bcr/abl*. The level of *bcr/abl* expression on days 0–1 is twice as low as that on day 5, which is also caused by proliferation inhibition in Ph^+ cells at an increased neutrophil concentration (*Figs. 5 and 6*).

Thus, the relative changes in the gene expression levels in Ph^+ cells correspond to stage alternation according to scheme 2/1 (from maturation to proliferation). Gene expression is in agreement with the inhibition of proliferation in immature cells by neutrophils maturating without dividing. Gene expression under the maturation stage with a maximum content of neutrophils (in the form of segments under a small content of proliferating cells in the S and G2/M phases) is several-fold lower than that in types 1–3 Ph^+ cells with a maximum proliferative cell pool. In these cases, there is an unambiguous increase in gene expression in actively proliferating cells during the S and G2/M phases, whereas neutrophils as nondividing cells are absent in these phases.

A low expression level of the genes studied in neutrophils can be seen under the maturation stages of types 2 and 3 Ph^+ cells (*Figs. 3–6*), which agrees with the diminished production of a number of proteins and growth factors in neutrophils [51, 57, 64, 65, 68, 69].

It can be seen (*Fig. 6*) that the expression levels of all genes in type 3 cells with the alternating 2/1 stages are diminished under maturation stage upon increased neutrophil content. The expression levels of the *p21* ~ *mdm2* ~ *p53* > *gapdh* > *c-myc* genes under the maturation stage are 3–5-fold lower compared to the proliferation stage (*Figs. 5 and 6*). The character of *p21* and *mdm2* expression is altered. The peaks of expression of these genes, which attains its maximum on days 1 or 2, become narrower, followed by a decrease to the minimum value, along with termination of the S and G2/M phases of the cell cycle.

It should be noted that *bcr/abl* expression considerably increases under proliferation stage with myelocyte accumulation. The *bcr/abl* expression during the neutrophil maturation stage is twice as low as during myelocyte accumulation. The *bcr/abl* expression during the proliferation stage depends on the type and concentration of proliferating myeloid precursor cells (blasts), in which *bcr/abl* expression is presumably suppressed by active expression of *p53, mdm2,* and *p21*. In addition to proliferation inhibition in type 3 Ph^+ cells with alternating scheme 2/1, inhibition of *bcr/abl* expression to its minimum (1.5–3 times lower than that of *gapdh*) occurs under the maturation stage (*Figs. 5–6*). Meanwhile, *gapdh* and *actin* are the only genes that are noticeably expressed in neutrophils in the maturation maximum (days 1–2, segments being the major components). The minimum levels of *c-myc, bcr/abl, p53* > *p21* > *bcl2* > *bax* are 2–10 times lower compared to those of *gapdh* (*Fig. 6A,B*), which is in agreement with the low cell content in the S and G2/M phases (< 12%).

Upon prolonged alternation of the 2/1/2/1 stages for Ph^+ cells with a very low cell content in the S and G2/M phases (2–5%) and active apoptosis, gene expression also correlates with the alternation of the maturation and proliferation stages. Expression of *bcr/abl* > *gapdh* ≥ *c-myc* genes is increased, whereas the expression level of the *mdm2, p53, and bcl2* genes remains low in both the maturation and proliferation stages (*Figs. 7A,B*). The *bcr/abl* expression is characterized by two peaks that are larger than those for the *gapdh* and bcr/abl^1 > bcr/abl^2 genes under the maturation and proliferation stages, respectively (Fig. 7). The maturation stage with a high level of neutrophil accumulation is accompanied by the expression of *bcr/abl* > *gapdh* > *c-myc* > *p53* > *mdm2*, which approaches a minimum by day 5. Under the proliferation stage (days 5–7), the expression levels increase again to their maximum value (on days 7–8), to decrease subsequently with a clear order. Thus, the maxima and minima of Ph^+ cell accumulation during the maturation and proliferation stages alternate in the same manner as the maxima and minima of gene expression in the range *c-myc*, *bcr/abl, gapdh*, *p53*. They correspond to high levels of *bcr/abl* and *c-myc* expression and very low levels of *bcl2* and *mdm2* expression. Rather quick neutrophil accumulation induces suppression of immature cells proliferation, expression of their genes, and a decrease in cell content in the S and G2 phases to 3–5% (*Figs. 7C,D*). The sequence of these events affects the gene expression level in the range *c-myc* ~ *gapdh* ~ *bcr/abl* > *p53* > *mdm2* on days 1–9 (*Figs. 7A–D*).

Two maxima of gene expression can be seen in another example of BM-derived Ph^+ cells (*Fig. 8*) with alternating 2/1/2/1 stages and an increased cell content in the S and G2 phases (~ 30%, with two maxima on days 2 and 6): the first maximum corresponds to the maturation stage (*p21* > *bax* ~ *c-myc* ~ *actin* >> *bcr/abl* and *gapdh* ~ *bcl2* ~ *p53* > *mdm2*), and the second maximum corresponds to the proliferation stage (*c-myc* ~ *p21* >*bax* >> *bcr/abl* > *actin* and *gapdh* > *mdm2* > *p53* > *bcl2*). The second maximum of the expression levels of *p21* > *bax* ~ *c-myc* is an order of magnitude higher than the first one. In Ph^+ cells derived from PB cells (*Fig. 9*) isolated from the same CML patient, the expression of *p21* > *bax* ~ *c-myc* genes was significantly lower than the expression levels in BM-derived cells (*Fig. 8*) and remained high under the proliferation stage

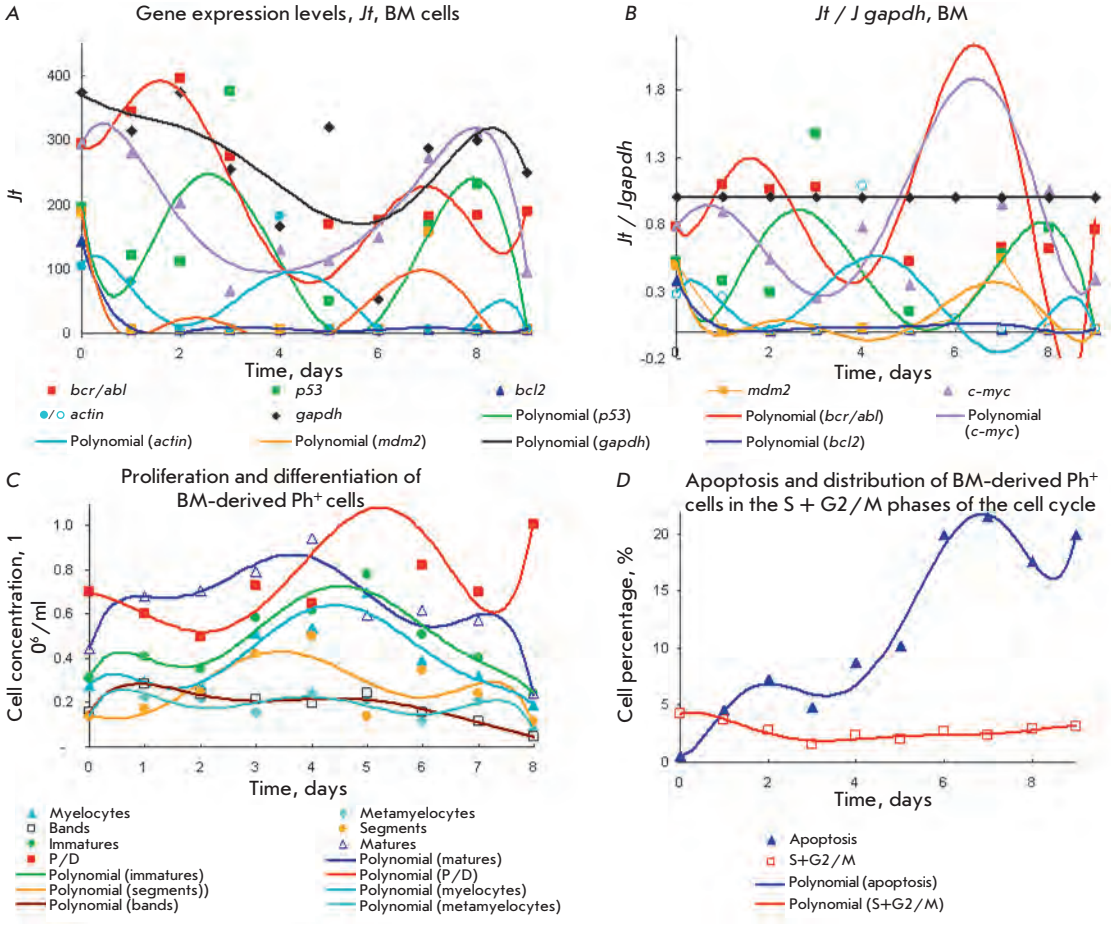

Fig. 7. Expression levels of the *p53*, *mdm2*, *c-myc*, *bcr/abl*, *bcr*, *bcl2*, *gapdh*, and *actin* genes (*a*, *b*) for CML type 3 Ph^+ cells from BM with stage alternating according to scheme 2/1/2. Comparison with the kinetic plots for cell proliferation and differentiation (*c*), apoptosis and cell distribution in the cell cycle (*d*). Details are identical to those in Fig. 1. *Jt* (*a*) and *Jt / Jgapdh* (*b*). Maturation stage with [matures] > [immatures] cells on days 0–5 and days 6–8. Proliferation stage with [immatures] > [matures] cells occurred on days 5–6 and day 8.

after thrice-repeated accumulation of PB neutrophils during the maturation stage. In other words, a significant level of neutrophil accumulation suppresses gene expression during the maturation stages even if the cell content in the S + G2/M phases is increased.

The results shown in *Figs. 7–9* are notable for the fact that *Fig. 7* demonstrates the effect of a long-term excess of neutrophils over immature cells on gene expression and complete suppression of the proliferating cell pool in the S + G2/M phases under a low level of apoptosis. *Figures 8–9* show the suppression of gene expression by neutrophils at the maturation stage, almost coinciding with the cell maxima in the S + G2/M phases (30%). However, proceeding to proliferation with a significant accumulation of immature proliferating cells under conditions of 50–80% apoptosis induction (which previously was 10–20%) results in the formation of a second maximum corresponding to the accumulation of the proliferating pool in the S + G2/M phases. Expression of the *p21* > *bax* ~ *c-myc* > *bcr/abl* > *mdm2* genes increases by an order of magnitude at the minima of *p53* > *bcl2* expression. In other words, neutrophils are capable of suppressing and delaying the formation of the proliferating cell pool in phases of the cell cycle and/or suppressing the expression of proper genes. These terms can also be used to interpret the results shown in *Figs. 4–6*.

Thus, gene expression in neutrophils and myelocytes under proliferation and differentiation with stages alternating according to the scheme 2/1 – 2/1/2/1 is in agreement with the types of cell regulation by stage alternation, apoptosis, and distribution of CML Ph^+ cells in the cell cycle phases. This provides additional support to the argument that neutrophils block apoptosis and inhibit Ph^+ cell proliferation. The gene expression levels under the maturation stages are determined by the maximum level of cell accumulation in the S and G2/M phases of the cell cycle and by inhibition of proliferation by neutrophils. The coincidence of the maxima of cell accumulation in the S + G2/M phases and during the proliferation stage attests to their contribution to the 1.5–7-fold rise in the expression levels of *p21*, *mdm2*, *p53*, *bax*, *c-myc*.

The expression levels of the other genes in neutrophils under the maturation stage are 2- to 10-fold lower than that of *gapdh* gene. This expression level is

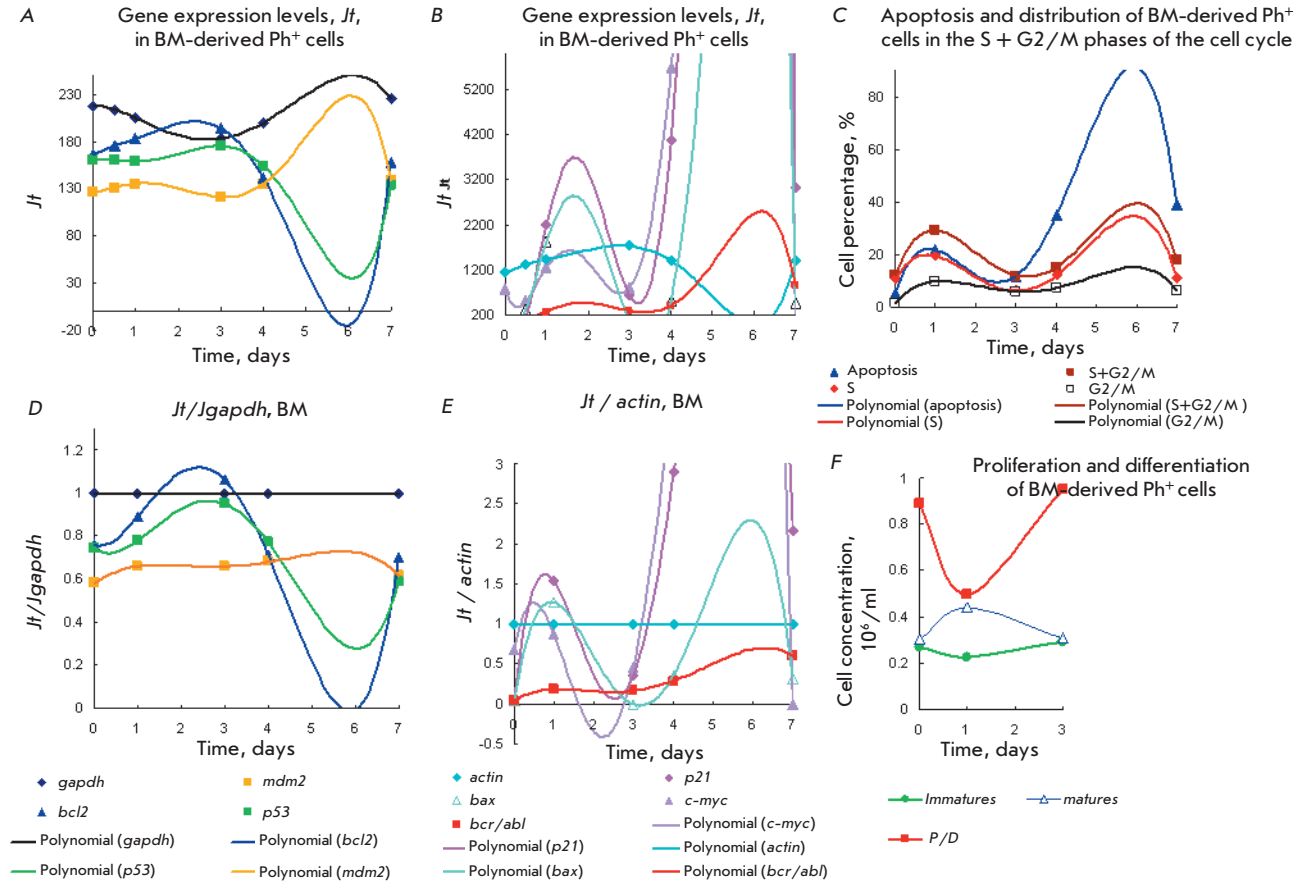

Fig. 8. Kinetic plots for the gene expression levels of *p21*, *c-myc*, *bcl2*, *p53*, *mdm2*, *bcr/abl*, *bax*, *gapdh*, *actin* (*a*, *b*, *d*, *e*) for type 3 CML Ph+ cells from BM with stage alternating according to scheme 2/1 in comparison with the kinetic plots for apoptosis and cell distribution in the cell cycle (*c*), as well as that for cell proliferation and differentiation (*f*). Details are identical to those in Fig. 1. *Jt* (*a*, *b*) and *Jt / Jgapdh* (*d*), *Jt / Jactin* (*e*). Maturation stage with [mature] > [immature] cells occurred on days 0–3. Proliferation stage with [immature] > [mature] cells occurred on days 3–7.

comparable to those in type 2 cells and is 5- to 10-fold lower than the expression levels in type 1 immature cells.

DISCUSSION

The kinetic curves of the expression of 10 genes that regulate the proliferation and differentiation, the cell cycle, and apoptosis were determined in hematopoietic cells containing the Ph chromosome and the *bcr/abl* oncogen, which were derived from CML patients. The expression of the main cell cycle regulators (*p53*, *mdm2*, *p21*, *c-myc*, *bcr/abl*, *bax*, *bcl2*, and *gapdh*) in differentiating proliferating myeloid Ph+ cells and neutrophils maturating without dividing correlates with the regulation of proliferation and differentiation processes, with apoptosis induction, and distribution in the phases of the cell cycle *ex vivo*. It has been demonstrated by comparing the kinetics of gene expression and regularities of the regulation of the proliferation and differentiation of Ph+ cells *ex vivo* with the functions of these genes that the genes participate in the regulation of the proliferation and differentiation of three main types of Ph+ cells, as well as in the alternation of the proliferation (1) and maturation (2) stages.

The gene expression levels can be regarded as estimates that are only demonstrating the general trend, since the RT-PCR data were compared with the expression levels of the *gapdh* and *actin* genes, which can be changed themselves (measured in the same probes) upon cultivation instead of using internal reference standards for each individual gene.

It has been revealed that gene expression changes synchronously with proliferation and differentiation regulation, cell cycle phases, and apoptosis. This fact demonstrates that the genes under consideration participate in the regulation of the proliferation and

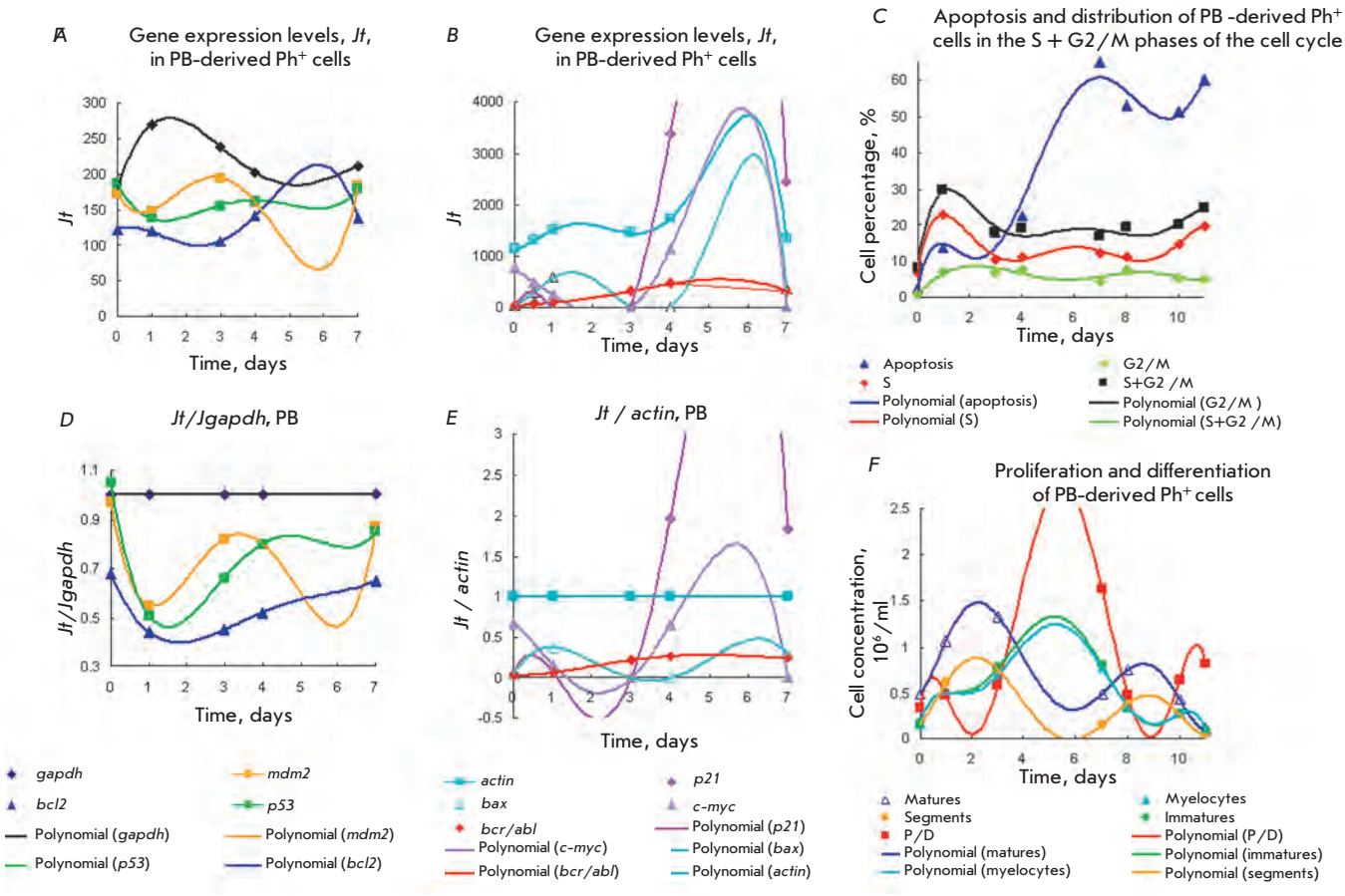

Fig. 9. Gene expression of *p21*, *c-myc*, *bcl2*, *p53*, *mdm2*, *bcr/abl*, *bcl2*, *bax*, *gapdh*, *actin* (*a*, *b*, *d*, e) in comparison with the kinetic plots for apoptosis and cell distribution in the cell cycle (*c*), as well as that for cell proliferation and differentiation (*f*) for CML type 3 Ph^+ cells from PB with stage alternating according to scheme 2/1/2/1. Details are identical to those in Fig. 1. *Jt* (*a*, *b*) and *Jt / Jgapdh* (*d*), *Jt / Jactin* (e). Maturation stage with [matures] > [immatures] cells occurred on days 0–4. Proliferation stage with [immatures] > [matures] cells occurred on days 4–7.

differentiation of proliferating myeloid Ph^+ cells and maturating neutrophils. The results obtained are in agreement with the available data pertaining to the regularities of these genes' expression in other cells. The data also correspond to the regularities of proliferation and differentiation, cell cycle, and apoptosis in other systems. This attests to the fact that these methods and the kinetic plots obtained by RT-PCR can be used to study gene expression. A low level of gene expression in neutrophils is in agreement with low production of the p21 protein, a number of specific proteins, and a number of factors in haematopoietic neutrophils [51, 57, 64, 65, 68, 69].

The kinetic approach to the study of gene expression using RT-PCR by comparison with the kinetics of cell proliferation and differentiation in a polynomial approximation appears to be a rather informative approach to investigating the regulation of proliferation and differentiation, the cell cycle, and the apoptosis of haematopoietic cells proliferating with differentiation and maturing without dividing. The results obtained allow one to ask new questions that are important for gaining further insight into the gene expression and CML mechanisms. One such question is whether the *p53*, *mdm2*, *p21*, and *c-myc* genes participate in the inhibition of *bcr/abl* expression. The second question is whether *bcr/abl* expression is genotoxic or cellular stress for hematopoietic cells and what is the response of the *p53*, *mdm2*, *p21*, and *c-myc* genes.

The results obtained in this study indicate that a diminished expression of the *p53*, *mdm2*, and *p21* genes, which creates conditions for the uncontrolled expression of *bcr/abl*, promotes an increase in the rate of proliferation and aggressiveness of proliferating Ph^+ cells with a high level of *bcr/abl* expression. On the contrary, overexpression of the *p53*, *p21*, *mdm2*, and *c-myc*

genes (the major cell cycle regulators) presumes suppression of *bcr/abl* expression in Ph^+ cells and formation of bcr/abl^+ cells.

CONCLUSIONS

1. Expression of the *p53, mdm2* and *p21, c-myc, bcr/abl, bcr, bcl2, bax, gapdh, actin* genes contributes to the total program of *ex vivo* regulation of the proliferation and differentiation of CML Ph^+ cells.

The expression of these genes is in agreement with the proliferation and differentiation of Ph^+ cells of three types and their regulation via alternation of the proliferation (1) and maturation (2) stages according to the schemes 1/2/1 and 2/1/2 and with proliferation and differentiation at either the proliferation (type 1) or maturation (type 2) stage.

2. The *p53, p21, mdm2* >> *gapdh* genes are overexpressed in the actively proliferating myeloid precursor cells accumulating in the S and G2/M phases of the cell cycle. Overexpression of these genes is observed in type 1 cells and when the cell maximum during the S and G2/M phases coincides with the proliferation stage in types 2 and 3 Ph^+ cells. Gene expression is significantly diminished upon maturation and repeated alternation of the proliferation and maturation stages, where neutrophils and myelocytes are accumulated. Alternating according to scheme 2/1/2 results in a decrease in cell content in the S and G2/M phases of the cell cycle.

3. The expression level in neutrophils under the maturation stage decreases in the range *gapdh* > *actin* > *c-myc, bcr/abl, p21* > *p53* > *bcl2* > *bax*; the expression level of these genes in myelocytes is also lower than the expression level of *gapdh*.

4. Expression of the *bcr/abl* gene in types 2 and 3 Ph^+ cells has two peaks, decreasing under the maturation stage as apoptosis is blocked and neutrophils accumulate and increasing 2- to 3-fold under the proliferation stage with myelocyte accumulation. Overexpression of the *p53, mdm2, p21,* and *c-myc* genes and cell maximum in the S and G2/M phases of the cell cycle correspond to a minimum level of *bcr/abl* expression.

5. The maturation stage involves apoptosis inhibition, neutrophil accumulation, and a decrease in the expression level of the *p53, mdm2* and *p21, c-myc,* and *bcr/abl* genes. Apoptosis in Ph^+ cells is induced by gene expression of *bax* > *bcl2, p53, p21, c-myc* and *gapdh*.

6. Overexpression of the genes *bcr* > *gapdh* > *bcr/abl* and diminished expression of *p53, bcl2, mdm, p21* < *gapdh* are observed in type 1 Ph^+ cells derived during the blast crisis and the CML acceleration phase with the efficiency indices P/D ~ 5–20 and a high $CD34^+$ cell content. Overexpression of *bcr/abl* in myeloid precursors is accompanied by low expression of the *p53, p21, mdm2* genes. It was assumed that the decrease or absence of control over the genes encoding the regulators of proliferation, differentiation, and the cell cycle promotes *bcr/abl* overexpression and active production of bcr/abl^+ cells.

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Experimental articles should not exceed 30,000 symbols (20 pages in A4 format, including tables and references). They should contain no more than ten figures. Lengthier articles can only be accepted with the preliminary consent of the editors.

A short report must include the study's rationale, experimental material, and conclusions. A short report should not exceed 12,000 symbols (8 pages in A4 format including no more than 12 references). It should contain no more than four figures.

The manuscript should be sent to the editors in electronic form: the text should be in Windows Microsoft Word 2003 format, and the figures should be in TIFF format with each image in a separate file. In a separate file there should be a translation in English of: the article's title, the names and initials of the authors, the full name of the scientific organization and its departmental affiliation, the abstract, the references, and figure captions.

MANUSCRIPT FORMATTING

The manuscript should be formatted in the following manner:

- Article title. Bold font. The title should not be too long or too short and must be informative. The title should not exceed 100 characters. It should reflect the major result, the essence, and uniqueness of the work, names and initials of the authors.
- The corresponding author, who will also be working with the proofs, should be marked with a footnote *.
- Full name of the scientific organization and its departmental affiliation. If there are two or more scientific organizations involved, they should be linked by digital superscripts with the authors' names. Abstract. The structure of the abstract should be very clear and must reflect the following: it should introduce the reader to the main issue and describe the experimental approach, the possibility of practical use, and the possibility of further research in the field. The average length of an abstract is 20 lines (1,500 characters).
- Keywords (3 – 6). These should include the field of research, methods, experimental subject, and the specifics of the work. List of abbreviations.
- INTRODUCTION
- EXPERIMENTAL PROCEDURES
- RESULTS AND DISCUSSION
- CONCLUSION

  The organizations that funded the work should be listed at the end of this section with grant numbers in parenthesis.
- REFERENCES

  The in-text references should be in brackets, such as [1].

RECOMMENDATIONS ON THE TYPING AND FORMATTING OF THE TEXT

- We recommend the use of Microsoft Word 2003 for Windows text editing software.
- The Times New Roman font should be used. Standard font size is 12.
- The space between the lines is 1.5.
- Using more than one whole space between words is not recommended.
- We do not accept articles with automatic referencing; automatic word hyphenation; or automatic prohibition of hyphenation, listing, automatic indentation, etc.
- We recommend that tables be created using Word software options (Table → Insert Table) or MS Excel. Tables that were created manually (using lots of spaces without boxes) cannot be accepted.
- Initials and last names should always be separated by a whole space; for example, A. A. Ivanov.
- Throughout the text, all dates should appear in the "day.month.year" format, for example 02.05.1991, 26.12.1874, etc.
- There should be no periods after the title of the article, the authors' names, headings and subheadings, figure captions, units (s – second, g – gram, min – minute, h – hour, d – day, deg – degree).
- Periods should be used after footnotes (including those in tables), table comments, abstracts, and abbreviations (mon. – months, y. – years, m. temp. – melting temperature); however, they should not be used in subscripted indexes (T_m – melting temperature; $T_{p.t}$ – temperature of phase transition). One exception is mln – million, which should be used without a period.
- Decimal numbers should always contain a period and not a comma (0.25 and not 0,25).
- The hyphen ("-") is surrounded by two whole spaces, while the "minus," "interval," or "chemical bond" symbols do not require a space.
- The only symbol used for multiplication is "×"; the "×" symbol can only be used if it has a number to its right. The "·" symbol is used for denoting complex compounds in chemical formulas and also noncovalent complexes (such as DNA·RNA, etc.).
- Formulas must use the letter of the Latin and Greek alphabets.

- Latin genera and species' names should be in italics, while the taxa of higher orders should be in regular font.
- Gene names (except for yeast genes) should be italicized, while names of proteins should be in regular font.
- Names of nucleotides (A, T, G, C, U), amino acids (Arg, Ile, Val, etc.), and phosphonucleotides (ATP, AMP, etc.) should be written with Latin letters in regular font.
- Numeration of bases in nucleic acids and amino acid residues should not be hyphenated (T34, Ala89).
- When choosing units of measurement, SI units are to be used.
- Molecular mass should be in Daltons (Da, KDa, MDa).
- The number of nucleotide pairs should be abbreviated (bp, kbp).
- The number of amino acids should be abbreviated to aa.
- Biochemical terms, such as the names of enzymes, should conform to IUPAC standards.
- The number of term and name abbreviations in the text should be kept to a minimum.
- Repeating the same data in the text, tables, and graphs is not allowed.

GUIDENESS FOR ILLUSTRATIONS

- Figures should be supplied in separate files. Only TIFF is accepted.
- Figures should have a resolution of no less than 300 dpi for color and half-tone images and no less than 500 dpi.
- Files should not have any additional layers.

REVIEW AND PREPARATION OF THE MANUSCRIPT FOR PRINT AND PUBLICATION

Articles are published on a first-come, first-served basis. The publication order is established by the date of acceptance of the article. The members of the editorial board have the right to recommend the expedited publishing of articles which are deemed to be a priority and have received good reviews.

Articles which have been received by the editorial board are assessed by the board members and then sent for external review, if needed. The choice of reviewers is up to the editorial board. The manuscript is sent on to reviewers who are experts in this field of research, and the editorial board makes its decisions based on the reviews of these experts. The article may be accepted as is, sent back for improvements, or rejected.

The editorial board can decide to reject an article if it does not conform to the guidelines set above.

A manuscript which has been sent back to the authors for improvements requested by the editors and/or reviewers is reviewed again, after which the editorial board makes another decision on whether the article can be accepted for publication. The published article has the submission and publication acceptance dates set at the beginning.

The return of an article to the authors for improvement does not mean that the article has been accepted for publication. After the revised text has been received, a decision is made by the editorial board. The author must return the improved text, together with the original text and responses to all comments. The date of acceptance is the day on which the final version of the article was received by the publisher.

A revised manuscript must be sent back to the publisher a week after the authors have received the comments; if not, the article is considered a resubmission.

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The journal uses a numeric reference system, which means that references are denoted as numbers in the text (in brackets) which refer to the number in the reference list.

For books: the last name and initials of the author, full title of the book, location of publisher, publisher, year in which the work was published, and the volume or issue and the number of pages in the book.

For periodicals: the last name and initials of the author, title of the journal, year in which the work was published, volume, issue, first and last page of the article. Must specify the name of the first 10 authors. Ross M.T., Grafham D.V., Coffey A.J., Scherer S., McLay K., Muzny D., Platzer M., Howell G.R., Burrows C., Bird C.P., et al. // Nature. 2005. V. 434. № 7031. P. 325–337.

References to books which have Russian translations should be accompanied with references to the original material listing the required data.

References to doctoral thesis abstracts must include the last name and initials of the author, the title of the thesis, the location in which the work was performed, and the year of completion.

References to patents must include the last names and initials of the authors, the type of the patent document (the author's rights or patent), the patent number, the name of the country that issued the document, the international invention classification index, and the year of patent issue.

The list of references should be on a separate page. The tables should be on a separate page, and figure captions should also be on a separate page.

The following e-mail addresses can be used to contact the editorial staff: vera.knorre@gmail.com, actanaturae@gmail.com, tel.: (495) 727-38-60, (495) 930-87-07