A Letter from the Editors

We are pleased to offer you the fourth issue of *Actae Naturae*. Soon we will be celebrating our first anniversary, so we can sum up the results of that year. According to our colleagues from Russia and abroad, our journal is getting more and more attention. The two-language format makes the journal available to readers from abroad. We have tried to balance the amount of basic research articles and methods articles. Several of the published reviews have brought considerable interest. We also hope that the material from the Forum section will find readers. Because we want to improve the quality of our journal, any comments and ideas are welcome.

In this volume we are publishing a review concerning the new concept of calcium signaling and its major role in the development of neurodegenerative processes. Enzymological articles devoted to the medical aspects of biocatalysis are also of interest.

The Forum section looks at the problems of financing science in Russia. We hope to receive some feedback on the published articles and think that the material in this section will allow Russian scientists and our compatriots working abroad to have a better understanding of the Russian grant system.

Editorial Board

Docking approaches are further improved by implementing new algorithms of the conformational search and new scoring functions (methods to estimate the free energy of ligand binding). Scoring functions may include either components of molecular mechanics force fields [2] or empirical terms, e.g. hydrogen bonds described by their geometrical parameters [4]. In this work we studied stacking interactions, which usually are not properly taken into account in widely used scoring functions.

THE PARAMETERS OF STACKING INTERACTIONS

Of all the various types of interactions in biomolecular complexes (such as hydrogen bonds, salt bridges, etc.), the stacking of aromatic substances deserves special attention. Most drugs include aromatic fragments in their chemical structure, and stacking often plays a notable role in their recognition by protein-targets. We have recently shown that an explicit account of stacking in scoring functions increases the efficiency of ATP docking [5]. The aromatic interactions were identified by the mutual orientation of two cycles described by geometrical parameters: the height h and displacement d of one cycle relative to the other, and the angle between their planes (Fig. 1).

However, the range of these parameters, which corresponds to the presence or absence of a stacking contact, is still not very well defined and usually taken as arbitrary [6, 7]. Defining it more accurately would assist in developing more efficient scoring functions and should increase the prediction quality of the spatial structures of protein-ligand complexes by molecular modeling methods. With this aim in view, we performed an analysis of the spatial structures of protein-ligand complexes determined experimentally with atomic resolution where ligands contained adenine or guanine as a substructure.

One well-known example of stacking interactions is the parallel packing of purine and pyrimidine nucleobases in DNA [8, 9]. Some aromatic compounds tend to orient perpendicular to each other (T-shaped stacking), as has been shown for amino acids in proteins [7, 10] and for model systems of carbon aromatic cycles (benzene and naphtalene) [11–14]. Besides, such compounds participate in cation–π interactions, where a positively charged group interacts with the negatively charged cloud of aromatic π-electrons [15–17].

Taking all that into account, we analyzed the distribution of geometrical parameters h, d, and α for contacts of adenine and guanine moieties of ligands with the aromatic side chains of receptor amino acids Phe, Tyr, Trp, and His, as well as with the positively charged guanidine group of Arg and amino group of Lys. The results obtained for guanine are presented in Fig. 2.

It can be seen that two distinct orientations are typical for Phe: parallel and perpendicular to the guanine plane (Fig. 2, shown in red and green, respectively). The displacement d lies in the same range 0–3 Å for both types of contacts. Meanwhile, they clearly differ in the value of height h, which is <4.5 for parallel Å and <5.5 Å for perpendicular orientation. Similar distributions were obtained for Tyr, Trp, and His, though the data are scarcer in these cases. However, the T-shaped contact is not as typical for Tyr, Trp, and His as it is for Phe.

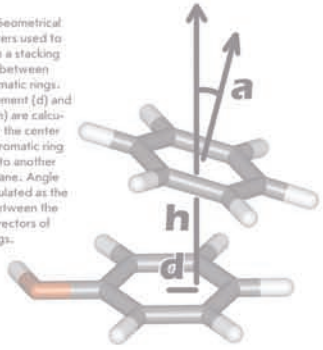
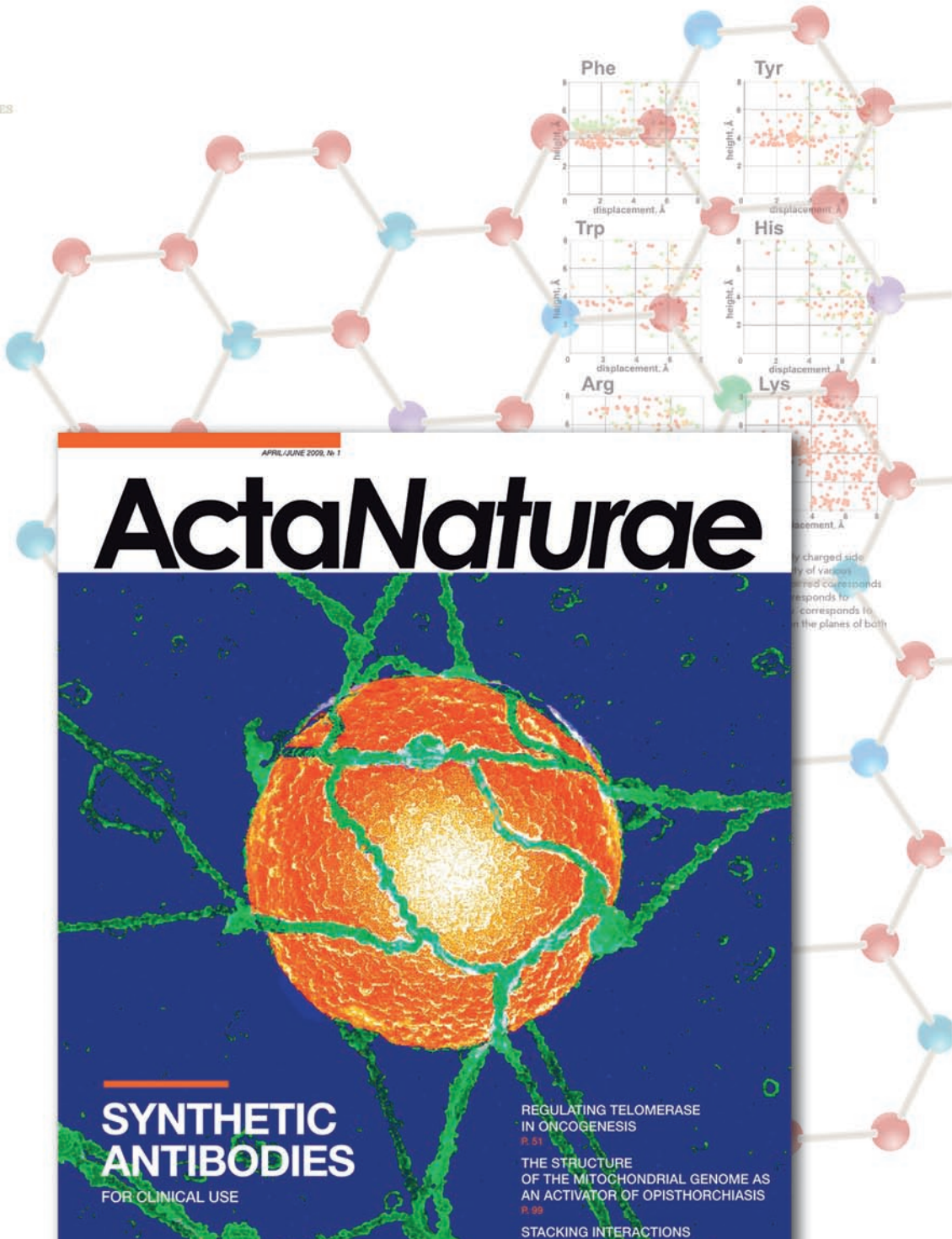


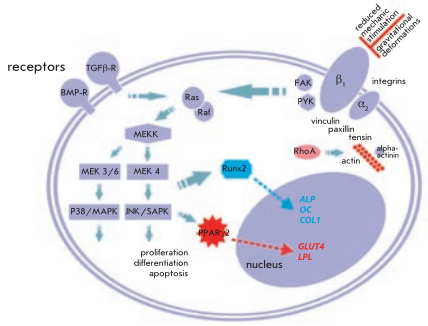
Fig. 1. Geometrical parameters used to describe a stacking contact between two aromatic rings. Displacement (d) and height (h) are calculated for the center of one aromatic ring relative to another ring's plane. Angle (α) is calculated as the angle between the normal vectors of both rings.



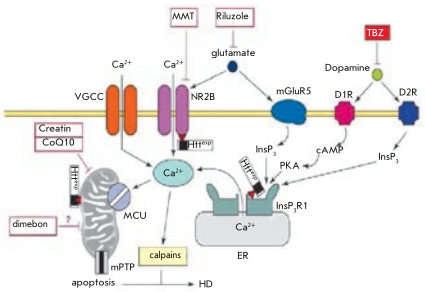
L.B. Buravkova, P.M. Gershovich, J.G. Gershovich, A.I. Grigor'ev

Mechanisms of Gravitational Sensitivity of Osteogenic Precursor Cells

This review presents a thorough analysis of current data concerning the mechanical and gravitational sensitivity of osteoblasts and osteogenic precursor cells *in vitro*



Molecular regulation of the proliferation and differentiation of MMSCs under the control of an extracellular mechanical field



A model of calcium deregulation during Huntington's disease

I.B. Bezprozvanny

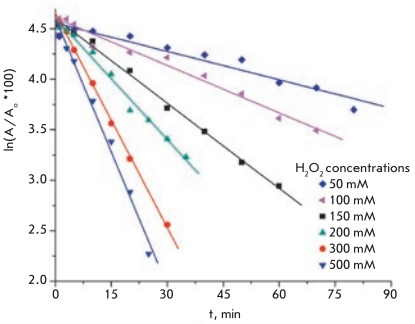
Calcium Signaling and Neurodegenration

This review discusses the results in favor of the calcium hypothesis of neurodegenerative diseases. According to this hypothesis, atrophic and degenerative processes in the neurons of patients with Alzheimer's, Parkinson's and Huntington's diseases and with amyotropic lateral sclerosis and spinocerbellular ataxias are accompanied by changes in calcium homeostasis.

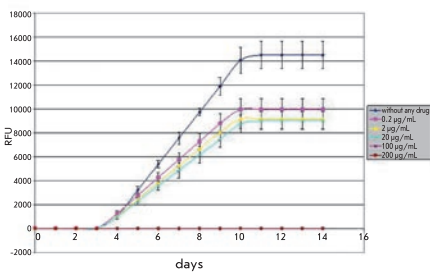
S.S. Savin, V.I. Tishkov

Assessment of Formate Dehydrogenase Stress Stability in vivo using Inactivation by Hydrogen Peroxide

The data obtained in this study confirm the hypothesis that FDH, whose production is increased under stress conditions, should display increased stability against inactivation by hydrogen peroxide. Moreover, this stability is in direct proportion with the severity of the stress experienced by the cell.
The data on the inactivation of purified FDH by hydrogen peroxide can be used for comparative analysis of the stress stability of formate dehydrogenases *in vivo*.



Inactivation of mutant PseFDH Cys255Ala at different hydrogen peroxide concentrations



Experiment on testing the antimycobacterial activity of one of the pyrimidine nucleoside analogs

L.A. Alexandrova, E.R. Shmalenyuk, S.N. Kochetkov, V.V. Erohin, T.G. Smirnova, S.N. Andreevskaya, L.N. Chernousova.

New 5-Modified Pyrimidine Nucleoside Inhibitors of Mycobacterial Growth

This work studies the ability of new pyrimidine nucleoside analogs to inhibit the growth of M.tuberculosis. It demonstrates that 2'-deoxy-, 3'-azido-2',3'-dideoxy and 3'-amino-2',3'-dideoxy derivatives of uridine and cytidine, which bear lengthy methyloxyalkyl sidechains at the 5' position of the base can inhibit the growth of a M.tuberculosis H37Rv culture in vitro.

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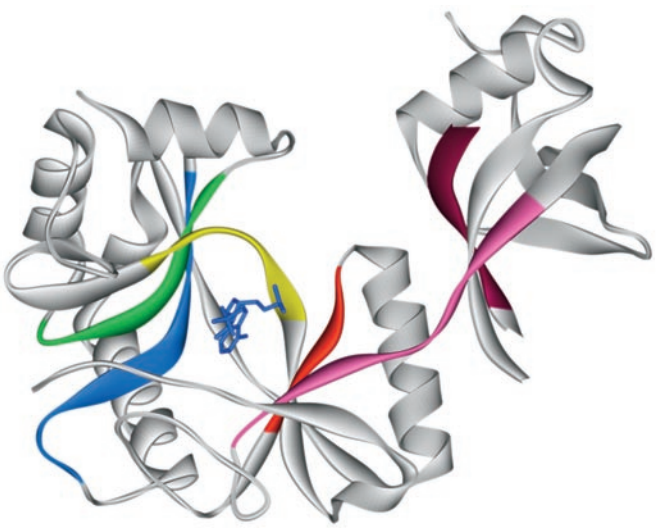

IMAGE ON THE COVER PAGE

The "Molecular and Cell Biology" Program of the Presidium of the Russian Academy of Sciences as an Effective Format for the Support of Promising Scientific Research Groups

V.V. Sychev

There are various ways to finance science in Russia, both governmental and private. Financial support can range from tens of thousands of rubles up to several million in stipends and grants. One of the questions most often addressed to the heads of agencies or funds is about the level of transparency and objectivity when selecting groups which receive financial support. Few well-known financing organizations have avoided criticism regarding this issue.
Nevertheless, there is one scientific financing program that has earned the right to be called one of the most transparent and objective programs of its kind. This is the "Molecular and Cell Biology" program of the Presidium of the Russian Academy of Sciences (RAS), which is commonly named the Georgiev program after its coordinator, RAS academy member Georgiy Georgiev. According to numerous specialists, this is the best program in our country.

It has been 8 years since the Presidium of the RAS decided to launch an experimental program for awarding the most promising research groups with large sums of money. The idea behind this plan was to support leading scientific groups and bring the working conditions of these groups closer to international laboratory standards. The plan was to finance specific laboratories and not whole institutes, because it was clear that each institute had laboratories that differ in their success rates. Therefore, it was prudent to finance specialists which have proven their high qualifications. There was another factor in support of such an experiment. Science receives much more financial support in the United States and Western Europe than in Russia, which means that, in order to keep the scientific potential and talented (especially young) minds in the country, scientists must be offered financially comfortable conditions. It was suggested that the principal investigators could decide if their priorities were selectively high wages or reagents and equipment. Where should this new system of scientific financial support begin? The founders of the program had a clear understanding that they needed to avoid repeating the situation with the planned budget financing of research institutes. First of all, the division of finances between different organizations was somewhat arbitrary. Secondly, after the money was received by the granted organization, it was completely controlled by the administration. Both of these circumstances were considered unacceptable. However, the key issue was the same as in every competition. How and by whom would the selection of worthy research groups be accomplished? Various fields of science have fairly objective criteria for such a selective process, which are scientometric parameters: the impact-factor (IF) of the journal in which the author publishes his or her articles and the citation index (CI). The citation index shows how often a certain article is referenced by other researchers, thus quantifying the informational consequences this article has achieved. Of course this index is influenced by many random factors and it often biases the real value of an article. The impact factor shows how often an article published in a certain journal is cited annually, thus establishing a hierarchy of scientific journals. The higher the IF of a journal, the stricter the requirements towards the articles to be published in it are, thus making it harder to get published and increasing the estimated value of a work published in such a journal. Strict reviewing of articles in such

journals is, for all practical purposes, an international expert committee for the evaluation of scientific merit, which is a very actively discussed topic. One of the fields where scientometric indices can be used as an objective indicator of the "promise" of a certain author or group is physico-chemical biology, which is a major part of modern life sciences. This is why the RAS Presidium created a special program in order to support scientists in this field. The program was initially called "Physico-Chemical Biology" (the name was changed in 2006 to "Molecular and Cell Biology" (MCB)), and RAS member Georgiy Georgiev became its coordinator. "We have three types of competitions" explained Georgiy Georgiev on the principles of finance distribution. "One competition is strictly for fundamental research, another is for socially oriented projects. These are aimed at solving important practical problems, but the final result remains unpredictable, as in any fundamental research project. Finally, the third competition is for the support of so-called new groups." The award for new group is a special part of the program. Commonly, a certain bright and productive young researcher emerges from a laboratory and makes a bid for independence and for the fulfillment of his own projects and ideas. Such a researcher can make arrangements with the administration of any RAS institute to provide him with a work space and a slot (slots) if he wins the MCB grant. In this case we mean supporting the work-force reserve (or life science "growth points" so to speak). This type of competition is also available to our fellow countrymen who reside abroad, although they do not receive any preferential treatment. The program also requires the head of the project to spend no less than 9 months of the year in a Russian laboratory, which practically means that the scientist in question must repatriate.

The amount of funds granted to specific projects varies depending on the type of competition. Until recently, fundamental and fundamentally-oriented

SPECIALIST COMMENTARY

Arseniy Kaprelyants: "The unique thing about the Georgiev Program is its openness"

Kaprelyants Arseniy Sumbatovich is a PhD in Biology, Head of the Microorganism Stress Biochemistry laboratory in the Bach Biochemistry Institute, RAS, and head of the project "Metabolic Pathways of Latent Tuberculosis Reactivation."

The program coordinated by RAS academy member Georgiy Georgiev is unique. There are no other programs like it. Its uniqueness is foremost the objectivity and transparency of the expert assessment. Of course other programs have expert assessment procedures, but they lack transparency. The applicant does not know the reasons for which his grant request has been denied, while another person wins the funding.

The "Molecular and Cell Biology" program is formalized, but you know the rules of the game. In my opinion, this is the correct approach in our current circumstances. In our country, when there is no objective mechanism of bid selection, all sorts of conflicts which have nothing to do with science begin to crop up, as well as other subjective factors.

In your opinion, is the know-how of the Georgiev program useful for the support of other fields of scientific research?

Yes, I think that in the current circumstances in Russia it would be advantageous. Many features of the expert assessment could be used in other RAS programs. They might not solve all the problems, but openness and transparency must certainly be incorporated.

What are your main achievements? What have you accomplished with the help of funding from the RAS Presidium's "Molecular and Cell Biology" program?

Even before we took part in this program, we discovered the resuscitation promoting factor (RPF) protein family in collaboration with English scientists from Wales University. These proteins are secreted by various bacteria (including mycobacteria, which cause tuberculosis). This awakens hibernating bacteria and induces them to shift from latency to active functioning. This was a very important discovery and we decided to continue this line of research. When we discovered these proteins, we did not know anything about them, how they worked or the mechanisms through which they functioned.

Using the program's funding we made one of our most important discoveries. We found that these proteins were enzymes that cleave specific molecules in the bacterial cell wall. In fact, we are very near to understanding how these proteins really work. We had a certain idea. We knew that the latent bacteria had increased cell-wall thickness, similarly to sporogenous bacteria. It seems that the RPF proteins cleave some type of bonds in the cell wall, thus softening it, which triggers active division. Before this, the hard outer "shell" prevented the cell from dividing. Thanks to this grant we have documented this mechanism and published the result in public journals.

The second achievement was the experimental proof of the importance of RPF proteins in the tuberculosis development in laboratory animals. In collaboration with our colleagues from South Africa, we studied the behavior of

M. tuberculosis strains with inactivated RPF genes (five genes). Knocking out no less than three of the five RPF genes lowered the virulence of *M. tuberculosis* strains in the tested animals. Moreover, we discovered that these strains could not be revitalized from their latent condition *in vitro*. Inactivating four RPF genes caused an almost complete loss of virulence in these strains. This lead to the idea that this strain could be used as a vaccine, since an avirulent strain presents no danger but the immune response could be strong. We could produce this strain in laboratory conditions, in a test tube, and then try to vaccinate laboratory animals with it. Which is what we did, with the help of our colleagues from the Central Scientific Research Institute of Tuberculosis at the Russian Academy of Medical Sciences (RAMS).

It turned out that these strains really do have a strong protective effect. Firstly, we vaccinated mice and then infected them with active tuberculosis. And most of the mice survived! Which meant that we had a high-efficiency vaccine that is no worse and maybe even better than the well-known Callmete-Guerin vaccine. Thus, the funding of our work by the Georgiev grant resulted in a very promising line of research.

Anna Gorbatova, STRF.ru, for Acta Naturae

Yulia Kravchenko: "I could feel that it is really possible to do science in Russia."

Kravchenko Yulia Evgenyevna, PhD in Biology, head of the Genome Translation Regulation group at the Engelhardt Institute of Molecular Biology, RAS, and head of the project "A Study of the Functions of the Nuclear RNA-polymerase spRNAP-IV and Systems Providing Genome Stability" (new group).

Yulia Evgenyevna, how was this group created and what results have you obtained using funding from the "Molecular and Cell Biology" program?

The basis for the creation of this new group was the discovery of a new fourth nuclear RNA-polymerase in eukaryotes (the so-called spRNAP-IV, from the English single-polypeptide RNA polymerase IV). This was the subject of my doctorate thesis, which I successfully completed in 2004; in the following year I applied for the "New Groups" competition of the MCB program and received a grant for the development of a new scientific group.

The main theme of our group's research is the study of transcriptional processes. We need to find out which transcriptional factors are involved in the functioning of the new nuclear RNA-polymerase, what the precise structure of the transcriptional complex is, which genes are transcribed by this polymerase, and which regulatory elements these genes use.

We have identified some components of the transcriptional complex. These are two of the three transcriptional factors of the mitochondrial RNA-polymerase. RNA-polymerase IV is an alternative splicing product of the *POLRMT* gene for the mitochondrial RNA-polymerase. We have demonstrated that two of the three transcriptional factors can be present in the nucleus; we also showed the direct interaction and complex formation between the novel polymerase and these factors. In other words, the mitochondrial RNA-polymerase and RNA-polymerase IV share these transcriptional factors. We are currently working on discovering the mechanism of transcription factor distribution: is it a competitive mechanism, or is it a switch from some proteins to others?

On the issue of which genes are transcribed by RNA-polymerase IV, currently we have identified more than 50 of these genes. However, we cannot find any common function for these genes. Some of them are involved in metabolic pathways (such as glycolysis in mammals and yeast), others are involved in the remodeling of heterochromatin, DNA-reparation, conserving methylation status, encode heat-shock proteins, etc. Also, our data indicate that this new RNA-polymerase transcribes genes whose products are involved in signaling pathways, one of which is the signaling pathway of the p53 tumor suppressor protein. Thus, we can already assess the role of spRNAP-IV in the organism's life supporting processes just by analyzing the functions of spRNAP-IV-transcribed genes. Notably, we discovered a very high degree of similarity in the regulatory regions of all the identified genes. We found a functional motif in the promoters of all the studied genes, and this motif was not present in any of the promoters belonging to the genes which were transcribed by other RNA-polymerases. Notably, this unique conserved motif was found to be present in all mammals.

Another line of research conducted in our laboratory with support from the "Molecular and Cell Biology" program is the study of antioxidative protective mechanisms of the cellular genome. This work is done in collaboration with the Cellular Proliferation Laboratory headed by Petr Mikhailovich Chumakov. Several years ago, Petr Mikhailovich's lab discovered the sestrin-2 protein, one of the three sestrin family proteins, which are activated by the p53 protein and which play a key role in the processes that protect the cell from excessive concentrations of active oxygen forms. Specifically, we demonstrated that sestrins act in conjunction with the sulphiredoxin protein to restore the oxidized forms of another component of the cellular oxidative protection system (the peroxiredoxin protein).

Until recently there has been no complex understanding of the functioning of the antioxidative protective system. There were only disjoined data on the functioning of separate proteins. However, we are the first to demonstrate the existence of a single multicomponent antioxidative protein complex, which includes sestrins, sulphiredoxin, thioredoxin reductase, and a number of other proteins which we have found to be a part of this structure. Interestingly, cells assemble this complex only in response to oxidative stress. While normally the components of the antioxidative complex are all localized in the cytoplasm, under stress the whole complex is transported into the nucleus, where it fulfills its main function: the protection of the genomic DNA from oxidation. We have also shown that this process is controlled by the p53 protein, which fulfills its "genome keeper" function, preventing damage to the DNA molecules.

How do you feel, being the head of a new scientific group?

After working for several years in the United States on a joint project and writing and completing my doctorate, I had the choice to find further work abroad or to stay here. To tell the truth, I didn't want to leave. Even though the work conditions for scientists abroad are great. Then I heard about the possibility of participating in the MCB program. I thought, OK, let's take a shot and apply, and I won a grant. The first year was mainly organization. We renovated the laboratory rooms which we got from the Institute using the funds from the program, bought equipment and reagents. As the head of the group, I had to delve into various organization issues, including the very peculiar way in which equipment and reagents would be bought in Russia. During this first and most difficult year, our group managed to slide on thanks to some earlier preliminary studies. On the other hand, I started feeling like you really could do science in Russia. I had a completely different outlook, much more optimistic. Moreover, being a part of the MCB program gives you the chance not only to work on scientific projects, but also to support people's way of life. MCB grants allow you to pay your workers a large enough salary that they do not need additional income. This creates fairly comfortable conditions for scientific creativity. Apart from myself, my group includes a PhD (who completed his doctorate during the work of this new group), three postgraduate students, and two undergraduates. Apart from that, I regularly receive letters from students asking if I take on postgraduates or undergraduates to complete their diploma project. Currently, I am thinking about taking on another undergraduate student; we have an interesting project. So this program is a good start for young and ambitious specialists. In addition, it is a good stimulus for a future career, since the work rate under the MCB program does not allow you to sit back and relax. The fact that you have to conform with the requirements of the Program gives you strength and keeps you on your toes (this mainly means obtaining results which are published in prestigious journals). However, the main motivating factor is still scientific curiosity, since without that you cannot be successful.

V. Sychev

projects received up to 4 million rubles annually over the course of 5 years (although if the project is purely theoretical, the amount of allocated funds is, of course, considerably less). New groups could receive up to 2 million annually over the course of 3 years. "Of course these sums seemed rather good 7 years ago, but currently they call for doubling due to constant inflation," says Georgiy Georgiev. Instead, the financing of this program decreased in 2010 by practically a third (29%). Accordingly, the sums allocated for each of the three competitions decreased. This immediately put the program on the verge of collapse, since its main goal, the support of promising research groups, was lost. This immediately initiated a new cycle of emigrations of young postdoctoral workers for a number of good laboratories.

How are the winning bids selected? A Scientific committee is formed by actively working members of the academy. The goal of this committee is to approve the general rules of bid selection. The same committee elects an Expert Committee consisting of seven members who organize the competition but whose role in the selection is minimal. The program must be automated to the largest possible extent and must not depend on the personal preferences of the coordinator or the overseers. The initial stage of any MCB competition is the accumulation of bids and their processing by technical groups of 2--3 people from various institutes. This stage results in data summary sheets which demonstrate the objective indices of the laboratory's effectiveness or (in the case of new groups) the effectiveness of a single scientist. A lot of value is set on the sum of the IF of articles published in the last 5 years, factoring in the input of the laboratory into each of the publications and the IF factor per member of the group.

Nonscientometric indices are also used when assessing applications and the applicants must conform to these requirements. These include signs of acknowledgement by the Russian and international scientific community (various awards and titles), the age of the members of the project group (special attention is paid to young people and middle-aged researchers under 45). The training of workers in the group and patented works are also taken into account.

The summary data sheets are published on the program's website (each applicant can verify the data). Bids which show especially high objective indices (most of all, the impact factor) are automatically declared winners (these are usually about a third of the overall number of winners). The Expert Committee's task is to verify whether any violations took place in this process (for instance, if members of a group which are in fact emigrants were included in the publication count). Notably, bids from members of the Expert Committee cannot be winners in the first round, so as to prevent any question of their objective assessment.

The winners of the first round and the best (highest scoring) winners of previous competitions form the "top pool," which then conducts an independent expert assessment. Each of the remaining bids receives five independent reviews from well-known strong scientists in the field which have no personal interest in the scores of the bid in question.

"This 'top pool' reviews all the bids that did not win in the first round and also the bids from members of the Expert Committee," explains Georgiy Georgiev. "Experts can slightly change the scores based on objective reasons. For instance we increased the score for a person who had very good science but very poor knowledge of the English language. He had difficulties with being published abroad, but experts knew the real value of his work, and thus increased the score. Another example might be a person who works abroad but who already has no firm ties with Russia and who does not benefit Russian science in any way. But he is still nominally a member of a laboratory in our country and his publications are used to calculate the score of a bidding laboratory. Our experts watch out for such instances."

Bids for socially oriented projects are assessed with even more attention. The scores include not only the "strength" of the group, but also the scope (significance) and originality of the project and its potential for being fulfilled. After the expert assessment, these indices are added to the objective criteria. The Expert Committee then makes its final decision, which can vary only slightly from the objective scores and expert recommendations.

The results of the scoring procedure and the decisions are published at once, and the author can view the expert opinions (upon request) and appeal to the Expert Committee or the specially formed Controlling Committee, which consists of academy members who do not work in the RAS system or do not work in its Central Region. In several cases the initial decisions had to be reversed.

"How are the funds distributed? Usually our program has a limit in terms of the amount of funds available for wages; this conforms to general academic expenditures." says Gerogiy Georgiev. "In 2010 we were told that this sum should be about 50–55% of the overall funds. The specifics are decided by the head of the group. The head of the laboratory and the staff are all responsible for the result; if there are no publications in 5 years or if the group violates the rules of the program, funding will cease."

"Molecular and Cell Biology" has already proven its worth. The success of 7 years of work is indirectly confirmed by the fact that almost all of the research groups supported by its funding are top specialists in life sciences in Russia. Of course the distribution of funds in the "Molecular and cell biology" program can be discussed, but a vast majority of specialists in our country acknowledge that no other program does a better job. It would be a very good thing if other scientific fields in Russia were to implement similar ways to distribute funding. ●

Federal Target Programs: Targeted Funding of Priorities in Science and Technology

M. A. Murav'eva, STRF.ru, exclusively for Acta Naturae

A federal target program (FTP) is one of the most important tools that the government has to create an innovative economy in Russia. In particular, the development of Russian science and technology is currently supported through the "Research and Development in Important Scientific and Technological Areas of Russia, 2007–2012" FTP. Currently, the main governmental client of the program, the Federal Agency for Science and Innovations, is being reorganized, and this FTP will be managed by the Ministry of Education and Science; the essence of the program, however, will not change. Top managers of the Federal Agency for Science and Innovations have told us about the program's structure and management.

Federal programs are designed to reflect the priorities and goals of the social and economic development of Russia; areas of structural, scientific, and technical policy; the forecasted needs and resources of the country; and the external political and economical environment. All federal programs, although differing in subjects, are similar in their focus on priority areas and targeted funding concept. All programs are approved by the Russian government and reflect the state policy in corresponding areas. The programs are primarily funded from the federal budget (funding from other sources, such as regional budgets and nonbudgetary sources, being much smaller). The system of federal target programs (FTP) is one of research, development, industrial, social-economical, and management procedures with coordinated tasks, resources, and schedules effectively addressing the systemic issues of state, economic, scientific, social, and cultural development of Russia. In 2010, 801.7 billion rubles will be spent on 54 FTPs on various topics. This year the level of budget funding of high-tech oriented FTPs will be the same as in 2009: about 250 billion rubles. The Research and Development FTP funding totals 7364.05 million rubles, including 6656.33 million rubles allocated to R&D. All contracts on FTP projects are competitive.

An FTP for the development of science and technology in Russia has existed since 2002 (from 2002 to 2006, its title was "Research and Development in Important Scientific and Technological Areas"; since 2007 its title has been "Research and Development in Important Scientific and Technological Areas of Russia, 2007–2012"). This is the only program based on scientific and technological priorities. There are five fields: Information and Telecommunication Systems, Industry of Nanosystems and Nanomaterials, Environment Conservation, Living Systems, and Power Engineering and Energy Efficiency.

"The distinctive features of this program are its multidisciplinary nature and the fulfillment of the entire innovation chain from the generation of IP to its commercialization," reports **Sergey Mazurenko**, the head of the Federal Agency for Science and Innovations (Rosnauka). "For the first time, a national program comprises an entire innovation chain and the tasks are set to develop the research and innovation infrastructure. Currently, research and educational institutions and, most importantly, small and mid-sized businesses have demonstrated significant achievements within this program. It is obvious that, if we do not develop innovative businesses and innovative economics, we will reach a dead end; oil and gas tend to run out."

Originally the program was designed to perfect the partnership between private parties and the state, says **Alexander Klimenko**, deputy head of Rosnauka. To do that, at every stage of the innovation chain, the program requires nonbudgetary funding from those organizations that will commercialize the results. "With this program, the state offers a hand to the business, inviting it to work together on the search, establishment, and development of innovative technologies," concludes Klimenko.

The current management structure and ideology of the FTP formed in 2004, when a Government Decree was issued which substantially changed the preceding 2002–2006 "Research and Development in Important Scientific and Technologial Areas" program. At the beginning, the FTP would often work as a number of uncoordinated projects,

EXPERTS' OPINIONS

Sergey K. Vartapetov, PhD (Physics and Mathematics), director of the Center for Instrument Engineering at the Prokhorov Institute of General Physics, Russian Academy of Sciences

As one of the "Research and Development" FTP participants, do you believe this program is an effective tool for the support and development of science?

I believe that the FTP a very effective tool, but there are defects in every tool; therefore, this system has to be further improved and perfected. Let us assume the program intends quick commercialization of the results. This does not take into account the specific character of the "Living Systems" projects. One cannot begin the commercialization of medical equipment or pharmaceuticals 2–3 years after the start of the project. Medical, clinical, and technical tests must be performed, and they require time, too. Now the FTP is designed in such a way that immediately after the R&D phase one has to begin the commercialization phase. That is not right. We should correct this misunderstanding. If a research team begins commercialization right after the end of the project, I can tell you for sure they have proposed an existing development for the FTP.

Two to three years are needed to develop a high-tech product; in the case of medical devices, at least 12–18 additional months are required for testing. Thus, the development cycle of a new medical product is about 5 years. The FTP deficiency is that these specifics are not taken into account in the program, hence the discrepancy between the requirements and obligations of the contractor.

How in principle should the state support science: where should it stand aside and where should it be active?

I think the answer to this question is clear. As in the rest of the world, the state should provide most funding to the R&D phases, especially high-risk ones. On the other hand, the state should stand aside at the commercialization phase. The Russian government is reluctant to do that; it seems expensive for the government. Many regulatory issues remain unsettled. The FTP provides that both the state and the author have the right to commercialize, but when two parties own the rights, no one owns them.

I have asked lawyers a number of times if I, as an author, can transfer the rights to an invention to a third party or should I ask the government for release? Whom exactly in the government should I talk to? There has been no answer.

The state has to launch a project in order to create jobs and manufacture machines. However, in fact the state acts as a businessman who has developed something and now wants to sell it. The state should not sell.

Besides creating a lot of confusion with legal issues, commercialization requires a large amount of money. If one secured funding for the R&D phase and it was successful, one would have to spend ten times more on commercialization to build manufacturing capabilities, enter the market, provide customer service, advertise, etc.

To raise money, one needs to tell the potential investors about his technology and who owns it. When I showed investors what is written in my government contracts, they become confuzed. Businessmen will not risk getting involved in commercialization under uncertain conditions.

Prof. Valery N. Danilenko, Doctor of Science (Biology); Head of the Department of Genetic Foundations of Biotechnology in the Vavilov Institute of General Genetics, Russian Academy of Sciences; and Secretary of the Council at the General Committee of the Russian Academy of Sciences for coordinating research in the "Medical Equipment, Technologies and Pharmaceuticals" area.

What do you think about the effectiveness of the "Research and Development" FTP as a tool for the support and development of science?

This program, especially making "Living Systems" a priority, is a very timely step. The FTP has made a significant contribution to the development of science in Russia. Without this program, we would not have seen the positive changes in this area occurring in the Academy of Sciences and higher educational institutions.

At the same time, the program does not address many important issues. One of these issues is that some projects funded by the FTP, including so-called integrated projects, ultimately hang in the air. The developed technologies and products are not manufactured, do not enter the market, i.e., there is no continuity, no chain from the basic research to a finished innovative drug or technology. This is because of a lack of integration among the state's departments: there is still poor interaction among the specialized programs of Rosnauka, The Ministry of Public Health and Social Development, and the Ministry of Industry and Trade. In addition, the projects supported by the FTP are not coordinated with those supported by the programs of the Russian Academy of Sciences, RFBR and other foundations.

Finally, when the project topics were created, especially at the first stage of the program, not all of the priorities were chosen well, since there were not enough experts in some areas, for example, in innovative drugs. I must admit our expert community is weak. Another important point: administrative staff should not play major role in project assessment. They should help the well-selected experts work efficiently and the researchers and business interact.

Eventually, within the "Living Systems" area of the "Research and Development" FTP, professional mechanisms of expert review, project progress, and quality monitoring have been formed. In my opinion, experience developed within this program on the infrastructural support, coordination board, and workgroup operation should be actively adopted in other FTP, in particular, in the Ministry of Industry and Trade's "Development of Pharmaceutical and Medical Industry of the Russian Federation until 2020 and Onward" Program.

Speaking strategically about supporting science, where should the state stand aside and where should it be active?

It is obvious that, in order to change Russian science, the state must implement certain policies and provide support.

Rapid changes are impeded by the conservatism of the existing system and the bad heritage of the nineties. The state needs to take part in setting priorities, creating efficient mechanisms for building those priorities, and in targeted financial support. This work has been initiated. Thus, last year the Presidential Committee on the Modernization and Technical Development of Russian Economy was established. It is important to keep working on the integration of Ministries, Departments, and business; ensure the effective use of innovation capabilities of the Academy of Sciences and the Academy of Medical Sciences; and control the selection and progress of large innovation projects. In his speech to students and at the meeting of the Committee on Modernization in Tomsk, President Medvedev emphasized again the importance of innovations, the active participation of the business in innovations, and the creation of adequate conditions for research work in Russia. I completely agree with the president that a decent salary for scientists is a necessary, but not the most critical, condition for the success of science in Russia.

In general, the approaches and principles claimed when creating research and technological programs are correct. How they are implemented is a different story. Science is an important part of the economic system, scientists are a part of Russian society, and all the problems in the country are present to a certain extent in science. For example, it is mainly bureaucrats again who formed the above mentioned "Development of Pharmaceutical and Medical Industry of the Russian Federation until 2020 and Onwards" FTP, but not the community of scientific experts, which must be involved more actively. Not only should scientific experts should be engaged, but also manufacturers and technologists who understand how this area works. In Russia, like in the rest of the world, in recent years, scientists, technologists and experts have been minimally used in making decisions on innovations and the technological modernization of certain areas, in this particular case in the pharmaceutical industry. Everything was farmed out to so-called new generation managers, often not very professional and not quite ready for certain tasks and projects. The international community is now criticizing that approach.

Irina G. Dezhina, Doctor of Science (Economics) and Head of the Sector for Economics of Science and Innovation Processes at the Institute of World Economy and Foreign Relations, Russian Academy of Sciences.

Government support for the implementation of scientific and technological innovations is one of the main tasks of the "Research and Development in Russia" FTP. Are the mechanisms of the government support plain, transparent, and efficient? Expert Irina Dezhina shared her views.

The areas of development of science and technology that are considered "high priority" were set without the extensive participation of the business community. Was that right?

Those areas are so broadly phrased and in such general terms that one can tailor to them almost any needs. In the United States, for example, instead of areas of high priority, there are critical technologies vitally needed by the government. The business community, however, takes part in creating a list of critical technologies, since private companies are among the contractors developing those technologies for the government.

I think that projects that are beneficial only for business should not be financed from the state budget. The replacement of private funds with governmental funds eventually reduces the investment activity of entrepreneurs. Certainly, business should participate in the selection of projects they want to co-finance; the share of business funds, however, should depend on the type and stage of the project.

What are the international practices of running similar programs; how are they different from ours?

I can tell you about the American Advanced Technology Program (ATP) finished in 2007. This program supported R&D performed by companies or consortiums of companies, universities, and/or national laboratories.

There were two basic selection criteria: first, there had to be a high likelihood of creating new technologies, which could result in broad cross-industry applications or in creating new markets, and second, the projects had to be in the early stage of development.

The government and the business shared the costs: consortiums of two or more companies had to finance at least half of the project cost, while large companies had to come up with at least 60%. The remainder was financed by the government, but the funds went to national laboratories only; private contractors could not receive budget funds.

We can see two major differences from FTP here: first, no money from the budget was to be given to private parties and, second, the business shared at least 50% of project costs with the government, whereas the minimum nonbudgetary investment share in large projects is just 30%.

The progress of ATP projects was regularly monitored, and statistical reports were published. In general, the program had a positive impact on the behavior of American companies: 61% of them increased spending on R&D, 67% increased investment in long-term high-tech projects, and 71% showed more interest in collaborating with governmental research institutions. What is happening in Russia? FTP has been active for years, and it has been modified a few times. Do we have information available that would show that some mechanisms of cooperation between the government and business used within the program worked better than others? The answer is no, and this creates the impression that it is a rather formal mechanism of distributing budget funds here and now which has not been assessed seriously.

Currently, technological development in Russia is in a miserable state. Engineering Science is still in crisis, the number of engineers working in science is going down. Our funding share in basic research has been stable (14–15% of total science funding), a large portion has been funded to development (almost 70%), but a very scarce share to applied research (15–16%; before the collapse of the USSR it was 33%). This means that, while our applied science is in ruins, only small technological improvements are being made which are not based on real scientific breakthrough results, hence the corresponding state of technologies.

not meeting the expectations of the level of result integration and program coordination. The state took an active part in increasing the efficiency of the FTP by improving the program implementation mechanisms and changing the management concept from the "control of budget funds" to the "management of results." The new paradigm of state management enriched the FTP with a number of qualitative and quantitative metrics for monitoring and controlling the progress and results.

One key FTP task is to initiate and optimize the mechanism for the competitive funding of research projects. This requires, first of all, creating a community of experts in Russia and perfecting the competition procedures and the examination and funding mechanisms. The new management system divides areas of accountability: the scientific community is responsible for the research part, while the administrative staff is responsible for compliance to formal regulations.

Research teams themselves determine the topics of the future funding opportunities of the Federal Program. The proposed R&D topics undergo a transparent and democratic review and approval process by workgroups involving reputable scientists. After that, applications are invited and proposals are submitted (by any institutions, regardless of the type of ownership and departmental affiliation) and reviewed by experts.

"When the FTP was launched, after Russian science had suffered for 15–20 years without funding, no one knew of the actual state and capabilities of the research groups or which areas are still alive," explains **Andrey Petrov**, director of the Government Office of Scientific and Technical FTPs. "Then the program's task was to actively monitor the state of research teams, understand the needs of the business and the nonfinancial sector, and assess the capabilities of the research teams. The first two years of the program were dedicated to this task."

This work resulted in the first "well-focused landscape of science and technology in Russia" according to Sergey Mazurenko. That is, we now realize the status of various research and development areas in Russia and the capabilities of those who may commercialize them. The best examples, Mr. Mazurenko believes, are the projects in nano- and biotechnology.

PRIORITY TO LIVING SYSTEMS

Effective diagnostics, agricultural crops enriched in vitamins, new drugs and vaccines, and many other technologies belong to living systems. This area has been undergoing a revolutionary development in recent years, being perhaps the most dynamic field of modern natural science. Life sciences have brilliant prospects of application in biology, medicine, agriculture, materials science, etc. This potential to create high technologies and improve the health and quality of life of humans makes living systems one of the key government priorities in science and technology, and it is actively supported by FTPs, including the "Research and Development" FTP.

"Living Systems," a priority area of the FTP, is generously supported by the state, receiving more than 30% of the total funds of the program (2.8 billion rubles in 2009), being second in funding only to "Industry of Nanosystems and Nanomaterials" (41.4% of the total FTP funding, 3.8 billion rubles) but still first in the importance of its results.

Today, living systems attract the attention of scientists all around the world. This field is at the forefront of science, bringing together biologists, chemists, physicists, and mathematicians. Many experts believe that it is living systems that will become the foundation of the new technological revolution, the basis for the new technological way of living. Therefore, the large number of participants in the "Living Systems" area does not look surprising. In 2007, 717 proposals were submitted (13.6% of the total number of proposals), 284 contracts were signed with a total budget of over 2.6 billion rubles. In 2008, 939 proposals were submitted to "Living Systems" and 179 contracts were signed (reaching 731 in total together with continuing contracts). In 2009, work was being performed within 282 projects, including 225 projects on basic research in life sciences and technology development, performed by institutions of various ownership types and departmental affiliations from seven regions of Russia.

"We support the most interesting and promising projects," says **Inna Bilenkina**, deputy head of Rosnauka. "Russian scientists have made a good start; there are strong research teams capable of creating effective drugs and advanced medical equipment. The problem is that these developments have not been actively sought by business yet. In my opinion, overcoming the barrier between basic science and industry is a vital task."

The program has been seriously criticized, in particular, for the mismatch between the research carried out and the needs of industry. Rosnauka admits that this is a problem and is addressing it.

"The topics proposed by scientists do not always push the frontiers," thinks **Gennady Shepelev**, head of the Department of Programs and Projects of Rosnauka. "It is easier for scientists to request funding for something they already know well and will surely achieve and, as a result, get money for without much effort. I suggest that, instead of using topics proposed by scientists, we invite proposals addressing real business tasks supported by the industry."

Inna Bilenkina thinks that, ideally, there should be two parallel ways of managing the program. The first would be to let the scientific community set the topics, and the second would be to create specific tasks set by the government.

Since 2009, part of the FTP funding has been spent on targeted contracts. Last year, a quarter of the total funding of the program was spent on 50 targeted projects. It had been planned to spend half the money on targeted projects in 2010–2011 and in 2012–2013 allocate 75% to large tasks combining a few dozen projects.

However, 70% of the funding for the 2010 program was cut back, which made it impossible to fulfill those plans without jeopardizing three-year projects that started in 2008 and were to continue in 2010. Nevertheless, as Andrey Petrov confirms, Rosnauka will keep launching large centralized tasks, so to speak, to create governmental contracts aimed at ambitious goals. ●

ADDITIONAL INFORMATION

The "Research and Development in Important Scientific and Technological Areas in Russia, 2007–2012" FTP is a continuation of the analogous 2002–2006 Program.

The Ministry of Science and Education of the Russian Federation as a customer and coordinator of the FTP sets the rules of the game (provisions about the program and the management structure, as well as procedures for evaluating and accepting work). Rosnauka is the main government customer; it manages budget funds on the government's behalf, accepts proposals for the FTP topics, and conducts their preliminary evaluation.

This program is managed by the Scientific Coordination Board led by the Minister of Education. This board establishes the schedule and amount of funding of projects and organizes expert groups in priority areas. The groups are made of qualified experts in the areas, representatives of various departments, scientists, businessmen, and technologists. There are more than 2000 experts in total. The aim of the expert groups is to help the board create topics based on proposals from the scientific community. The proposed topics are assessed for their relevance, originality, and scientific importance of possible results. The group members analyze the anticipated results, compare the funding requested to the amount of work proposed, and make a decision about initiating the project. Then Rosnauka holds competitive tenders and awards government contracts.

The activity units of the FTP structural elements:

(i) "Generation of Knowledge"

(ii) "Development of Technologies"

(iii) "Commercialization of Technologies"

(iv) "Institutional Basis for Research and Development"

(v) "Infrastructure of the Innovative System"

Research and development in living systems and the commercialization of the results is carried out in many projects within various units and activities of the FTP:

(1) Unit 1, Activity 1.2 "Perform problem-targeted exploratory research and accumulate scientific and technical knowledge in technologies of living systems": 1–2 year projects with annual funding of up to 10 million rubles per project. The project results must have sound market potential.

(2) Unit 2, Activity 2.2 "Perform integrated projects, including the development of competitive technologies in living systems intended for future commercialisation": 2–3 year projects, including experimental and process development with annual funding of up to 100 million rubles per project and a nonbudgetary funding share of at least 25%.

The integrated project result must have a significant potential for commercialization, and the government contractor is often obliged to utilize the result in its business activities.

(3) Unit 2, Activity 2.7 "Perform experimental and process development jointly with foreign research organizations or in areas proposed by the business community": annual funding of up to 50 million rubles per project and nonbudgetary funding share of at least 50%. The project result must meet the requirements of the business that initiated the project, or the result must be achieved with the direct participation of a foreign research organization, but it must be commercialized strictly in Russia.

(4) Unit 3, Activity 3.2 "Perform technology commercialization projects on topics proposed by the business community": innovation projects including R&D, manufacturing, and sales of high-tech products.

This category is similar to the previous one, but it differs in the scale and clear orientation towards the commercialization of the results. Projects of this type can be initiated by high-tech industrial organizations of any corporate form and type of ownership, as well as innovative industrial companies jointly with research and educational organizations. The government partly finances the R&D phase of these projects, while other costs (preproduction, advertising, marketing, etc.) are paid by the business.

The project term is up to 3 years, and the annual budget funding reaches 100 million rubles per project, with at least 70% of additional funding from nonbudgetary sources.

(5) Unit 3, Activity 3.1 "Perform nationally vital innovative projects (VIP) in the high-priority areas of the program": large innovative projects that will provide the following:

(i) The required level of national security,

(ii) Economic impact important for the entire economy, as well as for large economy sectors,

(iii) Solutions to key social issues, first of all, to increase the quality of life.

Each project will be run from the development of an advanced innovative product with a significant commercialization potential to industrial production and the successful market launch of new high-tech products.

Priority projects are based on partnership and risk sharing between the state and the business community. Budget funding of such a project is 1–2 billion rubles with at least 60% of additional nonbudgetary funding. The project term can reach 4 years.

The government contractor is obliged to provide for 5 times more money in revenue on sales of the developed high-tech products than the amount of budget money spent on the project.

Submitted projects must be in the framework of the critical technologies in the "Living Systems" area:

- Cell Technologies,
- Bioengineering Technologies,
- Genome and Postgenome Technologies for Drug Development,
- Biocatalysis, Biosynthesis, and Biosensor Technologies,
- Biomedical and Veterinary Technologies for Human and Animal Life Support and Protection.

Program funding by areas

	Budgetary funding in 2007 (million rubles)	Budgetary funding share (%)	Budgetary funding in 2008 (million rubles)	Budgetary funding share (%)	Budgetary funding in 2009 (million rubles)	Budgetary funding share (%)
Living Systems	2657.88	25.10	3242.63	24.50	2836.95	30.32
Industry of Nanosystems and Nanomaterials	4245.35	40.09	5458.61	41.24	3877.51	41.44
Information and Telecommunication Systems	840.959	7.94	1161.17	8.77	734.18	7.85
Environment Conservation	944.99	8.92	1220.29	9.22	573.74	6.13
Power Engineering and Energy Efficiency	1900.00	17.94	2154.89	16.28	1333.88	14.26

Expenditure from the federal budget on Rosnauka in 2004–2009

The Russian Foundation for Basic Research: Concern for the Future of the Largest Grant Fund.

I.A. Sterligov, STRF.ru, exclusively for Acta Naturae

The Russian Foundation for Basic Research is the oldest and most highly regarded source of scientific grants in Russia. It was created by a presidential decree of Boris Yeltsin "On Urgent Measures for Conserving the Scientific and Technical Potential of the Russian Federation" in 1992. Among other lines of research, the foundation is an active supporter of life sciences, which receive approximately 20% of the available funds.

AIMS, SCALE, AND MECHANISMS

In these times, when innovations are being implemented constantly, the Russian Foundation for Basic Research (RFBR), which is run by the government, is still an organization which first and foremost supports basic research. There is a very substantial reason for this. According to the head of the foundation's Biological and Medical Project Management Division, **Valery Smirnov**, "Science should not be divided into important and unimportant categories. We should always keep in mind that the foundations of modern genetics and molecular biology were all set during the course of experiments on wrinkled peas by Mendel and studies of the eye color of fruit flies."

This is why the RFBR supports projects which are not so much aimed at implementing practice (however, there is an "implementation-oriented" section, which will be mentioned later), but instead aimed at understanding and clarifying the laws and forms of nature.

Those researchers that are adept at solving such issues are offered a variety of competitions, the main one being a competition between initiative projects. Applications for conducting scientific research by individual scientists or groups of less than ten people, irrespective of titles, degrees, age, etc. are accepted. The only requirement is that the applicant must work in an organization in Russia whose charter stipulates that the organization conducts scientific research.

The funding is fairly moderate; for instance, in 2009, the average grant in biology and medicine was 380 000 rubles. The maximal grant was 750 000 rubles. "The funds we allocate to the researcher depend on the expenses required for his project," explains Smirnov. The foundation's staff acknowledges that the funding is not on par with international standards. An average grant should be approximately 1 million rubles; however, the foundation cannot accomplish this because of its own insufficient funding.

In 2010 the overall funding for biological and medical projects will be only 612 million rubles. This sum will be evenly spread between three sections: general biology, physico-chemical biology, and basic medicine and physiology. According to the directives from the board of the foundation, 30% of all the received applications must be granted. The results of the expert assessment will be published in March.

The expert assessment itself involves the following procedures. The biology and medicine department appoints an expert committee consisting of 64 people, 21–22 people for each of the three sections. This committee consists of leading Russian specialists, usually doctors of science. Directors and deputy directors of scientific organizations, state academies' staff members, etc., cannot be members of this committee. Each section consists of 8–10 theme divisions. One or two members of the committee are experts in each of these themes.

The expert committee is approved by the foundation's council, which is, in turn, appointed by the government. The members of the expert committee can serve a maximum of two 3-year terms. These people select a pool of external specialists (for biology and medicine, this is approximately 300 people) without any intervention from RFBR staff. Currently, the foundation is ready to include members of the Russian scientific community in an assessment of project applications. A key role in the expert assessment is played by the section head. This individual forwards applications to specific experts. Each application is analyzed by two or three specialists, who then rate the project on a nine-point scale. After all the reviews have been received, the projects are discussed at a meeting of the expert committee. Sometimes this meeting can decide to grant a project with a lower score in place of a bid with a higher score. "Publications in peer-reviewed journals with high impact factors (which, sadly, are mostly foreign journals) make the scoring much easier," adds Smirnov. However, there are no formal bibliometric requirements. The expert assessment is a lengthy process: decisions on applications which have been received

up until September 15 are announced in early March. The reviews are not disclosed to the applicants. The RFBR admits this is a serious drawback; however, the staff workload and problems with the contents of the reviews do not permit this to be rectified at this time. For instance, an expert can write that the work is interesting, the group professional, etc., but still score the project only 5 points. A system for sending reviews back to the applicants is in the works, but as of now there are no specific dates. A somewhat separate aspect of RFBR activity is the applied research support program, which finances studies aimed at practically implementing their results. Agreements with various government academies and institutions are the basis for a competition of implementation-oriented projects (code "ofi-c"). This program is aimed at helping leading scientific groups develop their promising results. The first interdisciplinary competition of implementation-oriented projects was conducted in 2009 ("ofi-m"). According to the head of the Oriented Research Department of RFBR **Sergey Tsiganov**, biological and medical applications in 3 of the 18 RFBR-approved fields of research received funding: "A Scientific Basis for the Creation of New Drugs and Vaccines," "Fundamental Aspects of the Genomics and Proteomics of Eukaryota" and "Cognitive Studies." The largest number of projects (28) involves the creation of new drugs. The average funding of an implementation-oriented grant is approximately 1.6 million rubles per year. All the bids for the "ofi-m" and "ofi-c" competition are assessed by an expert committee. Only those groups that have previously received RFBR or other well-respected grants and/or those that have publications in high-rated journals are allowed to bid for this type of grant. In all other aspects, the conditions are the same as in the initiative project competition. The foundation also has a small program for the targeted funding of projects which receive interest from the Russian Academy of Medical Sciences (RAMS).

FUTURE AND PROSPECTS

Almost every active scientist in Russia agrees that RFBR is the most effective mechanism of competition-based funding in a wide range of basic research fields. Notable factors are the high quality of expert assessment, clear rules for applicants, and limited amount of bureaucracy. The main problem is the size of the grants, which is not on par with the current needs of experimental science. In these circumstances, the government initially approved the doubling of the foundation's budget in 2010 but later cancelled these plans, thus not just stopping RFBR growth, but setting it back. The 2009 budget of the foundation was 7.1 billion rubles, while in 2010 the foundation received only 6 instead of the pre-crisis planned 12 billion. There is no way the foundation can grow under such extreme external sequestering. Currently, the foundation can only reminisce about the planned increase of the average grant to 1 million rubles, the creation of start-up grants for young researchers and the joint funding of offers for implementation-oriented projects from abroad, etc.

The remaining hopes of RFBR staff are on the innovational plans of the Presidential Modernization Committee. The concept of an "innovation elevator" was presented on one of the meetings of the committee, and this involved some of the studies supported by the foundation. The RFBR itself was named an "institute of development." Possibly these words will be followed by decisions that can help resuscitate this development. The chances that the government will unexpectedly realize the importance of basic research *per se* are much slimmer. ●

Nevertheless, despite the external limitation of the development of the Russian grant system, we have asked leading Russian biologists to share their ideas on the activity of the RFBR by answering the following three questions:

1. What can be done with the funding for an RFBR grant, what does it help achieve?

2. What are the pros and cons of the foundation's activity, apart from the amount of granted funds?

3. What steps should be taken to improve the support of biological projects by the RFBR?

Elizaveta Bonch-Osmolovskaya, *Doctor of Biological Sciences and Head of the Hypertermophilic Bacterial Communities Laboratory of the Vinogradsky Institute of Microbiology, Russian Academy of Sciences (RAS)*

"RFBR GRANTS ARE VERY WELL SUITED FOR STARTING A NEW GROUP OR LABORATORY."

1. RFBR grants really are pretty small. But they can support the work of a small group. They are very well suited for when a group or laboratory is just starting to take off. This was the case for our laboratory; for several years the RFBR funding was the only thing we had, and then we managed to move on to larger projects. Our laboratory has grown so much that RFBR funding is not enough, but we still apply for these grants and receive funding for small-scale pilot projects which may yield data useable for larger projects.

2. One advantage is the fairness of the granting system (compared with most other competitions and programs). Of course some people will surely receive funding even if they have a bad project, but in the RFBR this does not prevent researchers that really do work at a modern level from receiving support. A definite disadvantage is the absence of feedback; people should be informed on why their project was rejected, which means seeing the reviews.

3. My opinion is that regional competitions (in Russia) should be abolished, since the level of the research there is

RFBR: Opinions of Expert Science Theorists

Tatiana Kuznetsova, PhD in Economical Sciences, Director of the Scientific, Technical, Innovational, and Informational Policy Center for the Institute of Statistics and Economics of Knowledge at the State University of the Higher School of Economics.

"SMALL GRANTS SUPPORT SCIENTISTS, NOT SCIENCE."

Developed countries have arrived at an effective system of scientific funds, which usually have a special legal status. Since these funds are widely acknowledged as institutes of development, they are constantly experiencing various complex changes, widening of activities, direction of efforts (reorientation for multiprofile research, diversification of theme research, support of innovation projects, facilitating the transfer of scientific results, investments into small science-intensive companies, support of cooperation, increasing information, and expert functions).

What we see in Russia is quite different. Generally speaking, scientific funds in Russia have problems which can be separated into four aspects connected with scope, variety, time limitations, and regulations on their founding and activities.

(1) Since the funds allocated from the government are small, the average size of a grant is also moderate. This makes these grants less attractive and prevents increased efficiency: small grants mostly support the scientists, not the science.

(2) The conditions, mechanisms, and allocation and distribution of funding for the unreciprocated support of projects, including grant support, still remain unclear. Since the funds are participants in the budget process, they must obey budget law. The conflict between their status as head distributers of budget funds and their nondepartmental granting of funds still remains unresolved. The government does not fully understand the specifics of the activities of such foundations, which is competition-based funding of initiative projects selected on the basis of qualified expert assessment. Many issues on the selection of projects, forms for financing contracts of competition winners, etc., remain unresolved.

(3) The issue of which type of legal entity must be used for scientific foundations still remains unclear. The project for changing the legal status of some institutions which is currently being drafted will seemingly only worsen the situation.

4) RFBR and the Russian Humanitarian Scientific Foundation (RHSF) are aimed at financing basic research and are limited in their ability to support other types of projects, sometimes in conjunction with allegations of embezzling the budget funds.

There are still no special normative regulations for the activity of the foundation, which creates a number of problems (the legal status of the organization, the legal status of the allocated funds, and the conditions and procedures for their distribution).

The legal status of the grant itself is also an issue (the conditions of the granting agreement; right to grant funds to both individuals and legal entities). It is important to widen the rights of the foundations to use various financial schemes (government contracts, granting agreements, loan agreements, etc.). It is also worthwhile to allow foundations to receive funding not only from the federal budget, but also from other sources.

The problem of Russian scientific foundations is not only in the fact that the legal basis for their activity is being incorporated into the existing laws at such a slow rate. This problem is being solved and will be circumvented at some point. The main issue is the understanding and development of the ideology of research and innovation support via a system of various foundations and the development of these foundation's missions.

Currently, the capabilities and authority of the existing foundations and their role in the support of science and innovations are being diminished in a regular and methodic manner, which obviously conflicts with the current trend around the world.

Irina Dezhina, Doctor of Economic Sciences and Section Head at the Institute of World Economy and International Relations

"FOUNDATIONS NOT ONLY SUPPORT SCIENCE, THEY ALSO SUPPORT THE WHOLE RESEARCH PROCESS."

Governmental scientific foundations (RFBR and RHSF) are "by default" considered the most transparent and fair mechanisms for the distribution of budget funding for scientific research. Their funds are allocated not only for basic research, but also for processes which are needed for productive research: RFBR has efficiently financed and administered a program for the support of leading scientific schools and developed systems for buying and (most importantly) using scientific equipment for the benefit of all the grantees. This includes support in the creation of non-commercial collective-use centers.

RFBR and RHSF are very popular among scientists since they are just about the only institutions which can grant funds for participation in conferences, including those that take place abroad. Scientific organizations and universities rarely have the funds to pay for the science-related travel of employees that do not occupy high administrative positions.

Nevertheless, scientific foundations continue to be affected by the not-so-friendly policy of the government. First of all, these foundations are underfinanced on a regular basis. The law stipulates that RFBR and RHSF should receive 7% of the budget funds allocated for civilian science. According to information from Rosstat (Russian Statistic Agency), in 2007 only 6.1% of the funds were allocated through grants (this includes the Foundation for the Support of Developing Small Enterprises in Scientific and Technical Fields, which should receive 1.5% according to Russian Law), in 2008 it was 6.7%, and in 2009 (preliminary data) it was 5.6 %.

Secondly, scientific foundations have a very unstable legal status. This issue was supposed to be resolved in 2009. Ideally the status should be such that the foundations could grant funding to any grantee that wins the competition. This is the way in which they currently function, but this was legal only due to a delay stipulated in the Budget Code and which was in effect only up to January 1, 2010. In December 2009, the existing order of the financial support of grants was prolonged; in other words, a decision on the foundations was just delayed for another year. The optimal solution would be to amend the budget code so that the foundations would have a special status which would allow them to be the head distributors of the budget funding and distribute these funds in the form of grants. Obviously, the budget of the foundations should be increased and the administration of some programs should be transferred (returned) to the foundations. In the absence of other effective mechanisms, the foundations can also become a major organizational center for the expert assessment of scientific projects (which can even involve international specialists).

much lower than in the main competition for initiative projects. It might be prudent to temporarily suspend other types of RFBR competitions (publishing, participation and organizing of conferences and expeditions) in order to increase the grants for initiative projects. It is important to understand that, if there is no real scientific research, everything else will be irrelevant. Personnaly, I would close the implementation-oriented basic research programs. The Ministry of Science can handle this issue (The author is referring to the Federal Targeted Program "Research and Development in Priority Directions." This program has been suspended in 2010 – I.S.).

Vladimir Gvozdev, *RAS member and Head of the Molecular Genetics of the Cell Section of the Institute of Molecular Genetics, RAS*.

"A RESEARCHER WITH PUBLICATIONS IN DECENT JOURNALS WILL ALMOST CERTAINLY RECEIVE A GRANT."

(1) You can buy some reagents. Add a little to a very meager salary. The size of the grant certainly does not allow you to buy any serious equipment.

(2) A researcher with publications in decent journals will almost certainly receive a grant. The forms for the grant application are acceptable and are not as intimidating as those you can see in the lots in the Ministry of Education and Science and RosNauka. The forms could be further simplified by excluding the addresses and phone numbers of the grant participants.

It is a pity that the RFBR no longer supports scientific schools. Applying for the funding of a scientific school is now a most excruciating affair, and officials which do not seem to have any other work to do demonstrate their "active participation" by thinking up newer and newer forms and requiring extremely long reports. My opinion is that the expert assessment in the RFBR is well-qualified.

(3) The foundation needs money, which is used up by other programs that lack qualified expert assessment or which promote false "innovations."

Alexey Bogdanov, *RAS Member and Deputy Director of the Belozersky Scientific Research Institute of Physico-Chemical Biology at Moscow State University*.

"THE RFBR HAS CHANGED THE FACE OF RUSSIAN SCIENCE."

(1) The average RFBR grant can support the work of one post-graduate student (which includes a moderate addition to his or her meager scholarship) and buy some reagents and materials. However, it is important to bear in mind that large laboratories which have several groups with independent lines of research usually receive more than one grant. RFBR has always approved of this practice; this strongly differs from the policy of RosNauka, which states "one application for one lot" for each organization, irrespective of its size. This allows individual grantees in a laboratory to pool their resources for a common task.

Moreover, almost all the organizations to which the grantees are affiliated require the allocation of 15–20% of the grant for the support of the infrastructure (for instance, the support of a computer server) of the institute or department. This is also beneficial for the researchers. Another important factor is the moral support a researcher receives together with an RFBR grant. His project has been approved by a serious and qualified expert committee and his reputation with his superiors has obviously increased.

(2) Complimenting the RFBR is not very appropriate on my part, since I have been lucky enough to work for the foundation since its beginning. Nevertheless, based on my experience in many other expert committees, both Russian and international, I can confidently say that there is nothing better or more beneficial for Russian science than the RFBR. The main thing about the RFBR is the high professionalism of the people who are involved in workings of the foundation (beginning with expert reviewers and ending with the foundation's staff). The Russian scientific community has been schooled by the RFBR. It has been learning to write grants, to realistically judge the perspectives and results of their work, and to value international publication. The RFBR has changed the face of Russian science.

(3) My opinion is that the RFBR could improve the mechanisms of support for young scientists. Currently the foundation helps young researchers participate in international conferences (where else can such funding be obtained?) and facilitates the organization of young scientists' conferences. However, the so-called "Mobility for Young Scientists" program which supports trainee programs in various organizations inside the country is not working to its full capacity. The financing of the program is adequate. In my opinion, these funds would be better suited for supporting the first independent grants of young researchers (when a grant is allocated as an advance payment for a good idea).

Olga Lavrik, *Corresponding Member of RAS and Head of the Bioorganic Chemistry of Enzymes Laboratory of the Institute of Chemical Biology and Basic Medicine, RAS*.

"THE REVIEWING PROCEDURES IN THE RFBR ARE ON PAR WITH INTERNATIONAL STANDARDS."

(1) The size of RFBR grants is indeed small, and it has become even worse since the funding of grants in 2010 has been reduced instead of being increased, as would have been appropriate, because the role of RFBR in the development of basic research in Russia and in developing scientific personnel is hard to overestimate. The RFBR is practically the only scientific research foundation which has a serious and qualified expert assessment procedure for projects and which also makes annual reviews of the project reports. If the work is conducted in an unsatisfactory manner, the funding is stopped prematurely. There are, of course, other programs (such as the RAS "Molecular and Cell Biology" program) which are equally serious about objective ratings and judging the quality of publications, but the RFBR is still very important.

We mustn't forget that the RFBR has existed and functioned for many years; it has accomplished an enormous amount of work and has established a good set of principles for the selection of the best projects. This is great experience which should be used for the further development of basic research. Indeed, it would be criminal to negate all these results.

Another important aspect of RFBR grants is the fact that funding can be obtained not only by the head of a laboratory, but also by other leading personnel such as independent researchers and even young scientists. This is a very important issue; RFBR grants support pilot projects, which are the essence of basic research.

(2) As was mentioned previously, the foundation has a notable system of grant review and the application and reporting procedures are relatively simple. Everything is easier to judge in comparison. There was a time when we thought the RFBR system was overly complicated. But now, when we see the horrible bureaucracy involved in applications for research support by RosNauka and their reporting procedures, the RFBR procedure seems relatively easy and simple.

My opinion is that the largest part of RFBR resources should be focused on the funding of basic initiative grants, in which case they could become much larger. The creation of several new programs and competitions in the RFBR, including a scientific collaboration with South Ossetia does not seem justifiable to me. Moreover, the financing of oriented projects is in a certain sense repeating the work of RosNauka. As is evident from its name, the foundation should consider basic research a priority.

(3) The funding of initiative projects should obviously be increased; an average grant should be close to 1 million rubles. The current system for reviewing projects should be conserved and developed. It is objective and on par with international standards.

Konstantin Severinov, *Doctor of Biological Sciences; Professor at Rutgers University (United States); Head of the Molecular Genetics of Microorganisms Laboratory in the Gene Biology Institute, RAS; Head of Regulation at Prokaryote Mobile Element Gene Expression group at the Institute of Molecular Genetics, RAS*.

"RFBR IS A RAY OF HOPE IN OUR SCIENTIFIC REALITY."

(1) I was very interested to know the average size of a grant (380 000 rubles). My RFBR grant is smaller than average. Since the sum I applied for was much larger than the mentioned average, I cannot but wonder about the reasons for such a sad fact. I do not know the real answer, but I consider the following possibilities:

(I) all the applications are reduced by a certain (very large) percent,

and I modestly asked for a lower sum than my colleagues;

(II) other researchers applied with better projects that deserved more funding (since we are talking about the average size of a grant, this means that the research in my group is conducted below the average level in Russia, or more correctly, the average level funded by the RFBR);

(III) the decision on the grant's exact size is not based on the quality of the project or the funding required by the researcher, but on some other factors (for instance "rich American guys" receive less money in order to keep up the social justice).

The main role of an RFBR grant is to pay additional salaries to postgraduate students (one or maybe two). The fact is that RFBR funds are considered nonbudgetary funding, and budgetary funding (such as the RAS programs) cannot be used to pay postgraduate students. This cannot be understood rationally; you just have to keep it in mind and accept it. These additional salaries take up about half of the grant. The rest is used to buy plastic tubes, pipette tips, etc. These are small things, but a laboratory cannot work without them. If there is a need, one can pay for a single business trip with the grant money. You cannot buy any equipment for these sums of course. Finally, 15% of the grant is overhead for the institute.

Generally, an RFBR grant is better to have than not, but it does not allow you to conduct full-scale research on the projects stated in the applications; additional funds are a necessity. If I have four articles in international journals after 3 years of RFBR support, all of which cite the grant as a source of funding, this is to be taken with a grain of salt. Yes, the grant's funding was a contributor in this result, but these results could be obtained without this grant. If this grant was the only source of funds, the work would not have been completed. I was also granted a larger RFBR grant (ofi-c). I obviously like it better in proportion to its larger size, as compared to my initiative grant. More funds give more possibilities. We even bought a microbiological shaker costing $10 000.

(2) The foundation has a wonderful website (the "Grant-Express" system started working before a similar system was implemented in the NIH in the United States, and the system works very well). The forms are fairly adequate in general, you don't get the feeling that people are trying to torture you with them. Well, maybe just a little, for instance when the reports need to include lists of references from articles supported by the grant.

I also cannot understand why the foundation needs the tax address information for the researchers involved, since the turnover of the participants is high. This means the grantee has to run around after people, asking for their ITN (Individual Taxpayer Number) and home address. It is obviously a disgrace that the applicants do not see the reviews for their projects.

The RFBR has a very good and needed system of support for trips to conferences; several researchers from my lab have used this option. It did involve several idiosyncratic requirements, and the staff of the foundation was not always polite when asked for information, but as they say, you gotta do what you gotta do.

The foundation has a series of joint competitions with various countries, starting with Kyrgyzstan and ending with the United States. I think this is not the best part of the foundation's activities, it is very much nontransparent. When I discussed the options with the program administrators on the American side, I received direct advice not to apply. I think all these programs should be discontinued and the funds should be allocated for increasing the average size of the grants, without decreasing the number of grants.

What I consider an utter anachronism is the requirement of a hard-copy version of the application. I need to take it all across Moscow, and others need to mail it across the whole of Russia just to drop it into the "letters and newspapers" box. What is the need for all this paperwork when the foundation has a wonderful website?

(3) What about further development? Introduce the practice of sending the

application reviews to the applicants, publish the lists of expert reviewers, introduce the rotation for these experts (*this mechanism is in place only for members of the expert councils, but not for external experts – I.S.*) and also publish the criteria which are used during the selection of these experts. These criteria are to be followed with all due accuracy and reason. Grants need to be increased, but without decreasing their number. All the attempts to select "priority lines of research" and introduce "targeted funding" are susceptible to abuse and are usually initiated by people whose ability to select priorities is very questionable.

In general, the RFBR is a ray of hope in our scientific reality. It needs some small improvements, but the main principles of its activity are sound.

Piotr Chumakov, *Doctor of Biological Sciences, Head of the Cell Proliferation Laboratory of the Engelhardt Institute of Molecular Biology, RAS.*

"RFBR IS THE ONLY HOPE SOME SCIENTISTS HAVE OF GETTING FINANCIAL SUPPORT."

The sad condition of Russian science is in part due to the ridiculous amounts of financing. The RFBR is not the only source of funding for basic researchers, but it is the most important one. For most scientists who are not a part of a powerful interest group but just doing science, the RFBR is the only chance of obtaining support. Of course, since the average size of a grant is 380 000 rubles, no serious projects can be initiated. This amount is 10 times less than is needed in order to support an experimental physico-chemical project. That is, if the appropriate equipment is already in place.

Basic research is valuable only if it yields new data. The price of obtaining new data is dependent on objective factors, such as the prices for reagents and other consumables. Throughout the world, researchers use common standard sources for most laboratory materials. Unfortunately, most of these materials are manufactured abroad and something that costs $1 in Western countries can cost $1.5–2 in Russia after all the custom duties and commissions. This means that the cost of scientific results in Russia is much higher. In the United States it is considered normal for an active researcher to use up to $15 000–20 000 worth of materials annually in the course of successful work. This figure does not include salary or equipment.

Which means that a Russian researcher must use $25 000–39 000 just to stay on par. Therefore, a 380 000 ruble grant can only be used to prolong the agony of science. Nevertheless, Russian scientists still exist and even yield results with these meager sums. However, the efficiency of this process is incomparable to the real potential these scientists have. Only the hope of a better tomorrow keeps these people going.

When a difficult financial situation occurs, there is hope that it will be circumvented and everything will be back on track. But when this insanity is still going on after 20 years, when it is getting worse and the growth of systems for supporting basic research and open competitions is being negated, a pressing question comes to mind: What have we been waiting for all these years? The constant calls for "modernization" and "innovation" sound especially funny in this situation. Where will these things come from when the tree is being chopped off at the roots?! What kind of innovations can come from dead science? Or did somebody invent a new, "innovative" method of getting innovations out of thin air?

2–3. Pros:

(I) Applications for funding are sent in by researchers, without any go-aheads from their superiors. This system lets a researcher feel like a real Russian Scientist who is venturing into the Secrets of Existence;

(II) The applications are assessed by scientists and not by officials. The main criterion for the grant is the scientific value of the proposed project;

(III) The fact that a researcher has an RFBR grant gives him or her some protection against abuse from officials or institute administrators, who do not always work in the interests of scientific development;

(IV) Each researcher can decide what he or she will do with the grant money; nobody asks why the money was used in such and such a way. This system is good, because the researcher feels trust, while the institute's accounting system is still there to safeguard against misuse of funds.

(V) The foundation sets a limit for the funds that can be used by the institute without consent from the grantee. This gives the researcher an additional degree of protection against local administrator abuse;

(VI) Applications and reports are sent in via the internet using unified application forms. The applications are relatively short, which is adequate, considering the moderate size of the grant. If the funding were to be increased, the small amount of paperwork would be a drawback because a more strict review would be in order.

Cons:

(I) The procedure for decisions on whether to support or decline a project is somewhat nontransparent. What is missing is feedback on the results of the project's review, which should be available to the researcher. Publishing a list of projects and the grant sums for ongoing and completed projects would also be good, as well as a list of the members of the themed expert committee who take part in the meeting where the review results are discussed;

(II) the scientific community is a very tight-knit group, which creates a basis for conflicts of interest and personal conflicts to affect the course of science. The existing expert assessment procedure still has room for manipulation and subjective decisions. A correct move would be to involve both Russian experts and specialists from abroad, taking into account that a large portion of Russian speaking scientists are working in other countries. This potential should be used, and their involvement in Russian matters should be stimulated;

(III) the funding is allocated with delays, and scientists are left without any money in the first months of the year. Our inflexible financial system does not allow the creation of legal "stashes" for such times, pushing us towards unavoidable (if the work is to be continued) violations;

(IV) it would be good to have two or three types of grants which would differ in their timeframe and amount of funding. For instance, many initiative projects are not concluded in three years. Five-year grants are also necessary. ●

"Basic Sciences for Medicine": A basic-research program of the Russian Academy of Sciences Presidium

Grigoryev A.I.
Program Coordinator
Doctor of Medical Sciences, Member of the Russian Academy of Sciences (RAS) and Russian Academy of Medical Sciences (RAMS), Vice-President of RAS.

The RAS Presidium program for basic research "Basic Sciences for Medicine" was started in 2002 and is approved annually by the RAS Presidium. Over the 8 years of its existence, this program has demonstrated that it is an essential and effective part of RAS innovation activities. It is an interdisciplinary program conducted annually on a competitive basis. The aim of the program is to use the results of basic research obtained in exact and natural sciences in order to solve the high-priority practical problems of healthcare, especially those under research in RAS medical facilities. The main aims are (a) to study the etiology and pathogenesis of a variety of widespread and socially important diseases; (b) to develop new medical technologies such as diagnostics, medical equipment, and drugs; (c) to encourage RAS medical facilities in their attempts to develop new technologies and conduct clinical trials for these technologies; (d) to introduce tested results into the practice of RAS medical facilities and into the Russian Healthcare system as a whole; and (e) to increase the quality of medical services provided to RAS employees. The WHO predicts that over the next 10–15 years, neurological and psychological conditions will be the most common form of illness, overtaking the cardiovascular diseases and cancer which are currently dominant. Taking this into account, a new section has been created inside this program in 2010 to stimulate brain research.

The importance of this program is based on its wide possibilities for social use and its unique ideology aimed at introducing the achievements of exact and natural sciences such as biology, chemistry, physics, and mathematics into practical medicine by pooling a major part of the resources of RAS-affiliated scientific research institutes.

The multidisciplinary proficiency of RAS institutes and their high scientific and staff potential allow a very wide scope of research and implementation activities, starting with obtaining deeper or new knowledge on the etiology and pathogenesis of socially-important diseases and ending with the development and manufacture of new drugs or materials. Moreover, the unique technical and material base of RAS facilities specializing in physics makes the construction of medical equipment and its adaptation for mass production possible. One of the tactical objectives of this program is to fund projects which can be practically carried out in the course of a few years.

THE SCIENTIFIC ORGANIZATIONAL STRUCTURE OF THE PROGRAM

The scientific organizational management of the program is provided by the coordinator and the scientific council, which is headed by a chairman appointed by the coordinator (Fig. 1). The scientific council includes leading scientists, mainly members of RAS and RAMS.

The coordinator and the scientific council for the ideology of the program's development, selects projects to be funded, establishes the extent of the funding, and assesses the results obtained during the course of the projects. The work of the coordinator and the scientific council is mostly based on the opinions of the expert council, which they appoint. This council includes leading scientists from RAS, RAMS, the Ministry of Healthcare and Social Development, and other organizations. This council performs an expert assessment of projects submitted for the competition and the results obtained by the funded projects during the course of the year, firstly as sectional moderators on the annual program conferences and secondly as reviewers of the annual reports. The connection between the coordinator and, on one hand, the scientific council and, on the other, scientific organizations is the coordination center (Fig. 1). Its functions are the following:
(i) conducting the competition,
(ii) organizing the annual reporting conference,
(iii) collecting annual project reports,
(iv) reporting the Program's activity in the media and on the program's website.

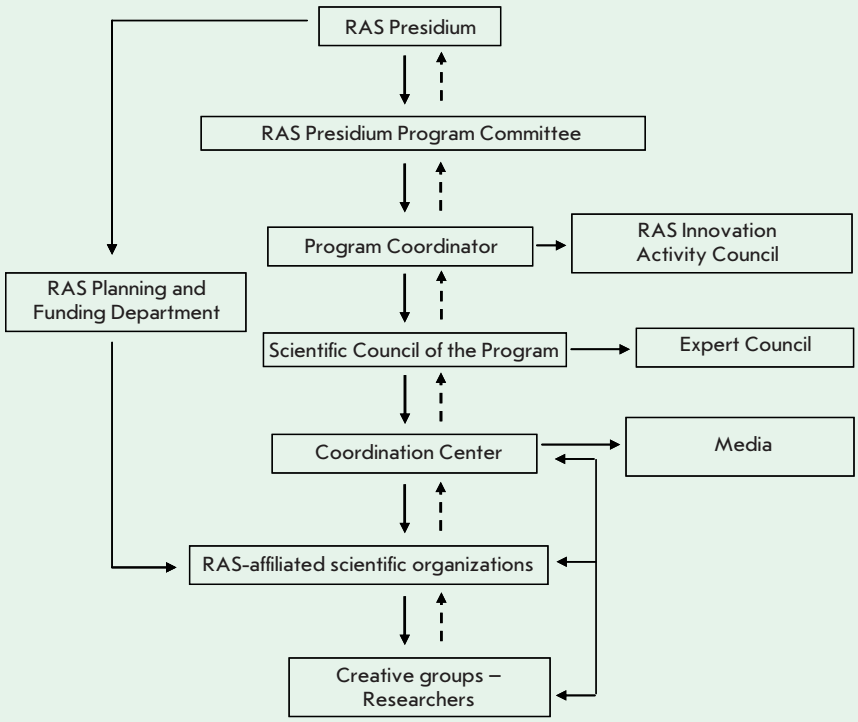

Fig. 1. Schematic of the organization of the "Basic Science for Medicine" Program

The program is developing very quickly and exhibiting very dynamic growth. For example, in 2002–2003, research was conducted in only three fields:
(i) A study of the etiology and pathogenesis of widespread and socially important diseases;
(ii) The development and improvement of diagnostic methods and equipment;
(iii) The development of medical materials and equipment and new drugs.

In 2004, a new line of research was introduced under the title Current Problems of Radiobiology; within the original fields, the most promising projects concerning chronic neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, etc.), biophotonics, and informatics were formed. As of 2005, a number of innovation projects have been joined into groups which are now the basis for trials and implementation of new medical technologies in RAS medical facilities; the RAS Central Clinical Hospital; and other RAS hospitals for scientific centers in St. Petersburg, Pushino, Chernogolovka, Troitsk, Kazan, and the Kola Scientific Research Center.

A new line was also formed in 2006 under the title Human Polymorphisms. It was developed and financed in conjunction with Moscow State University. Finally, in 2010, in accordance with a decree from the RAS General Assembly (dated December 2009), a group of projects concerning brain research was started. The further development of the program has the virtually unlimited resources of RAS institutes at its disposal, but unfortunately many tens of projects have to be declined due to the limited funding of the Program, even though these projects are of high theoretical and practical interest.

The dynamic development of the program is easily quantified. From 2002 to 2010, the number of funded projects has increased from 37 to 187, which is 5-fold; the number of organizations taking part in the projects has increased from 20 to 72 (3.5-fold); and the funding has increased from 30 to 100 million rubles (over 3-fold) (Fig. 2). Currently eight branches of the RAS are taking part in the program in many Russian cities (Moscow, St. Petersburg, Pushino, Nizhniy Novgorod, Samara, Kazan, Chernogolvka, and Ufa). Taking into account the innovational direction of the program, one of the key indexes of its effectiveness is the number of patent applications and granted patents. In 2002–2010 these indexes have increased more than tenfold (Fig. 3).

One of the most important elements in the program of the development of the Central Region branch of the RAS was the creation of branch offices in the regional centers of the RAS (Siberian, Ural, and Far East centers).

As was mentioned earlier, the results of the program's work are summarized annually during conferences which take place first in all the branch offices of RAS starting with the Far East, Siberian, and the Ural RAS regional branches; then they are followed by a joint conference that includes all the institutes of the Central Region branch of the RAS. A new approach was taken in 2009: the conferences were organized according to specialization. These conferences attract a lot of interest from the scientific and medical communities, and the conferences are always summarized at special press conferences for media such as ITAR-TASS, *Meditsinskaya Gazeta* (Medical Gazette), *Poisk* (Search), *Radio Rossii* (Radio Russia), TV media, etc.

A SHORT SUMMARY OF THE PROGRAM'S RESULTS

1. BASIC RESEARCH INTO THE ETIOLOGY AND PATHOGENESIS OF WIDESPREAD AND SOCIALLY IMPORTANT DISEASES

The biggest progress in this line of research has been achieved in the study of the pathogenesis and etiology of neurodegenerative diseases (NDD).

NDDs include Parkinson's disease, Alzhemier's disease, hyperlactatemia, etc., all of which result in the death of certain neuron populations. These diseases are currently as common as cardiovascular and oncological diseases. Neuronal death causes a deficiency in the amount of chemical signals synthesized by these cells and disrupts the functions which they regulate. The first symptoms of an NDD usually emerge after the death of most of a certain population of neurons. This process usually

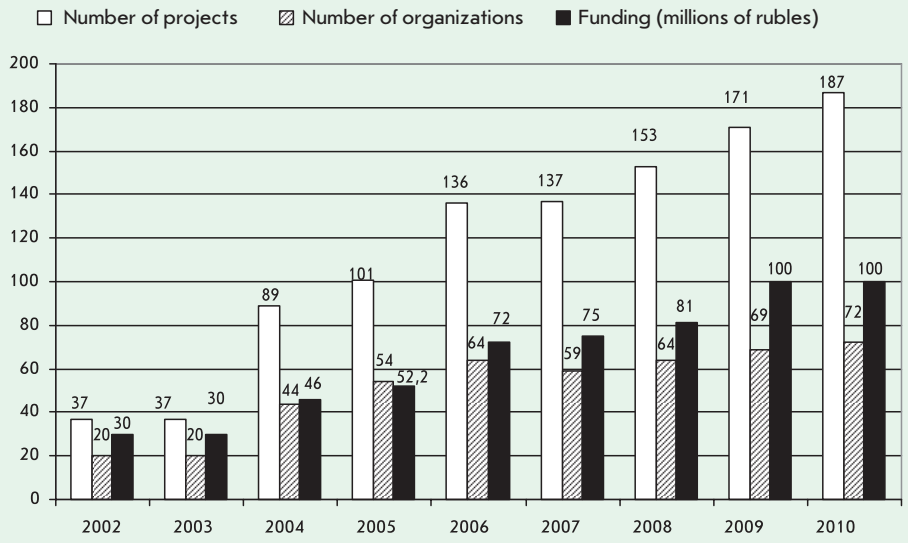

Fig. 2. Major indicators of the Program's development

begins at the age of 25–35 and can continue for 20–30 years. Treatment usually begins only after the emergence of symptoms and does not lead to patient being cured, so the patient gradually becomes incapacitated and dies.

A project supported by the program showed for the first time that a deficit of signal molecules caused by the death of the neurons that synthesized these molecules was compensated by changes in the synthesizing pathways of other neurons, which allowed the additional synthesis of these signals and explained such a long asymptomatic period for NDDs (Koltsov Institute of Developmental Biology, RAS).

In this context, the emergence of symptoms indicates the irreversible destruction of the relevant regulatory system and the depletion of the compensating mechanisms. These factors explain the ineffectiveness of current treatments. This creates a rationale for developing NDD diagnostics, which could identify the disease long before the emergence of symptoms, as well as for prophylactic treatment, which would stop or at least delay the death of neurons.

New and important data were also obtained in the course of studying the etiology and pathogenesis of other socially important diseases, such as lung and cardiovascular deficiency, diabetes, and tuberculosis.

2. DEVELOPMENT AND IMPROVEMENT OF DIAGNOSTICS AND TREATMENT

Diagnostics. New and improved diagnostic and monitoring methods for a wide range of neurological, endocrine, cardiovascular, lung, and digestive conditions have also been developed. These studies were successful due to the focus on the metabolic dysfunctions in the damaged organs and tissues. Such dysfunctions can be detected by testing for specific endogenous markers. One of the most important priorities of this line of research is the development of noninvasive diagnostic approaches. Among these, biophotonics seem to be very promising, including termography, endofluorescence, and coherent optical tomography, all of which seem to be especially promising for the diagnosis of oncological conditions.

Thus, researchers working with support from the program created and implemented a noninvasive diagnostic method based on coherent tomography, which makes targeted biopsy, identifying the borders of the pathological zone during operations, and monitoring wound healing possible. A portable, high-resolution tomographic scanner and microscope were created for use with this method at the Institute of Applied Physics, RAS.

Studies of NDD pathologies conducted within the program allowed researchers to understand the importance of preclinical diagnostic methods (see earlier). It is clear that a presymtomatic diagnosis can be made based on positron-emission tomography. This method is expensive and is therefore hardly accessible during a large-scale prophylactic medical examination; however it can be used for diagnosing patients from a small risk group. For this, researchers involved in the Program are searching for peripheral markers of NDD both in

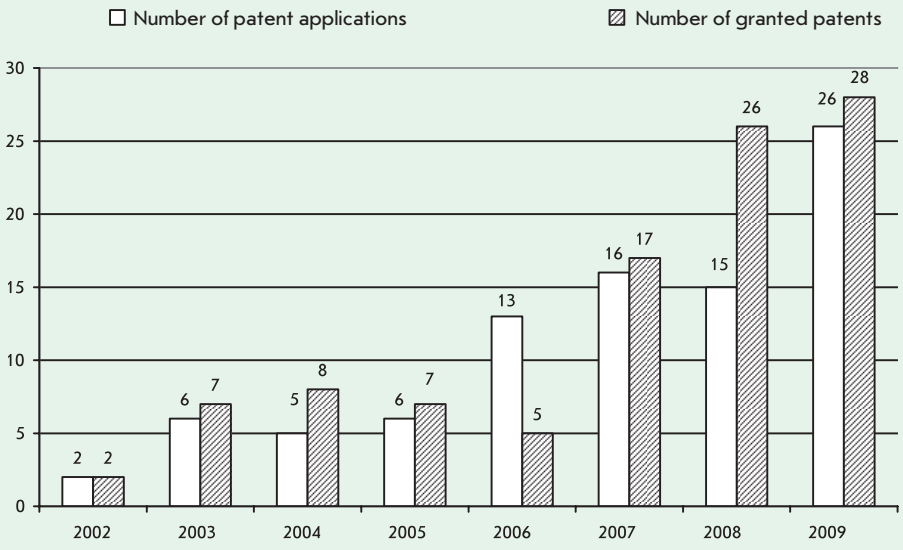

Fig. 3. Major indicators of the Program's effectiveness

experimental systems and in patients who have only recently started manifesting symptoms.

In the future this work may make it possible to identify risk groups based on the results of a routine large-scale prophylactic examination (Koltsov Institute of Developmental Biology, RAS, and the Central Clinical Hospital of the RAS). The development of preclinical diagnostics for NDD will open new possibilities for the development of prophylactic therapy.

One of the achievements of the program is the development of an early-stage diagnostic method for eye diseases based on the appearance of arestin, a protein involved in the phototransduction cycle. It has also been demonstrated that the detection of antibodies against the S-antigen, one of the retina proteins, in the blood correlates with the development of diabetic retinopathy in diabetes patients and posttraumatic complications in eye burn trauma (Biochemical Physics Institute, RAS).

In recent years, new informatics approaches that can increase the predictive power of diagnostics for a wide range of diseases have been developed. The creation of a basic telemedical network for patients and prophylactic centers (Fig. 6), including mobile functional diagnostics that can be performed at a distance (Institute of Biological Instrumentation, RAS), is of great importance. This network will be used for express diagnostics and for monitoring the patient's condition; it will involve the best specialists from Moscow and St. Petersburg.

The projects on the development of information technologies for supporting diagnoses and treatments and the creation of an integrated information system for medical facilities (based in the Central Clinical Hospital, RAS) have also been competed (The Research Center of Medical Informatics at the Institute of Program Systems, RAS).

Treatment. Based on the results of experiments that showed the selective effect of transcranial electrostimulation on the brain's protective systems (which react by releasing endorphin and serotonin), specialists developed a therapeutic method involving transcranial electrostimulation (TES therapy). This method uses the surface electrodes and currents of a specific form to induce the activity of the brain's protective systems. This method can be used for treating a wide range of neurological conditions (Pavlov Institute of Physiology, RAS).

3. THE CREATION OF MEDICAL EQUIPMENT, MATERIALS AND NEW DRUGS

Equipment. In the course of our program, researchers have improved several methods of therapy by developing the necessary instrumentation, including that utilizing the effect of lasers on biological tissues. Some of the projects have moved into the clinical trial phase and some new drugs are being implemented. For instance, a periodical-impulse CO2 laser was created for the treatment of skin diseases, such as for the dissection of papillomas and malformations and for the removal of burn marks (General Physics Institute, RAS). This device has completed clinical trials in the three leading dermatological clinics in Russia. It conforms to the best technical standards seen in similar devices around the world, but it costs about ten times less.

A compact infrared computer tomographic scanner with enhanced metrological capabilities has also been constructed (Institute of Radiotechnics and Electrotechnics, RAS). Using this device, a number of medical institutions have created infrared diagnostic facilities and devised new methods for the differential diagnosis of tumors, diseases of the vascular system, motor apparatus, and mammography. In particular, an infrared thermographic imager was manufactured with the help of the program and became the base for an infrared diagnostic facility in Clinic no. 2 at the Central Clinical Hospital of the RAS.

The widespread use of cryotherapy and cryosurgery in Russian practical medicine (dermatology, gynecology, otolaryngology, general surgery, oncology, and microsurgery) is obstructed by the absence of simple and reliable cryomedical instruments intended for rapid and contact-free freezing (liquid nitrogen sprays) and the deep contact freezing of select surfaces or volumes of tissue (active-tip cryodestructors with liquid nitrogen cooling). The following has been achieved in the course of the Program: (a) a critical analysis of the practical experience in developing and using liquid nitrogen sprays and cryodestructors with interchangeable tips; (b) the modernization of portable cryoinstrument construction; (c) prototypes of portable cryosprays and cryodestructors have been manufactured (Institute of Solid State Physics of RAS) and are being tested in clinics at the scientific facilities of the RAS in Chernogolovka, Saint-Petersburg, and several clinical facilities in Moscow.

Materials. Researchers have obtained new results which pertain to the use of chemical or biological materials for replacing damaged tissues in the organism and for the endoprosthetics of joints and blood vessels. New matrix materials based on natural polysaccharides have been created for the cultivation of human skin cells, which can then be transplanted to facilitate the healing of wounds (the Institute of High Molecular Weight Compounds, RAS, and the RAS Cytology Institute). It has been demonstrated that the most promising polymer materials for the cultivation of human fibroblasts and keratinocytes are filmlike composite matrices formed by chitozan and modified by collagen. A method for perforating these filmlike matrices by a laser beam has also been developed.

Drugs. Research supported by the Program has yielded clinical experimental data which elicits the use of the Russian drug Xymedon for the prophylactic treatment of secondary immunodeficiency (Arbuzov Institute of Organic and Physical Chemistry, Kazan Scientific Center, RAS). This drug can normalize the somatic status of HIV patients by lowering HIV replication (viral load) and it can help improve the pathogenically relevant parameters of the immune system. Based on this, the criteria and schemes of drug administration have been fine tuned for the treatment of HIV infection. This is the first instance of treating HIV with a low-toxicity nonglycoside analog of pyrimidine nucleosides.

A novel group of original compounds for the treatment of Alzheimer's disease and other NDDs has also been created (Institute of Physiologically Active Compounds, RAS).

4. CURRENT PROBLEMS IN RADIOBIOLOGY

Studies on the effects of high energy nuclei and low-intensity irradiation of living organisms have been conducted in several RAS institutes (Emanuel Institute of Biochemical Physics, Institute of Theoretical and Experimental Biophysics and the Semenov Institute of Chemical Physics). These studies have developed new technologies for the irradiation of living organisms, including some methods that lower the negative effect of irradiation therapy (Trapeznikov Instiute of Control Sciences, RAS). Studies have led to the creation of drugs of natural origins that can effectively decrease radiation-induced damage in human cells (Institute of Petrochemical Synthesis, RAS, and Konstantinov Institute of Nuclear Physics, St. Petersburg, RAS). Researchers from the Emanuel Institute of Biochemical Physics, RAS, have developed criteria for determining the resistance of a human organism to extreme conditions. Considerable efforts are being put in to construct new instrumentation, such as a new generation of dosimeters and an irradiation source (Prokhorov Institute of General Physics, RAS). This line has recently been reorganized and the projects have been included into other lines of research according to their subject.

5. HUMAN POLYMORPHISM

Projects in this line of research are involved in basic scientific research concerning the individual variety of biomacromolecules in humans at the genetic and protein levels. A comparative study has been performed in the field of structural functional and evolutionary genomics, including cloning, sequencing and an analysis of the polymorphism of the prepromotor region of the ribosomal DNA of humans and higher primates (Gene Biology Institute, RAS). Both similar and highly divergent regions have been found, the latter being due to single substitutions, insertions, deletions, and structural reorganizations. Several hypotheses on the initiating mechanisms of genome evolution were formulated. Researchers have suggested a new experimental approach for the genome-wide identification of insertional retroelement polymorphisms which are characteristic of patients with oncological or autoimmune conditions. This approach was used for the first genome-wide screen and analysis of species-specific and polymorphic integrations of retroelements. This method is also being used for a study of the effect of retroelements on gene expression (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, RAS). This line has recently been reorganized and the projects have been included into other lines of research according to their subject.

6. INNOVATIVE CLINICAL PROJECTS

A large portion of the projects conducted with support from the program are performed in collaboration with RAS medical facilities. The results of these projects can then be implemented into everyday medical practice. For instance, the following projects were performed in collaboration with the RAS Central Clinical Hospital (Moscow): (a) the study and development of modern methods for the prophylactic and therapeutic treatment of prostate gland cancer using the new drug Mycolicopene; (b) the study of the polysaccharide complexes of mycelial fungi used as dietary fiber for the treatment of diseases of the digestive tract; (c) the development and implementation of the apparatus and software for the Vertikal diagnostic and therapeutic complex used for restoring the ability to walk; (d) the development and implementation of laser-induced hyperthermia of the thyroid gland in the treatment of the nodular goiter; (e) the development and implementation of methods for an individual diagnosis of oxygen side-effects, in which these effects are assessed in combination with the drug therapy; (f) the development and implementation of guidelines for equipping medical diagnostic thermographic facilities, including mobile varieties; (g) the implementation of immunochromatographical test-systems based on polymer membranes for the complex express diagnosis of viral hepatitis; and (h) the development of a complex of software and equipment for the 3D visualization of pathological zone images obtained from endoscopy of the small pelvis organs. One of the most important parts of the program is the creation of an integrated information system for medical facilities. Researchers of the program are the first to use the television method for noncontact endoscopic measurement of the linear parameters of the intracavity objects. The main principles on which integrated medical systems should be based have also been laid down. Specialists have also developed a concept model of a general electronic medical record.

Thus, the "Basic Science for Medicine" Program uses the resources of the eight branches of RAS and its regional branch institutes. It makes an important contribution into the study of the etiology and pathogenesis of socially important diseases, the development and implementation of diagnostic methods, the creation of medical instrumentation and materials, and a search for new medical technologies and drugs. Overall, this program strengthens Russian science and healthcare.

This article is dedicated to the memory of academy member O.G. Gazenko, one of the organizers and ideologists of the program and the constant Chairman of the Scientfic Council.

COMMENTARY FROM THE CURATORS OF INDIVIDUAL LINES OF RESEARCH OF THE BASIC RESEARCH PROGRAM "BASIC SCIENCE FOR MEDICINE" OF THE RAS PRESIDIUM

RAMS academy member Yurii Vladimirov Andreevich, curator of the Biofotonika line:

The "Biophotonics" line appeared because there is a need for and the possibility of using modern physical and engineering methods involving photonics for the diagnosis and treatment of human diseases. These methods included (1) the creation of various powerful lasers; (2) the creation of sensitive light sensors, both integral and with spatial resolution (matrixes); and (3) the development of computer technology for creating 3D images of living objects of macroscopic (tomography) and microscopic size (confocal microscopy).

The aim of this project was to coordinate research in this field and develop new medical technology based on these scientific achievements.

The first step was to inventory all the research on biophotonics by collecting and analyzing bids from laboratories in academic institutes. A large portion of these bids were supported, and initially the decision was almost solely made based on the general scientific quality of the projects. This yielded a very wide variety of projects; however, some projects were good science, but they did not seem to result in the creation of a specific product. After the coordination council was formed, the selection procedure and control of the work procedures became much more stringent. Specifically, a group of qualified independent experts on biophotonics from non-RAS affiliated institutes, including universities and medical institutions, were asked to participate. The overall number of experts was fairly large, so each expert ended up reviewing only 3–4 projects. The expert assessment was completely anonymous, the experts had no contact with each other, and their names were kept secret. Experience showed that, nevertheless, the opinion of 2–3 experts that received a project for review was often similar, so the Council did not have much problem making an unambiguous decision on the funding of a project. We also introduced a procedure for reviewing and discussing every project no less than twice a year, one of which took place during a large scientific conference or symposium. In 2008, after numerous discussions, the members decided upon a coordinated plan for further research which would include the following aims: the development of laser technologies for visualizing living structures, the creation of light-consuming nanomaterials for the photothermic destruction of cancer cells in patients and for biochemical analysis using plasmon resonance of nanoparticles, and the development of new sensitizing materials for the photodynamic laser therapy of cancer and for controlling its efficiency.

The "Basic Science for Medicine" program is interesting not only because of its achievements, which are important for both practical and basic science. This program shows the effectiveness of the new principles that underlie the work of the Academy of Sciences. These include democratic, rational, and effective channeling of funds into groups of scientists which have applied for funding, with the independent expert assessment of the projects on one hand and science-based planning of the projects and stringent control over the completion of the projects in all their stages.

RAS member Nikolai Alexandrovich Kuznetsov, curator of the Informatika line:

The RAS Presidium program "Basic Research for Medicine" coordinates the work of the institutes which belong to eight branches of RAS, as well as many institutes affiliated with the Russian Academy of Medical Sciences and educational institutions which are collaborators in the projects. This means that the importance of the Program is incredibly high. Let us see an example of this in the work of the Biomedical Informatics section. All the work in this line of research can be divided into two subclasses: (1) expert systems made for the diagnosis and prediction of treatment results and (2) computer genomics.

A wide range of expert (partner) systems for a large number of diseases are being developed for computer support of diagnostic and prognostic solutions. The central idea of such systems is the summation, generalizing, and sharing of the experience of highly qualified specialists by using information that has been accumulated by healthcare institutions and medical and biological research centers. This information includes data on patient examination and polling, as well as data on the study of various diseases and therapeutic schemes. The development of software for such expert systems requires the use of a whole range of mathematical methods, such as algorithm theory, recognition theory, mathematical logic, and evaluation and control in indeterminate conditions. The methods, algorithms, and software for these systems are mainly developed in RAS-affiliated institutes, such as the Federal Scientific Center of Russian Federation (RF), the Institute of Medicobiological Problems, RAS; the Computational Center of the RAS; the Institute of Applied Mathematics, RAS; Harkevich Institute of Information Transmission Problems, RAS; Kotelnikov Institute of Radiotechnics and Electronics, RAS; Institute of Program Systems, RAS; Trapeznikov Institute of Control Sciences, RAS; Institute of Calculation Mathematics, RAS; Institute of Calculation Technology, RAS; Institute of Automation and Electronics, Siberian Branch, RAS; Institue of Image Processing Systems, Siberian Branch, RAS; St. Petersburg Institute of Informatics, RAS; and in the universities and institutes affiliated with the Ministry of Education and Science of the RF:

The Moscow State University; St. Petersburg University; Moscow Institute for Physics and Technology; Bauman State Technical University, Moscow; etc., in conjunction with Russian Academy of Medical Sciences affiliated institutes, institutes of the biological section of RAS, and other medical and healthcare institutions.

In the field of computer genomics, projects include studies of genes and regulatory sites, the prediction of RNA structure, annotations of genes and genomes, an analysis of regulatory signals, an analysis of gene expression, modeling metabolic and regulatory networks, and rational drug development. These projects incorporate a wide spectrum of mathematical methods and various computational equipment, from PCs to supercomputers. These projects are performed in the institutes of the Russian Academy of Sciences, such as the Vavilov Institute of General Genetics, RAS; Engelhardt Institute of Molecular Biology, RAS; Institute of Protein, RAS; Institute of Cytology and Genetics, Siberian Branch, RAS; Harkevich Institute of Information Transmission Problems, RAS; Institute of Mathematical Problems in Biology, RAS; Institute of Cytology of RAS (St. Petersburg), Moscow State University; and St. Petersburg Polytechnical University, as well as universities and institutes affiliated with the Federal Healthcare and Social Development Agency, including the Institute of Physico-Chemical Medicine (RosZdrav) and State Research Institute of Genetics and Selection of Industrial Microorganisms.

The list of universities and institutes which are involved in biomedical informatics research is very long and is still incomplete. It is presented here to show the importance of coordinating research in this field. Unfortunately, because of the limited funds of the RAS "Fundamental Science for Medicine" Program, only eight projects are currently being supported, so the coordination efforts of the program are limited as well. Real coordination of research in this field is accomplished at the level of seminars, symposiums, and conferences initiated by the Council of the Program. Effective coordination of fundamental research in the medical field requires that the "Basic Science for Medicine" program be awarded interdisciplinary status and receive a considerable increase in funding.

Yurii Vladimirovich Obukhov, doctor of physico-methematical sciences, curator of the Pribory line

Institutes of the Russian Academy of Sciences have performed active research and developed numerous solutions for medical use since the 1970s. New methods such as laser treatment, radiophysical methods of diagnosis and therapy, magnetic cardiography, encephalography, and other innovative medical technologies have been implemented under the leadership of academy members A.M. Prokhorov, N.D. Devyatkov, Yu.V. Gulyaev, and others. By the mid-1990s, the development of medical instrumentation in the RAS became a very wide field of research and development, which is why the creation of RAS Presidium "Basic Science for Medicine" program was needed, so as to concentrate efforts and funds on the most important lines of basic research and innovation technologies in medical instrumentation.

Currently, the instrumentation section has 14 projects. These are conducted in six RAS organizations in Moscow; two institutes in St. Petersburg; the Pushinskii, Troitskii, and Noginskii Research Centers (four institutes); and in Kazan and Nizhniy Novgorod. This does not include projects conducted in the regional branches of the RAS. The projects are also conducted in conjunction with medical organizations, of which there are more than 30. The instruments and methods are subjected to trials in the clinics. The results of the projects have been presented at international and Russian conferences and demonstrated at specialized exhibitions. ●

Mechanisms of Gravitational Sensitivity of Osteogenic Precursor Cells

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ABSTRACT **This report is a detailed review of the current data on mechanic and gravitational sensitivity of osteoblasts and osteogenic precursor cells *in vitro*. It summarizes the numerous responses of cells with an osteoblastic phenotype and osteogenic precursor cells and especially their responses to the alteration of their mechanic or gravitational surroundings. The review also discusses the osteogenic cell's pathways of signal transduction and the mechanisms of gravitational sensitivity. It was shown, that the earliest multipotent stromal precursor cells of an adult organism's bone marrow can sense changes of intensity in a gravitational or mechanic field in model conditions, which may play a certain role in the development of osteopenia in microgravity.**

KEYWORDS **osteopenia, gravitational sensitivity of cells, microgravity, osteoblasts, osteogenic precursor cells, multipotent mesenchymal stromal cells, differentiation, cytoskeleton.**

During the evolution, the skeletal system of land vertebrates adapted itself to an environment, in which one of the most prominent and constant factors is gravity. This factor has determined the morphogenesis and structure of all land animals. Certain elements of the skeleton have evolved for maintaining posture and achieving active locomotion, and thus are constantly experiencing static and dynamic strain as a result of "defying the gravitational force". Since humans have started exploring the outer space, the effect of microgravity on the skeletal system became an important issue, as a lack of mechanic stress (microgravity, hypokinesia, hypodynamia, immobilization) can lead to the loss of bone mass caused by insufficient mechanic impulses and gravity-induced deformations, which are not capable to support the integrity skeletal remodeling processes [1, 2].

Studies conducted in the last decade have conclusively demonstrated that cultured cells of osteoblast phenotype are sensitive to microgravity [3–8]. However, one question remains unanswered: How can microgravity affect the numerous functional aspects of less mature cell forms, namely the progenitor cells.

During post-natal development the main source of precursor cells is the bone marrow, which is closely connected with bone tissue both in formation and in functioning. Among the numerous components of the bone marrow stroma, there is a minor population of cells, which is localized in the perivascular region of the marrow, but differs from endothelial or smooth muscle cell populations by the expression of several surface antigens and by the cell's ability to differentiate into tissue cells of mesenchymal origin. So these cells posses all the characterstics of multipotent mesenchymal stem/stromal cells (MMSC) [9, 10]. MMSC were first isolated from animal bone marrow in the 70's of the 20th century by A.Y. Friedenstein and his collegues. Later MMSC were found and extracted from human bone marrow. A large number of studies showed that *in vitro* MMSC can differentiate into the cellular elements of bone, cartilage and fatty tissues, as well as support and regulate hematopoiesis [11–13]. It is well known that osteoblasts of different stage of maturity have a different degree of gravitational sense [14, 15], however the mechanisms of gravitational sensitivity of less committed cells of the bone tissue have only recently started to be elucidated.

POSSIBLE MECHANISMS OF THE GRAVITY EFFECT ON THE CELLULAR LEVEL

Comparison of the results obtained in *in vitro* experiments, with the changes that take place in a human organism under the influence of microgravity provides an opportunity to differentiate and establish the role of cellular reactions in forming physiological responses, since it allows to factor out the effects of the integral regulating systems of the human organism. The development of the views on cellular gravitational sensitivity per se can be seen in a series of reports [16–20]. Discussions of whether an *in vitro* single cell or a cell population can sense changes in the gravitational field are still very heated. Despite this, an enormous body of experimental data undoubtedly indicates that several types of cultured cells are sensitive to gravity. In particular, it was demonstrated that microgravity cause multiple and often reversible morpho-functional alterations including remodeling of the cytoskeleton, change of gene expression and a mosaic rearrangement of the intracellular regulatory machinery. These alterations are reviewed in detail in [5, 19, 21, 22].

It seems that undifferentiated mammalian cells do indeed have structural elements that may play the role of «gravitational sensor» and «sense» the intensity of a mechanical ten-

sion, and that many intracellular processes can depend on the value of the gravitational force. The most probable candidates for the role of these structures are various elements of the cytoskeleton, the nucleus, intracellular organelles and also certain cell surface receptors (integrins), which interact both with cytoskeletal structures and extracellular matrix. These structures are able to sense strains and deformations in the matrix which are caused either by a gravitational or mechanical field, and transfer this signal to intracellular messengers, which then cause a cellular response to the gravity changes [18, 23, 24]. Based on several theoretical considerations and practical observations, it is supposed that the gravitational sensitivity of the cells which grow on a surface is a function dependant on two variable parameters: The level of cell adhesion to the substrate and the strength of the intercellular interactions, while the realization of these interactions is in direct proportion to the amount of invested energy [17]. The indirect effect of microgravity at the cellular level can manifest itself in changes of the physico-chemical parameters of the medium, especially the processes of convection, sedimentation and also concentration gradients, which are all gravity-dependant and can thus be altered in microgravity [20, 25].

MECHANIC AND GRAVITATIONAL SENSITIVITY OF VARIOUS TYPES OF BONE TISSUE CELLS: EFFECTS ON THE PROLIFERATIVE POTENTIAL OF CELLS

For a long time, osteocytes and the mature inactive osteoblasts were widely accepted to be the most likely candidates for a mechanosensor in the bone tissue [14, 15]. It was supposed that this process was performed via cell-cell junctions, formed by integrins, which interact with elements of the actin cytoskeleton (actin, vinculin, etc.) inside the cell and with various proteins of the bone matrix outside the cell, thus forming a continuous network which encompasses osteocytes and the bone matrix. It was thought that this ever present and all-encompassing structure could sense and potentiate the effect of even miniscule mechanical stimuli [26].

It was demonstrated on bone cell cultures that certain types of mechanic stimulation, such as pulsatile fluid flow or mechanic strain, can trigger a cascade of regulatory reactions. The latter included a transient increase in the production of low molecular weight messengers, such as NO, expression of the inducible prostaglandin synthase (Cox-2) and secretion of porstaglandins (PGE_2, PGI_2), which were involved in the increase of the intracellular calcium concentration, in the activation of the inositol-3-phosphate signal cascade [27], and in increasing cAMP and IGF-I levels, activation of proliferative and differentiation processes in bone cells [15], and activation of cytoskeletal remodeling [28]. Nevertheless, effects from different types of mechanic stimulation are not identical [29, 30], and cells at different stages of maturity can react to the same mechanical stimulus either in the same manner [28], or differently [14, 15]. Such selectiveness and variability of the bone cell responses towards various types of stimuli seems to be caused by the unalike distribution of differentiating and mature cells within *in situ* bone tissue, as well as by the differences in their maturity and their functions.

It is well known that the proliferative activity of osteoblasts is controlled by a wide range of bioactive compounds, as well as by mechanical signals. In particular, it was shown that Cox-2 expression and PGE_2 production increase in osteoblasts in response to the growth factor TGF-β and that this effect is required for the transition between the G1-phase and the S-phase, DNA replication and active proliferation [5]. Notably, different types of mechanic stiumuli, as well as hypergravity [31], can increase PGE_2 production, which implicates PGE_2 in the anabolic effects of mechanical stress. Surprisingly, the studies conducted in microgravity detected both an increase in PGE_2 production, and also a decrease of Cox-2 mRNA levels and PGE_2 production in conjunction with an overall decrease of cell growth under microgravity [5]. The latter effect was accompanied by alterations in the structure of the actin cytoskeleton.

Studies that analyze the effect of mechanical stress on progenitor cells are of special interest. It was determined that human MMSC express both Cox-1, and Cox-2, and produce PGE_2 at a higher level than osteoblast-like cells derived from them. It was also found that the increased production of this metabolite in MMSC was associated with an increase in the expression of a membrane-bound prostaglandin-synthase. Also endogenous MMSC PGE_2 production controls the synthesis of the osteogenic growth factor BMP-2 [32]. It seems that MMSC, as well as mature osteoblasts and osteocytes, can be thought of as mechanosensory bone tissue cells, since anisotropic single axis mechanic deformation of MMSC cultured on special elastic membranes causes overall changes in the gene expression pattern, lowers the activity of certain signal transduction pathways (Jagged1) and activates cell proliferation [33]. Thus, the view that has dominated for some time now, that bone tissue cells with low-level differentiation cannot or can hardly sense mechanic stimuli, must obviously be corrected. It is worth noting, that the data which show changes in the proliferative activity of cells with osteoblastic phenotype under altered gravity are fairly controversial. Inhibition of osteoblast cell proliferation has been shown both in microgravity and in experiments that modeled these conditions [6, 34, 35]. On the other hand, the use Random Positioning Machine (RPM) did not inhibit 2T3 mouse preosteoblast growth [36]. The proliferative activity of MMSC during osteogenic differentiation did not change in a rotational bioreactor [37], decreased after incubation in a clinostat [38] and actually even increased after cultivation in a 3D-clinostat [39].

It seems that the most probable effect of microgravity on the osteogenic precursor cells is a change in the normal cell response to the anabolic influence of growth factors. Currently many researchers are of the opinion that the observed cell reactions are not caused by physical loss of growth factor receptors (for instance EGF, PDGF), but more likely by a change in the signal transduction system caused by microgravity [5, 40]. This opinion has led scientists into thorough research of candidate intracellular mechanisms and signaling pathways. According to modern views, the major routes of all three main directions of MMSC differentiation include the activation/repression of MAP-kinase cascades (mitogen-activated proteinkinases) [41]. It was demonstrated that the activation of the well-known MAP-kinase cascade (ERK1/2) is mainly achieved through a Ras-dependant signaling pathway which is activated in response to binding of growth factors with their receptors [41]. It is supposed that growth factors

such as BMP-2 and IGF-I, cause their positive mitotic effect on MMSC via the activation of the MAP-kinase cascade. This process also includes proteinkinase D, but not protein kinase C [42]. Notably, the increase in MMSC proliferation observed under the effect of pulsatile fluid flow is also realized through the calcium signaling system and the MAP-kinase cascade, which indicates the existence of a general mechanism for transforming mechanical signals into biochemical ones in osteogenic precursor cell of varying degrees of maturity [43].

THE ROLE OF ADHESION RECEPTORS IN REGULATING PRECURSOR CELL FUNCTIONS AND IN SENSING MECHANICAL AND GRAVITATIONAL STIMULI.

The question of whether the immunophenotype of precursor cells remains intact under conditions of altered gravity may be of much importance for several reasons. First, the main CD-clusters, which are expressed on the MMSC membrane, regulate various aspects of precursor cell functioning. Since they are surface receptors for growth factors and thus mediate the interactions between MMSC and hemopoetic precursors and lymphocytes, they modulate the maturation and activity of the latter and take part in the interaction of cells with molecules of the extracellular matrix [11, 13, 44]. Second, the role of some antigens in the realization of unique stem cell differentiation potentials is still unknown. Instances of the effect of model microgravity on the expression of specific MMSC surface markers are rare and controversial. Specifically, one study determined that a 7-day incubation in a 3D-clinostat caused an increase of the population ratio of human MMSC cells expressing stromal cell antigens CD44+, CD90+, CD29+ [39]. Another study showed that a 6-day incubation in a horizontal clinostat decreased the number of cells bearing the CD105 and HLA A,B,C antigens in a culture of human bone marrow MMSC [45]. Our own studies show that a 5-day incubation of MMSC on a RPM causes an increase in the number of cells expressing integrin CD49b, but does not affect the percentage of cells, expressing CD29 [46].

Perhaps the most interesting aspect of the biological peculiarities related to osteogenic precursor cell immunophenotype is the potential role of certain antigens in the mechanisms of mechanic and gravitational sensitivity. The mechanochemical hypothesis proposes that integrins and other receptors on the cell surface play an important role in the physical interaction between the extracellular matrix and the cytoskeleton and in sensing gravitational signals [23, 24]. A complex study, which looked into the molecular functions of integrins, demonstrated that simple clusterization of integrins on the cell surface in response to signals from the extracellular matrix triggers the transmembrane activation of 20 major mediators of signal transduction including cytoskeleton effector proteins RhoA, Rac, Ras, Raf, and MAP-kinases MEK, ERK and JNK. Notably, the use of cytochalasin D and tyrosine kinase inhibitors did not abolish the aggregation of integrins with FAK and cytoskeleton proteins (vinculin, talin and α-actinin) [47].

The potential role of several mentioned antigens in the response of bone cells to a rapid decrease in the mechanical stress level is very intriguing. Proof of the fact that integrins (namely, β1-integrin or CD29) play a role in osteoblast mechanical signal sensitivity was obtained in a study conducted on mice, which expressed β1-integrin in the normal amount, and transgenic animals, which had a dominant negative *β1-integrin* gene introduced into their genome. Adult mice at 2 months of age exhibitied an osteopenic phenotype, displayed a characteristic decrease in the bone tissue mass of the hind limbs, and also decreased durability and robustness of the tissue, despite a normal level of bone remodeling [48]. Another study showed activated expression of the alpha2-integrin during the course of MMSC differentiation in conditions of simulated microgravity [49]. Recent studies in mechanobiology focus not only on integrins, but on other receptors of cell adhesion as well, especially CD44 (HCAM). A study on a MC3T3−E1 osteoblast culture showed that mechanic stress caused by pulsatile fluid flow led to an increase in the level of osteopontin mRNA. This protein is a major component of the bone tissue matrix and is a ligand of CD44 [50]. Mice lacking the *OPN* gene exhibit resistance to unloading by tail-suspension and loose less bone tissue mass than the wild-type mice. Bone marrow MMSC form suspended *OPN*-negative mice, cultivated *ex vivo*, are characterized by normal ability to form mineralized nodules, as compared to decreased mineralization in cultures extracted from wild-type mice after they were suspended [51]. In connection with this, it is interesting that a 5-day incubation of rat osteoblasts under microgravity led to a decrease in osteopontin expression levels, but increased the expression of CD44, while the expression level of β1-integrin (CD29) remained constant [7].

It is known that CD44 plays a role in binding and regulating matrix metalloproteinases (MPPs) [52]. MMSC express and produce various types of MPPs (- 2, 3, 10, 11, 13, 14), and mechanical stress causes increased activity of these enzymes, and of collagenase (MPP-13) in particular, and interestingly, this increase takes place on the post-translational level [53]. These studies demonstrate, that a change in the specific balance of collagenase activity level or expression can play a distinct role in the mechanisms of collagen matrix maturation and destruction, including changes caused by alterations in the mechanic field parameters.

MECHANIC AND GRAVITATIONAL SENSITIVITY IN DIFFERENT TYPES OF BONE TISSUE CELLS: EFFECTS ON OSTEOGENIC CELL DIFFERENTIATION.

Studies that focus on the various parameters of collagen biosynthesis of the so-called mechanocytes (fore mostly fibroblasts and bone cells) under conditions of elevated or decreased gravity are of especial interest. Hypergravity usually results in increased type I collagen biosynthesis [54], while microgravity or their modeling suppress the expression of this protein [4, 55]. Our study found that MMSC, which were committed to osteogenesis simultaneously with the transfer of cells into simulated microgravity, displayed a decrease in the production rate of extracellular collagen matrix (type I collagen) [56].

A correlation has been found between the level of collagen synthesis and the activation of MAPK-family kinases, and ERK1/2 in particular, since inhibition of this signaling pathway caused decreased gene expression levels and decreased protein production levels of one of the chains of type I collagen [54]. MMSC cells committed to osteogenesis displayed a complete lack of type I collagen expression coupled with changes in the expression levels of integrins, specific to collagen, after 7 days of cultivation in a rotational bioreactor.

They also exhibited decreased levels of ERK1/2^{MAPK} phosphorylation, as opposed to p38^{MAPK} phosphorylation levels, which were elevated [37, 49].

It was shown that during the course of induced osteogenesis in normal cells of osteoblastic phenotype the activation of type I collagen expression begin after 5-6 days of cell cultivation osteogenic medium, and the peak of protein expression was usually reached on days 9-14, which was the end of the proliferative phase and the beginning of the so-called matrix maturation period [57]. This indicated that a short-term exposure of cells to microgravity did not necessarily cause expression inhibition for relatively "late" phenotypic genes, such as collagen. This also probably means that the expression of any "mechanically sensitive" osteoblast protein product is most vulnerable to changes in the gravitational field at the peak of its expression, which is tightly connected with three distinct differentiation phases in cells of osteoblastic phenotype (*Fig. 1*).

Another important aspect of the effects of microgravity on osteogenic cell differentiation is the decrease of expression and activity levels of the alkaline phosphatase (APL), and the expression inhibition of the late mineralized bone matrix marker proteins, such as osteopontin and osteocalcin, which indicate slowing effects on both the early and the late phases of osteogenic precursor cells' differentiation into osteoblasts [4, 36, 37, 49, 55, 58]. APL definitely plays a role in the mineralization of the bone matrix in bone tissue, nevertheless, it is still unclear how this mechanism functions and the precise function of the enzyme remains an issue for discussion [59]. It is not always possible to see a direct correlation between the activity of this enzyme and certain observed physiological effects, which indicates that other pathways of mineralized matrix formation may exist. One study, in particular, demonstrates that the mechanic stimulation of osteoblasts by pulsatile fluid flow causes an increase in the cellular activity of APL, although it is not accompanied by an increase of matrix mineralization in the culture [60]. One possible explanation might be provided by the recently proposed hypothesis stating that the cells of the osteocyte lineage play a role in the formation of a stable morphologically structured bone matrix. The authors propose that depending on external factors bone cells regulate the formation, maturation and rate of crystallization of amorphous phosphate-calcium mineralization nuclei via non-collagenous proteins of the bone tissue (osteonectin, osteopontin, osteocalcin and bone sialoprotein) [61]. Interestingly, lowered expression of *osteocalcin* in cells under micrigravity is often accompanied by lowered expression of a key transcription factor, which regulates osteogenic differentiation of osteogenic cells. This factor is Runx2 (runt-related transcription factor 2), and it may will be one of the primary "targets" of microgravitational effects on the osteoblastic phenotype.

THE ROLE OF Runx2 IN THE REGULATION OF OSTEOGENIC DIFFERENTIATION OF MMSC AND OSTEOBLASTS AND ITS POTENTIAL ROLE AS THE MAIN "TARGET" OF ALTERED GRAVITY EFFECTS

Runx2/PEBP2aA/Cbfa1, the main regulator of mesenchymal cell osteogenic differentiation, which can respond to the effect of osteogenic growth factors, was first identified in the course of studies connected with osteogenic differentiation of pluripotent mesenchymal precursor-cells of the C2C12 mouse line [62]. Full-fledged osteoblast differentiation and expression of specific osteogenic genes requires the cooperation of Runx2 and Smad molecules, which are activated by BMP-2. It was

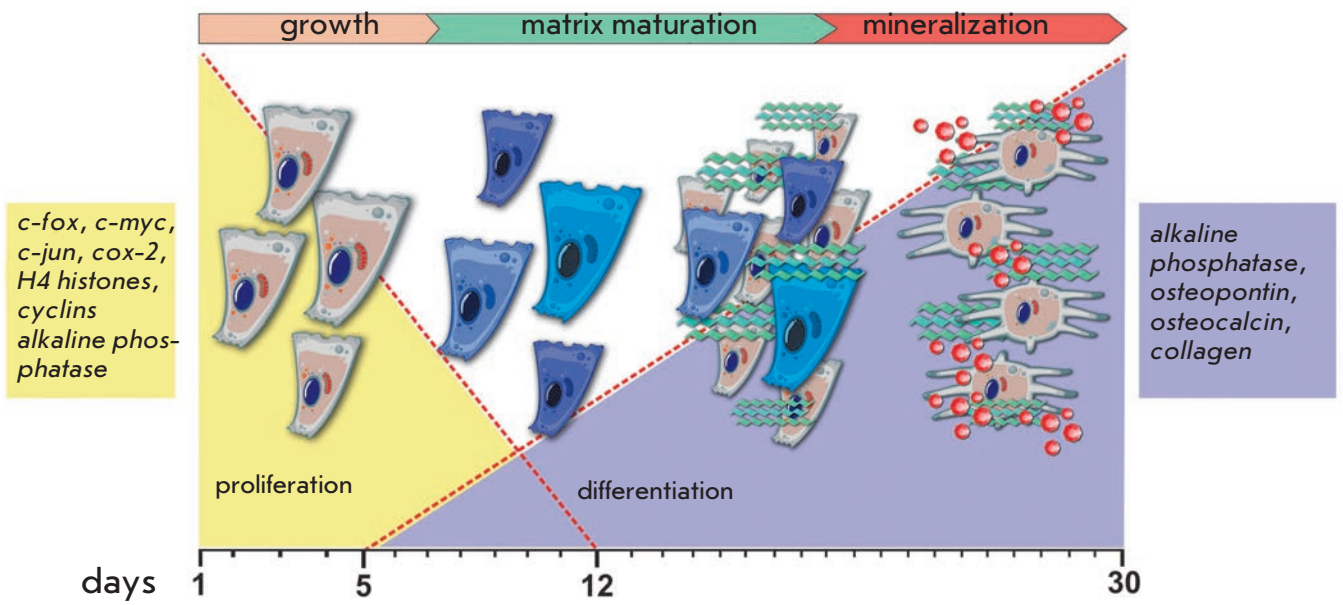

Fig. 1. Relationships between growth and differentiation stages of osteoblasts. Early stages of osteogenic cell growth are regulated by early response genes *c-fos*, *c-myc*, *cox-2* and *efr-1*, as well as by transcription factors which are activated in the cells that have commenced their cell cycle. During the very late proliferation stage and early differentiation stage, alkaline phosphatase, collagen, and fibronectin are upregulated. In the middle of the matrix maturation stage, mineralization genes and alkaline phosphatase are activated. Adapted from [5]

also discovered, that growth factor BMP-7 induced the expression of Runx2 mRNA earliear than osteocalcin expression, and furthermore, transfection by an isoform of *Runx2* led to the osteogenic differentiation of non-osteogenic cells [63].

It is currently accepted that Runx2 is an essential, but not the only needed osteogenesis transcription factor. It cooperates in the postnatal development of osteoblasts with other transcription factors (Osx, Msx, Smad, Dlx) and also plays a key role in the regulation of osteogenic differentiation of mesenchymal cells [64, 65]. Preosteoblasts which experience mechanical deformation, respond with rapid activation of *BMP-2, Runx2 u Smad5* expression, and this effect is later followed by increases of the expression of genes needed for the formation and maturation of the matrix: *ALP, COL1a1* and *OC, OPN* [66]. "Mechanically sensitive" genes were identified in osteoblasts under conditions of simulated microgravity, with *Runx2* being one of them [67]. It was also demonstrated, that LMHF (low magnitude and high frequency mechanical loading) of preosteoblasts could prevent the suppression of the osteogenic differentiation potential of cells under simulated microgravity. This was accompanied by restoration of the previously suppressed expression of *Runx2* [68]. Notably, *in vivo* models also showed that deactivation or lowering the expression level of *Runx2* were among the main mechanisms by which hypokinesia affected the osteoblastic phenotype. Partial *Runx2* heterozygous knockout mice were particularly sensitive to unloading, which provoked a more noticeable loss of bone tissue mass than in wild-type mice with a normal level of *Runx2* expression [69].

THE ROLE OF MECHANICAL SIGNALS IN DETERMINING THE DIFFERENTIATION FATE OF MESENCHYMAL STROMAL PRECURSOR CELLS: PPARγ2 VERSUS Runx2

The organism possess a highly surprising connection between osteogenesis and adipogenesis, which is preserved in the cultured precursor cells as well. Probably, these unusual reciprocal interactions between the two differentiation lineages of MMSC are determined by shared signaling pathways and regulating mechanisms, which prioritize the development of one differentiation path at the expense the other one, basing this choice on the signals received by the cells. At least some of these mechanisms have recently been elucidated.

PPARγ2 is a key adipogenic transcription factor and it functions as a negative dominant regulator of osteogenesis [70]. Specific activation of PPARγ2 by various natural and synthetic ligands leads to complete suppression of the main transcription factors of osteogenesis, cbfa1/Runx2 and Osterix, and also to increased conversion of bipotent mesenchymal precursors into adipocytes, without affecting the morphofunctional condition of osteoblasts, which are in the terminal stages of differentiation [71]. Chronic injection of a PPARγ2 antagonist rosiglitazone leads to a loss of bone tissue mass in mice, which is accompanied by an increase in the number of adiopcytes in the murine bone tissue and a decrease in the osteoblast to osteoclast ratio [72, 73]. Interestingly, as MMSC age, the activity of PPARγ2 increases, which correlates with the decrease of the pool of osteoblasts and the elevation of adipocytes numbers in the bone marrow. The cells also experience lowered expression levels of *Runx2 и Dlx5*, and also decreased production of collagen and osteocalcin [74].

Recently, the role of mechanical signals in determining and realizing various MMSC differentiation programs has been a highly discussed topic [75]. It was determined that mechanical stretching led to lowered PPARγ2 levels in a culture of bovine MMSC and in a C3H10T1/2 cell line [76]. It was also shown that mechanical stimuli led to elevated expression levels of Msx2, which activated osteogenic differentiation of cells, showed a synergistic effect with BMP-2 and inhibited PPARγ2 thus acting as a suppressor of adipogenesis [77]. Transitory activation of the Wnt/β-Catenin signaling pathway inhibited adipogenic differentiation of cells by suppressing *C/EBPα* and *PPARγ2* expression and activating the expression of osteogenetic transcription factors *Runx2, Dlx5* and *Osterix* [78]. Other studies demonstrated the possibility of the Wnt/β-Catenin-signalling pathway being implicated in the inhibition of adipogenesis and stimulation of cell osteogenesis in response to mechanical deformation. This process was realized via estrogen α-receptors [79] and insensitive to the powerful adipogenesis inducers which were present in the cell culture media [80]. Interestingly, mouse osteoblasts subjected to simulated microgravity were found to have suppressed levels of several components of the Wnt/β-Catenin-signalling pathway, such as *Sfrp2* and *Wisp2,* which may indicate, albeit indirectly, the activation of an adipogenic program under microgravity [67]. It was also shown that MMSC extracted from the bone marrow of unloaded rats and cultured *ex vivo* exhibited lowered levels of *cbfa1/Runx2* expression during the activation of osteogenic differentiation. On the other hand, these cells demonstrated an increased expression of *PPARγ2* during activation of adipogenic differentiation and generally differentiated more easily into the adipogenic lineage [81]. Similar changes were seen after short incubations of MMSC in a rotational reactor, which models the effects of microgravity [37]. However, studies of induced adipogenic differentiation of MMSC under prolonged incubations in simulated microgravity did not yield any phenotypic signs of increased adipogenesis in MMSC [56].

Microgravity can modify the differentiation potential of precursor cells through changes in activity of the major kinase signal transduction cascades (*Fig. 2*). It was determined, that MAP-kinases played an important, if not a key role in regulation of the differentiation potential of mesenchymal origin cells, including the cells under mechanical stress conditions [41, 82, 83]. For instance, it was shown that phosphorylation of Runx2 by MAP-kinases was needed in order for this protein to function in transcription activation [84]. Also, decreased/altered levels of MAP-kinase activity were an often seen cell response under simulated gravity conditions. Lowered levels of phosphorylated ERK1/2^{MAPK} during the process of MMSC osteogenic differentiation in a rotational bioreactor [37, 38] or decreased levels of phosphorylated p38^{MAPK} during osteogenic ostoeblast differentiation in a 3D-clinostat [58] have also been reported.

THE ROLE OF AUTOCRINE SIGNALS IN THE REGULATION OF THE MORPHO-FUNCTIONAL STATE AND THE COMMITMENT OF MESENCHYMAL STROMAL PRECURSOR-CELLS UNDER CONDITIONS OF MICROGRAVITY

The reciprocal suppression of the two differentiation pathways of MMSC may be attributed to the existence of other

regulatory mechanisms, including those of autocrine and paracrine nature. For instance, the products of one of the differentiation pathways may inhibit the production of compunds, which are needed for the formation of the other phenotype. Studies have shown that the lipoprotein lipase produced by adipocytes could bind sortilin, the expression of which was induced during osteogenic differentiation of MMSC, since this receptor protein was needed for the normal mineralization of the bone matrix. Moreover, sortilin itself was able mediate the endocytosis of lipoprotein lipase [85]. It has also been shown, that the increase in adipogenic differentiation of MMSC's obtained from osteoporosis patients was caused by an abnormal response of the cells to the leptin cytokine, which usually suppressed PPARγ by phosphorylation [86].

The functional role of most cytokines in the regulation of the MMSC lifecycle and in the adaptation of these and other osteogenic cells to microgravity has not been studied very deeply and requires further investigations. During recent years, researchers have paid much attention to the role of IL-8. It is known that the expression of the neutrophil-activating factor is regulated by IL-1β and TNF-a, and also by glucocorticoid hormones. Notably, IL-8 can regulate the expression of cell adhesion molecules, and also the excretion of several enzymes which can degrade the extracellular matrix [87]. These properties of IL-8 can be important for the local mechanisms of bone tissue remodeling, which are a part of several local cellular responses to microgravity. For example, bioptates of Macaca mulatta bones exposed to microgravity on the bio-sattelite «Bion-11» were found to exhibit activated resorption mediated by osteoclast resorption and osteocyte osteolysis. Neturophile activity was also elevated, and these cells excreted hydrolytic enzymes, which took part in the destruction of the mineralized bone matrix [88]. It was recently shown, that the production of IL-8 in MMSC increased in response to repeated mechanic stretching, moreover, cells cultured in osteogenic medium showed the highest increase of IL-8 production [89]. Interestingly, a manifold activation of IL-6 and IL-8 production was seen in endothelial cells, which were subjected to simulated microgravity, using an RPM [90]. It was shown that the cells exhibiting different levels of commitment (MMSC and their derivative osteogenic cells, and also osteoblasts) all responded to prolonged incubations in simulated microgravity in a similar manner, by an increased level of autocrine IL-8 production [91].

THE ROLE OF THE CYTOSKELETON IN GRAVITATIONAL SENSITIVITY OF MMSC UNDER ALTERED GRAVITY

Recently there are more and more observations giving strength to the idea that cytoskeletal structures and cell surface receptors connected to them play an imporatant role in the regulation of the differentiation potential of stem cells, which is affected by signals from an "external mechanical field" (*Fig. 3*). Also, changes of shape and of the inner cytoskeletal architecture are common cell responses under conditions of real [22] or simulated microgravity [26, 46, 92]. It has been determined that changes in the morphological characteristics of cells, or modulation of the Rho family pro-

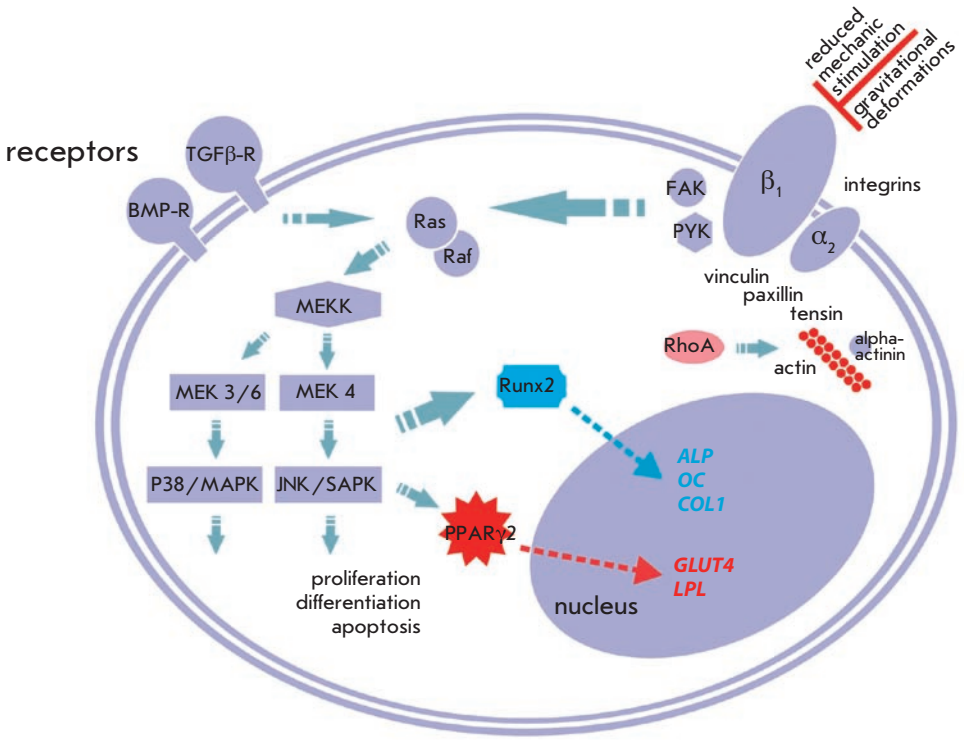

Fig. 2. Molecular regulation of the proliferation and differentiation of MMSCs under the control of an extracellular mechanical field. Extracellular signals and mechanical stimuli or their absence act via putative extracellular channels and receptors (e.g., integrins) and possibly trough other still unknown mechanisms. Signals are transferred from integrins to integrin-related focal adhesion kinases (FAK, PYK), which are, in turn, involved in multiple signal transduction pathways, including MAP-kinases, cellular cytoskeleton effectors (vinculin, paxillin, and talin), and Rho-kinases (ROCK). MAP–kinases regulate the main cell processes, such as proliferation, differentiation, and apoptosis. Rho-kinases perform actin cytoskeleton remodeling. Blue arrows indicate Runx2 signals to the nucleus; orange arrows indicate PPARγ2 signals

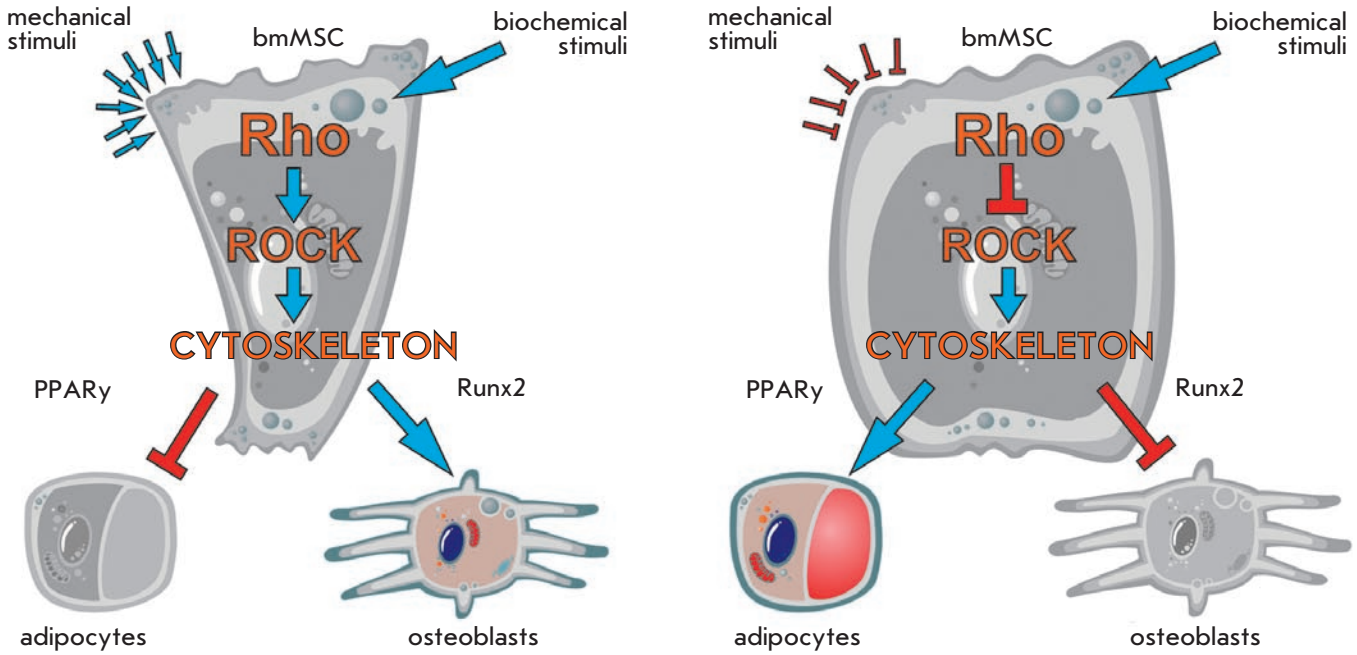

Fig. 3. Model of a mechanically mediated switch in MSC commitment. The cell shape acts as a mechanical cue, driving MSC commitment between adipocyte and osteoblast when RhoA signaling and cytoskeletal tension are intact. Well-spread cells prefer to differentiate into the osteogenic lineage and round cells prefer to differentiate into the adipogenic lineage. When the cell shape is changing active RhoA is sufficient to replace biochemical stimuli whereas RhoA effector - ROCK acts independently of cell shape. Interference with the cell shape, RhoA signaling, ROCK activity, or cytoskeletal tension alters hMSC commitment. Red arrows indicate blocking of differentiation, blue arrows – activation of differentiation

teins activity (GTPases that regulate actin cytoskeleton) can lead to the modification of the differentiation potential of MMSCs. For instance, activation of Rho-kinase (ROCK) by the upstream RhoA GTPase can induce the myogenic MMSC differentiation pathway and inhibit the adipogenic pathway even in the presence of the insulin-like IGF-I factor [93]. It is proposed that cell shape can act as a mechanical stimulus and plays an important role in the determination of the differentiation pathway of the precursor cells. It was shown that well spread cells were inclined to differentiate down the osteogenic pathway, while round unspread cells tended to take the adipogenic fate. Expression of the dominant negative *RhoA* caused differentiation into adipocytes, while overexpression of the wild-type gene led to osteogenesis. The authors found that normal actin-myosin tension was required for the correct activation of Rho-kinases by RhoA and suggested that the cytoskeleton and the regulatory proteins coupled to it could act as an integral regulatory system that controlled cell differentiation decisions, which were mainly defined through mechanical signals [94]. Interestingly, cultivation of MMSC under simulated microgravity caused changes in the actin cytoskeleton, up to a complete absence of filamentous actin in the cell after a 7 day incubation. Another effect was a strong drop in the activity of RhoA-kinase. Moreover, transfection of the cells by a viral vector, which expressed a constitutively active *RhoA*, prevented the described cytoskeleton alterations and neutralized the development of adipogenic features in the cells [92]. Direct interaction between ERK1/2^{MAPK} with the integrin-mediated signaling pathway and also with the activity of several cytoskeletal effector proteins was demonstrated by switching off of one of the actin cytoskeleton remodeling proteins (Rho), which caused the inactivation of the MAP-kinase cascade [95].

Studying all the complex factors that control the commitment of MMSC cell differentiation can help elucidate the mechanisms, which are required for maintaining the delicate equilibrium between the two stem cell differentiation pathways. Deregulation of this equilibrium during hypokinesia or microgravity can lead to severe medical conditions, such as osteopenia or osteoporosis. In conclusion it is worth repeating, that multipotent mesenchymal stromal cells of the human bone marrow are the population of cells with low-level commitment, which are sensitive to gravitational changes. Despite the growing number of reports on the effect of real or microgravity on the morpho-functional state of various types of cultured osteogenic cells, the precise molecular and intracellular mechanisms of the observed effects are still not fully understood. However, the overall phenomenology of responses from osteogenic cells of various levels of commitment indicates that common mechanisms for sensing and responding to alterations in the gravitation field do exist. Further comprehensive studies in this field will facilitate fundamental understanding of the mechanisms of gravitational and mechanical sensitivity of adult precursor cells and their possible involvement in the local cell reactions, which take place in the bone tissue in a microgravity. ●

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Selectivity of Enzymatic Conversion of Oligonucleotide Probes during Nucleotide Polymorphism Analysis of DNA

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ABSTRACT **The analysis of DNA nucleotide polymorphisms is one of the main goals of DNA diagnostics. DNA-dependent enzymes (DNA polymerases and DNA ligases) are widely used to enhance the sensitivity and reliability of systems intended for the detection of point mutations in genetic material. In this article, we have summarized the data on the selectiveness of DNA-dependent enzymes and on the structural factors in enzymes and DNA which influence the effectiveness of mismatch discrimination during enzymatic conversion of oligonucleotide probes on a DNA template. The data presented characterize the sensitivity of a series of DNA-dependent enzymes that are widely used in the detection of noncomplementary base pairs in nucleic acid substrate complexes. We have analyzed the spatial properties of the enzyme-substrate complexes. These properties are vital for the enzymatic reaction and the recognition of perfect DNA-substrates. We also discuss relevant approaches to increasing the selectivity of enzyme-dependent reactions. These approaches involve the use of modified oligonucleotide probes which "disturb" the native structure of the DNA-substrate complexes.**

KEYWORDS **DNA complexes, mismatch, selectivity, DNA ligase, DNA polymerase, modified oligonucleotide probes.**

ABBREVIATIONS **PCR – polymerase chain reaction, NA – nucleic acid, AdD – nucleotidyltransferase domain of DNA ligases, OB – oligonucleotide/oligosaccharide binding domain of DNA ligases, DBD – DNA binding domain of DNA ligases, HhH – motif of DNA ligases helix-hairpin-helix, Zn – zinc-fingers, BRCT – C-terminal domain of DNA ligases, PNA – Peptide Nucleic Acids, LNA – Locked Nucleic Acid, ENA – Ethylene Nucleic Acid, dNTP – deoxyribonucleosidetriphosphate, PPi – inorganic pyrophosphate, mc – main chain of protein backbone.**

INTRODUCTION

Single nucleotide polymorphism (SNP) is the most common form of genetic variations in the genome. Currently, the number of known single nucleotide mutations in the human genome is in excess of 9 million [1]. Such mutations are often important genetic markers that can determine the phenotypic and physiological traits of an individual and are also the molecular basis of certain diseases.

Detection of single nucleotide substitutions in nucleic acids (NA) using the most effective and simple methods which yield reproducible results is a problem of much practical interest. The development of methods for detecting point mutations which use oligonucleotide probes specific to complementary regions of NA-targets has lead to a whole range of approaches based on the use of DNA-dependent enzymes, most often DNA-polymerases [2, 3] and/or DNA-ligases [4, 5]. Currently, methods for detecting single nucleotide substitutions such as allele-specific PCR [2, 6], minisequencing [7, 8], oligonucleotide ligation assay (OLA) [9, 10], ligase chain reaction (LCR) [11], and other complex methods such as the modified ligase chain reaction (Gap-LCR) [12], which is based on the combined use of both enzymes, have firmly established themselves in practical applications. For most of these methods, the oligonucleotide probes are designed in such a way as to place the putative substitution on the NA-template into the hybrid complex, thus forming a non-complementary base pair (mismatch). Therefore, detection of a mismatch coupled to the duplex-dependent labeling of the probe can take place at either of two stages: firstly, at the hybridization step (because of the lowered stability of the imperfect complex) and, secondly, at the probe's enzymatic conversion step (because of the lowered effectiveness of the enzyme, caused by the presence of a mismatch in the DNA substrate) (*Fig. 1*). However, even such double control is not always sufficient for reliable DNA analysis. Single nucleotide mismatches which alter the complete complementarity of the DNA-duplex often do not provide sufficient specificity for a reliable diagnosis, even when DNA-dependent enzymes are used.

The search for enzyme-based ways to increase reliability in NA polymorphism analysis has been under way for sev-

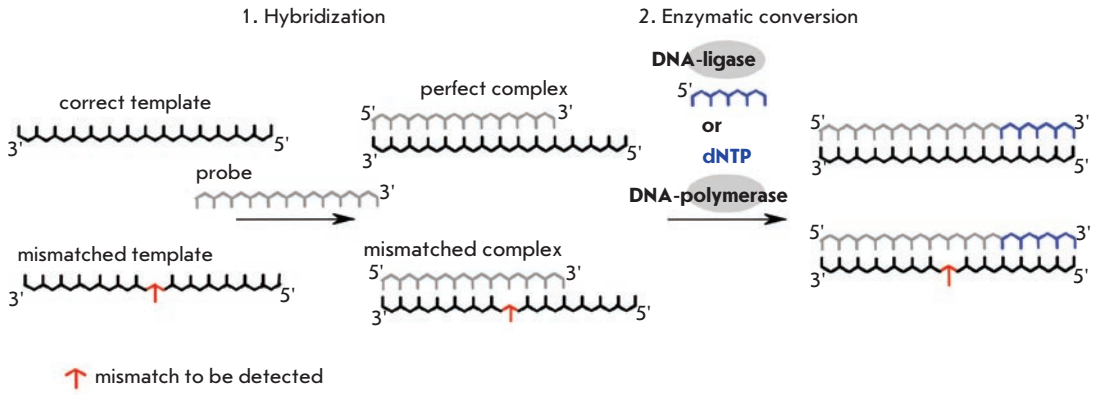


Fig. 1. Stages of DNA hybridization analysis using oligonucleotide probes and DNA-dependent enzymes

eral decades. But the issue is still unresolved, since the currently available methods for increasing the selective activity of DNA-dependent enzymes are often not universal and require preliminary screening in each specific case in order to optimize the procedure for the particular task.

This review summarizes data which characterize 1) The sensitivity of a range of DNA-dependent enzymes, which are used in NA analysis, to the presence and type of a single mismatch in the substrate complex; and 2) The structural traits of enzymes and substrate complexes which influence the selective activity of the enzyme. We analyze the peculiarities of the spatial organization of enzyme-substrate complexes, which encompasses a network of protein-nucleic junctions critical to the enzymatic reaction. We also review approaches to increasing the selectivity of enzyme-dependent reactions based on the introduction of additional "disruptive" elements into the structure of the imperfect DNA-substrate, such as artificial mismatches and synthetic nucleotide analogs.

SELECTIVE ACTIVITY OF DNA-DEPENDENT ENZYMES TOWARDS NON-COMPLEMENTARY PAIRS IN THE STRUCTURE OF A DNA-SUBSTRATE

This review uses the term "enzyme selectivity," which is the ability of an enzyme to detect a non-complementary base pair in a substrate complex under certain conditions, thus lowering the effectiveness of the enzymatic conversion of the imperfect complexes as compared to perfect (fully complementary) ones. It is known that the ability of an enzyme to identify a certain non-complementary base pair in a DNA-substrate depends on the type of base pair, its nucleotide surroundings, and the location in regard to the site of the enzymatic conversion. The selective activity of enzymes also depends on several external factors, such as the buffering quality of the environment, temperature, and temporal conditions, so an analysis of the literature does not lead to an easy establishment of the general mechanisms of enzyme discrimination in some mismatches and tolerance towards others. Some of the difficulties in the analysis and comparison of the effective detection of mismatches are due to the different methods used for measuring the selectivity of enzymes in various studies. Most often, the authors compare the following characteristics: yield of the products of the enzymatic reaction, initial rates of product accumulation, and the ratio between V_{max} and K_m. Usually, they consider the difference between the values of the threshold cycle (ΔC_t) during a real-time PCR reaction for a perfect and imperfect template, or they analyze the occurrence frequencies of the mismatch in the products of the enzymatic conversion of a random oligonucleotide library paired into complexes with a DNA-template of known structure.

In general, a significant decrease in the amount of the resulting target product during elongation of a normal oligonucleotide probe is usually seen when the mismatch is on the exact 3'-terminus of the elongating chain [13–17], and it is sometimes also seen with the mismatch in the next-to-the-last position from the 3'-terminus [15, 18]. However, even several mismatches in the central part of the substrate complex have no noticeable effect on product accumulation. One study [19] showed that the presence of 2 to 4 inside mismatches in a long (28÷30 bp) DNA-oligonucleotide complex has no noticeable impact on the yield of the PCR reaction product (*Thermus aquaticus* DNA polymerase *(Taq)* was used in this reaction). Only the presence of 5 and 6 non-complementary base pairs lowered the efficiency of the PCR 22- and 100-fold, respectively. According to other data, a single nucleotide mismatch located farther from the 3'-terminus (up to the 8th position) is enough to lower the product yield of a PCR-reaction by a factor of 10 or more (up to 1,000) for probes 17- to 19-bp long [20]. It is notable that this was not observed on all the DNA-templates tested in this study [20].

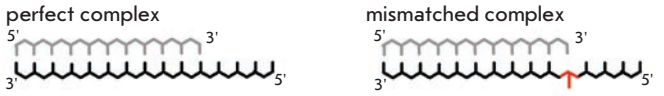
The study of the type of mismatch on the 3'-terminus of the elongating chain, which affects the polymerase reaction, showed some general patterns (*Table 1*). Polymerases from different organisms show decreasing discrimination of nucleotide mismatches in the following order:

Pur/**Pur** > Pyr/**Pyr** > Pur/**Pyr** = Pyr/**Pur** [2, 3, 14, 22, 23].

The normal font in the first position depicts the nucleotides from the oligonucleotide probe, while the second position bold text depicts bases from the template strand.

According to data from another study [3], the calculated relative elongation efficiency $(V_{max}/K_m)_{mismatch}/(V_{max}/K_m)_{complement}$ of a mismatch bearing DNA-substrate by *Taq* polymerase is less than 10^{-6} for a Pur/**Pur**, 10^{-4} to 10^{-5} for a Pyr/**Pyr,** and 10^{-3} to 10^{-4} for Pur/**Pyr** and Pyr/**Pur** mismatches. Such measurements have also been performed for the *Drosophila melanogaster* DNA polymerase α and for the reverse

Table 1. Relative elongation effectiveness for 3′-terminal mismatch bearing DNA-complexes; reaction catalyzed by DNA-polymerases



DNA-polymerase	Respective template nucleotide	primer, 3'-terminal nucleotide				reference
		A	T	G	C	
Taq DNA-polymerase	T	++++	+	++	+	[15]
	A	+	++++	+	+	
	C	+	+	++++	+	
	G	+	+	+	++++	
Taq DNA-polymerase	T	++++	++++	++++	++++	[14]
	A	++	++++	+	++++	
	C	++++	++++	++++	+	
	G	+	++++	+++	++++	
Taq DNA-polymerase	T	++++	+++	++++	+++	[21]
	A	++	++++	+++	+++	
	C	++++	+++	++++	+++	
	G	++	+++	+++	++++	
Taq DNA-polymerase	T	++++	+	++	+++	[3]
	A	+	++++	–	++	
	C	++	+	++++	–	
	G	–	++	–	++++	
Avian myeloblastosis virus reverse transcriptase	T	++++	++	++++	++	[23]
	A	+	++++	+	++	
	C	++	++	++++	+	
	G	+	+++	–	++++	
Drosophila melanogaster DNA-polymerase	T	++++	++	++	++	[23]
	A	+	++++	+	++	
	C	++	++	++++	+	
	G	++	++	+	++++	

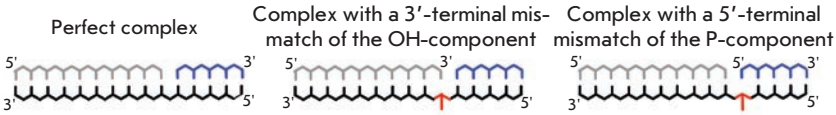
Comment. «–» means that there was no observable product of the conversion of the oligonucleotide probe. When the product does appear, the effectiveness of this process is rated by a four-step scale from «+» to «++++». The effectiveness of product formation from a complementary template was always taken as «++++».

transcriptase of the avian mieloblastosis virus (AMV RT) [23]. Discrimination efficiency for each mismatch type is about 10 times less for these enzymes. In general, accumulation of a mismatch-bearing product is in complete agreement with the presented scenario, but complete inhibition of the enzyme and absence of the full-size product can only, if rarely, be seen in the case of purine/purine mismatches [2, 3]. However, some exceptions to this general rule have been reported. Studies [3, 14] show that *Taq* DNA-polymerase does not elongate a primer if it has a pyrimidine/pyrimidine C/**C** mismatch at its 3'-terminus; however, it does efficiently elongate the primer if there is a C/**T** mismatch in the primer-template complex at the same position [3], or for that matter any other mismatch with a T residue on the 3'-terminus of the primer (T/**G**, T/**C**, T/**T**) [14]. According to another study [15], primers that have T, G or C on the 3'-terminus do not get elongated by *Taq* polymerase if the template bears substitutions in this position, while primers with 3'-A show low discrimination of any of the 3'-A/**N** mismatches, albeit with a decrease in the effectiveness of the whole PCR reaction.

As for DNA-polymerases, the position of an oligonucleotide mismatch in relation to the conversion site is a major factor in determining the effectiveness of an enzymatic reaction catalyzed by DNA-ligase. Close proximity of a mismatch to the ligation site between two oligonucleotides (single-strand break) increases the mismatch discrimination factor, causing very effective enzyme inhibition [13, 24, 25]. For instance, T4 phage and *Thermus thermophilus (Tth)* bacterial DNA-ligases show reaction yields 2.5 to 5 fold lower when the mismatch in the substrate is located one nucleotide off the conversion site, as compared to when it is squarely in the site [24]. Ligation of random sets of nanomers [26] and dodecamers [27] onto a DNA-template using *Tth* DNA ligase and *E.coli* DNA-ligase, respectively, showed, after sequencing of the long products, that the largest number of mismatched base pairs were located in the central parts of the complexes of ligated oligonucleotide blocks. Mismatches were primarily situated in the 5th position from the 3'-terminus in case of nanonucleotides [26] and in the 6th of 7th position from the 3'-terminus in case of dodecanucleotides [27]. Ligation of probes bearing random nucleotides in the first 5 positions from each side of the single strand break showed that mismatches were rarely found in the first two positions on either side of the nick in case of the T4 phage DNA-ligase. The largest number of mismatches was seen in the 3rd position, and the mismatch most often occurring was T/**G** [28]. It is worth noting that besides the proximity of a mismatch to the single-strand break, DNA-ligase discrimination is also affected by the component of the ligation in which the mismatch is situated: in the duplex part of the 3'-hydroxyl donor (OH-component) or in the 5'-terminal phosphate donor (P-component). DNA-ligases impose "extra" requirements on the structure of the OH-member of the substrate complex, and any disruption in this part of the DNA-complex has a much more pronounced effect on the enzymatic process than a similar disruption in the P-component of the duplex [26–32].

It is difficult to establish a general rule for the effects of mismatches of different types located in close proximity to the conversion site on DNA-ligases from various organisms (*Table 2*) based on the analysis of data in the literature. *Vaccinia virus* and *Chlorella virus* ligases, as well as human DNA-ligases I and III, can effectively discriminate only 3'-purine/purine mismatches located in the OH-component [29, 30, 33, 34]. The *Chlorella virus* DNA-ligase also exhibits a significant (100-fold) decrease in ligation efficiency, as compared to perfect substrates, in case of 5'-G/**A** and A/**G** mismatches in the P-component [29]. Most oligonucleotide mismatches in the P-component of the complex practically cannot be discriminated by the above-mentioned DNA-ligases. Thymidine-bearing mismatches 5'-C/**T** and G/**T** are the worst discriminated ones [33]. The archeal *Thermococcus kodakaraensis* DNA-ligase is sensitive to any 3'-mismatch of the OH-component and can only discriminate 5'-terminal P-component mismatches if they are purine/purine [31]. DNA-ligase from the African swine fever virus (ASF) is one of the least sensitive to 3'-mismatches in the OH-component when compared with the other DNA-ligases studied [35]. This DNA-ligase shows the highest fidelity (calculated according to $(V_{max}/K_m)_{mismatch}/(V_{max}/K_m)_{complement}$), which is 10^{-4} towards a substrate with a 3'-G/**A** mismatch, and the lowest

Table 2. Relative effectiveness of the ligation of DNA-complexes, which are either perfect, or bear a mismatch on the 3′-terminus of the OH-component or on the 5′-terminus of the P-component; catalysis by DNA-ligases



DNA-ligase	Respective template nucleotide	OH-component, 3′-nucleotide				P-component, 5′-nucleotide				reference
		A	T	G	C	A	T	G	C	
T4 DNA-ligase	T	++++	++++	++++	+++	++++	++++	++++	++++	[36]
	A	++++	++++	+++	++++	++++	++++	++++	++++	
	C	++++	++++	++++	+++	++++	++++	++++	+++	
	G	++++	++++	++++	++++	++++	++++	++++	++++	
T4 DNA-ligase	T	n/a	n/a	n/a	n/a	++++	+++	++++	+++	[37]
	A	n/a	n/a	n/a	n/a	++	++++	+	+++	
	C	n/a	n/a	n/a	n/a	++++	+++	++++	++	
	G	n/a	n/a	n/a	n/a	+	++++	+	++++	
T4 DNA-ligase	T	++++	+++	++	+++	n/a	n/a	n/a	n/a	[35]
	A	++	++++	+	+++	n/a	n/a	n/a	n/a	
	C	+++	++++	++++	++	n/a	n/a	n/a	n/a	
	G	++	++++	+++	++++	n/a	n/a	n/a	n/a	
Human DNA-ligase III	T	++++	+	+	++	n/a	n/a	n/a	n/a	[33]
	A	+	++++	–	+	n/a	n/a	n/a	n/a	
	C	+	++	++++	+	n/a	n/a	n/a	n/a	
	G	–	+++	–	++++	n/a	n/a	n/a	n/a	
Chlorella virus DNA-ligase	T	++++	+	+	++	++++	++++	++++	++++	[29]
	A	++	++++	+	++	++	++++	+	+++	
	C	+	++	++++	+	+++	+++	++++	++	
	G	+	++	+	++++	+	+++	++	++++	
Tth DNA-ligase	T	++++	–	+	–	++++	++	++	–	[30]
	A	–	++++	–	–	+	++++	–	+	
	C	–	–	++++	–	++	+	++++	–	
	G	–	+	–	++++	+	+	–	++++	
ASF virus DNA-ligase	T	++++	+++	+++	++++	n/a	n/a	n/a	n/a	[35]
	A	++	++++	++	++++	n/a	n/a	n/a	n/a	
	C	+++	++++	++++	+++	n/a	n/a	n/a	n/a	
	G	++	+++	+++	++++	n/a	n/a	n/a	n/a	

Comment. See comment to Table 1; n/a – effectiveness of product formation was not assessed.

(2.7) towards a complex with a 3′-T/**C** mismatch, which is converted more effectively than perfect substrates. In case of the *Tth* DNA-ligase, ligation of random selection of probes showed that the prevalent mismatches were those containing purine (G/**T**, G/**A**, G/**G**, A/**G**), which amounted to 86%, and 71% of the non-complementary pairs bore a guanine residue [26]. Also, a nonequivalence of isomismatches (G/**T** and T/**G**) was noted during library ligation. The occurrence frequencies were 14 and 2 times (the overall number of mismatches was 57) for guanines located in the ligated and the template strands, respectively [26]. A similar oligonucleotide library ligation experiment with *E.coli* DNA-ligase showed prevalent accumulation of G/**T** mismatches [27]. Also, several studies demonstrated that T4 DNA-ligase can ligate most mismatches irrespective of their location relative to the site of enzymatic conversion [32, 36, 37]. Nevertheless, [35] shows that 3′-terminal purine/purine mismatches, with the exception of 3′-G/**G** and the pyrimidine/pyrimidine mismatch 3′-C/**C**, can be discriminated, since the fidelity of their conversion by T4 DNA-ligase is approximately 10^{-4} to 10^{-6}, as calculated using the above-mentioned formula.

Literature sources on the identification of single mismatches in proximity to the enzymatic conversion site of DNA-polymerases and DNA-ligases are listed in Tables *1* and *2*, respectively. The data presented confirm all of the discussed peculiarities of the reactions catalyzed by DNA-dependent enzymes on DNA-substrates with a single mismatch.

Thus, the only fully confirmed fact is that DNA-polymerases and DNA-ligases do not always exhibit absolute selective activity when converting natural duplexes, which would be needed for the reliable detection of any nucleotide mismatches in a substrate complex formed from native oligonucleotides. This means that one of the foremost goals in the development of approaches to detect point mutations using DNA-dependent enzymes is a systematic analysis of the factors that influence the sensitivity of these molecular systems

to local disruptions in the probe-DNA complexes. In our view, specific data on the structure of the diagnostically useful enzymes, DNA-ligases and DNA-polymerases, as well as studies of the spatial structure of substrate complexes, will make it easier to explore possible ways to increase selectivity in DNA analysis and to gauge the effectiveness of such ways.

The specifics of the spatial structure of DNA-polymerases, DNA-ligases and their complexes with DNA-substrates

DNA-polymerases and DNA-ligases catalyze the formation of new phosphodiester bonds between the nucleotide precursor-components that make up two-strand DNA structures. Even though these are two separate classes of enzymes, they are similar in many ways, such as common structural elements and similarities in the interaction with the DNA-substrate.

COMMON CHARACTERISTICS OF THE DOMAIN ORGANIZATION AND ACTIVE SITE STRUCTURE OF DNA-DEPENDENT ENZYMES

The catalytic cores of DNA-polymerases extracted from different organisms have varying amino-acid sequences and belong to different families, but they still have a similar structure and consist of three domains, which are assembled in a structure reminiscent in shape of a half-open palm. The domains have appropriate names such as "palm," "thumb," and other "fingers" (*Fig. 2, A*) [38–40]. The domains of the A-family DNA-polymerases consist of six evolutionarily conserved motifs (A, B, C, 1, 2 and 6), which are thought to play the main role in the formation of the active site and the network of specific bonds with the DNA-substrate [39–42]. The most conserved motifs are A, B and C, two of which (A and C) are present in all the known DNA- and RNA-polymerases. Motifs 1, 2 and 6 also have a fairly conservative spatial structure, but they show more variety in terms of amino acid sequence. Compared to the highly conservative A, B and C domains, these other domains are less involved in forming bonds with DNA. To capture the dsDNA-substrate, the enzyme uses the "palm" (motifs A, 2, 6) and the "thumb" (motif 1) domains. The "fingers" domain closes above the "palm" forming a pocket (cavity) for the newly formed base pair. This pocket is mainly made up of motif B amino acid residues. The fragments responsible for the capture of the 3'-terminus of the primer, the inserted nucleotide, and the two magnesium ions needed for the catalysis are all localized on the inner surface of the "fingers" (motifs B, 6) and on the surface of the "palm" at the base of the "fingers" (motifs A, C). The polymerase active site, which accomplishes the addition of nucleotides to the growing strand, is situated in the "palm" domain [40, 42]. Some DNA-polymerases also have additional domains, which can, for instance, add 3' → 5' exonuclease activity.

Like DNA-polymerases, DNA-ligases extracted from various organisms have a common minimal catalytic site, which is formed by two distinct domains: the N-terminal (AdD) catalytic domain and the smaller C-terminal (OB) regulatory domain (*Fig. 2, B, 3*). The ligase active site is for the most part made up of six conserved motifs (I–VI). Five of them (I, III, IIIa, IV and V) are parts of the N-terminal domain 1 (AdD) and form the nucleotide-binding cavity. This domain is responsible for the identification and specific bonding with ATP (or NAD^+), and, there, motif I contains a lysine residue which is adenylated during the enzyme's activation. Through motif V, domain 1 binds to the oligonucleotide/oligosaccharide binding domain 2 (OB) [43–46]. Binding of the DNA-substrate to the enzyme takes place through the interdomain crevice including several structures, such as motif V [43]. Besides the regular domains, eukaryotic ligases carry an additional DNA-binding domain (DBD) on their N-terminus, which is vital for catalysis and allows a tighter "grip" on the DNA duplex [44, 47]. The C-terminus of NAD^+-dependent ligases carries the "zinc fingers" (Zn), "helix-hairpin-helix" (HhH), and other domains. These are analogous to the DBD-domain, and they increase the efficiency of substrate binding and/or the fidelity in discriminating disruptions in the substrate structure [44] (*Fig. 3*).

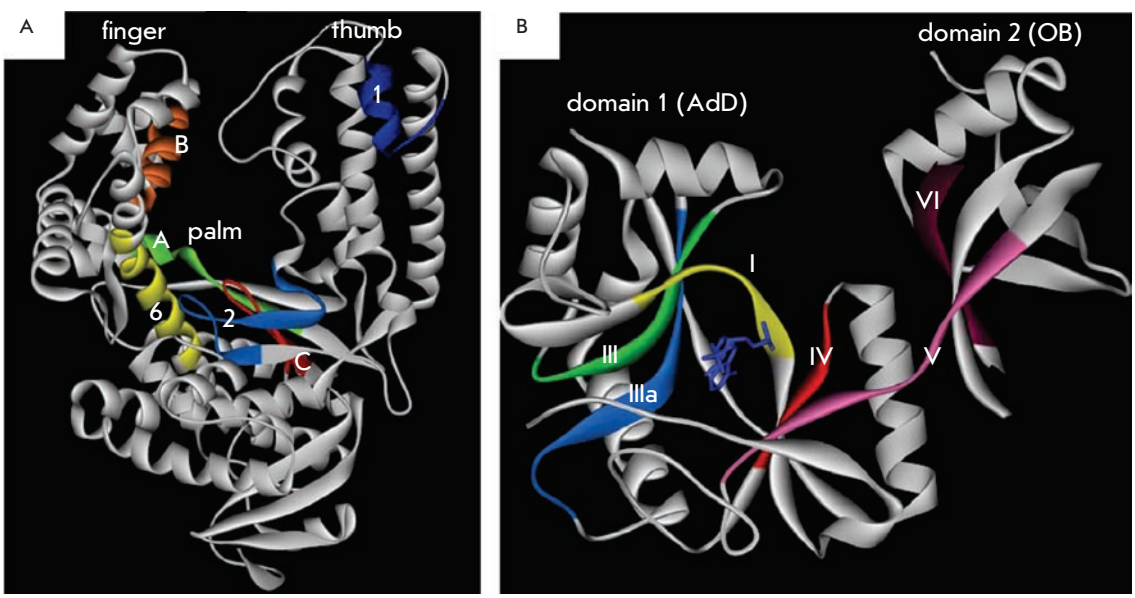


Fig. 2. Spatial and domain structure of *Taq* DNA-polymerase in its free state (A) and *Chlorella virus* DNA-ligase in adenylated state (B). Distinct motifs are highlighted with colors. Images were obtained using PDB 1TAQ [41] and 1FVI [54], respectively

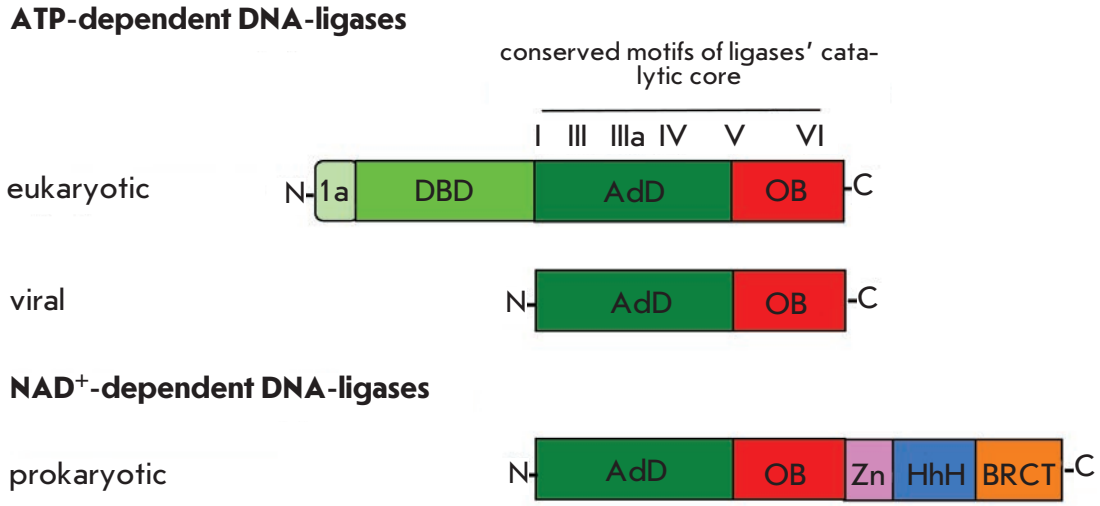


Fig. 3. Domain structure of DNA-ligases.

The structural organization of DNA-ligases, just like that of DNA-polymerases, is comparable to a hand. Domain 1 is called the palm; and domain 2, the fingers [48]. Such a metaphoric view of these domains of DNA-dependent enzymes is useful when analyzing the structural rearrangements they undergo during enzymatic reactions.

CONFORMATIONAL CHANGES DURING ENZYMATIC REACTION

In order to perform effective catalysis, the molecules of DNA-processing enzymes undergo conformational transitions. During a catalytic cycle, polymerases experience two main structural changes (*Fig. 4*). The first is coupled with the binding of the DNA-substrate, which enters the open crevice between the "thumb" and the "palm" of the enzyme. The upper edge of the "thumb" interacts with the substrate from the side of the minor groove of the double helix, and thus it bends towards the surface of the palm. This causes the "thumb" domain to form a hollow cylinder, which has a fragment of the DNA-helix firmly lodged inside. Then, the second conformational change occurs; the "fingers" turn towards the "palm," which is coupled with the binding of a nucleosidetriphosphate molecule in the polymerase's active site. This change is called the transition between the "open" and "closed" states of the enzyme, and it is the final positioning and binding of the substrate in the enzyme's active site. This is the step when the bonds between the "fingers" domain and the inserted nucleotide form, which allows to analyze the geometry of the transitional state, and thus the complementarity of the forming base pair [49–51].

Changes in the enzyme's structure are accompanied by adjustments in the DNA-substrates, which mainly take place at the stage where the duplex/polymerase complex is assembled. The vicinity of the DNA-duplex close to the enzymatic conversion site experiences changes of the carbohydrate-phosphate backbone: namely the transition of deoxyribose residues from the C2'-endo to the C3'-endo conformation. This causes significant changes in the shape of the minor groove; it becomes wider and shallower. The width of the groove increases significantly from 7 Å (DNA-helix in a free state) to 9–10 Å (in a complex with an enzyme). Thus, a fragment of the DNA-duplex helix is transformed from the B-form into an A-like form [49, 51, 52] (*Fig. 5*). These structural changes in the enzyme-bound DNA-substrate involve no more than 4–5 bp [49, 51, 52]. When deoxynucleosidetriphosphate is bound and the complex becomes "closed," the substrate experiences additional conformational changes that involve the single-strand piece of the template chain, which appears to be fixed [51].

DNA-ligases also experience a conformational transition from "open" to "closed" when performing their catalytic activity. "Closing" of the enzyme begins after the nucleotide cofactors ATP or NAD^+ have reacted, which causes the mobile domain 2 to come into close proximity with domain 1 (*Fig. 6*). An ATP (NAD^+) molecule is coordinated in a position favorable for a nucleophilic attack of the ε-aminogroup of the conserved lysine residue at the α-phosphate of ATP (NAD^+). Moreover, such a structural change leads to the formation of the catalytically active site and turns the DNA-binding domain towards the active site [44, 47, 53, 55]. Such changes not only cause the adenylated enzyme to take on a conformation which can recognize and capture DNA-substrates [44]. Final «closing» of the enzyme happens upon its binding to DNA and leads to the tight surrounding of the DNA-substrate by the adenylating and OB domains, as well as by the DBD or HhH domains, in the vicinity of the single-strand break.

Similarly to DNA-polymerase data, an opinion has been voiced to the effect that the DNA-substrate which is in complex with DNA-ligases switches from B- to A-form [48]. One of the proven facts bolstering this hypothesis is that the cavity between the DNA-ligase domains, where the binding of the duplex happens, is crooked, and in fact the warped B-A hybrid DNA-duplex would structurally match this site of the active enzyme [47]. These changes in the substrate have been confirmed by X-ray structural studies on DNA-ligase I complexes with human DNA [47]. In this case, deformation of the DNA helix into an A-like shape was only seen in part of the duplex on the OH-component side. Similar data obtained for

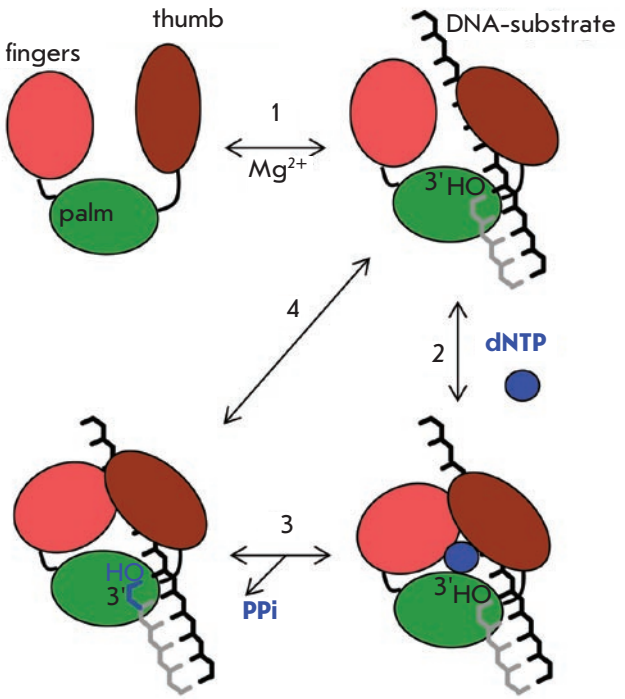


Fig. 4. Schematic representation of the conformational changes in DNA-polymerases during the enzymatic elongation of a DNA-substrate.

DNA-ligases from the *E.coli* and *Chlorella* viruses [56, 57] also suggest that, upon binding to DNA, the regions situated on both sides of the nick partially revert to the A-form (*Fig. 5, B*). The length of such partially unwound DNA fragments is dependent on the specific enzyme and varies from one to six nucleotide pairs [47, 48, 55–57].

Notably, the B → A transition of the DNA helix has been reported for other DNA-dependent enzymes (such as DNAse I), as well as for DNA-binding proteins [58]. The main reason for such a transformation of the substrate is thought to be rapid dehydration of the double helix in the hydrophobic DNA-binding cavity of the enzyme, which promotes this change in the dsDNA molecule.

PROTEIN-NUCLEIC INTERACTIONS IN THE REACTIVE ENZYME/DNA-SUBSTRATE COMPLEX

Changes in the structures of the enzyme and the substrate "tune" both of them to each other, creating a whole network of protein-nucleic acid interactions based on hydrogen and ionic bonds, as well as on Van-der-Waals interactions. This network of bonds is highly specific, and the residues of the active site, which are the most conservative ones, are often incorporated into this network. Unwinding of the DNA-duplex near the enzyme's active site increases the availability of various sites in the minor groove of the double helix, which can in turn interact with the protein structure. For the most part, these interactions are tight nonsequence-specific interactions, which are based on hydrogen bonds between the centers present in any canonic base pair (electron acceptors, which are in the N3 position of purine residues and in the O2 position of pyrimidine residues) and the conserved amino acids in the protein [51, 52, 59, 60]. In turn, the induced A-form of the duplex is stabilized by a network of Van-der-Waals interactions between the amino acid residues and the carbohydrate fragments and/or heterocyclic bases of the nucleotide [43, 47, 51, 52].

The region of the DNA that interacts with a polymerase via the minor groove of the duplex structure is about 4-5 base pairs long and is located on the 3'-terminus of the primer strand. X-ray diffraction analysis reveals which amino acid residues in the A-family polymerases (*Taq, Bst,* etc.) are involved in the formation of bonds in the groove (in case of the *Taq* DNA-polymerase):

– arginine, Arg573, which forms a hydrogen bond both with the 3'-terminal nucleotide of the primer and its complementary nucleotide in the template;

– glutamine, Gln754, which interacts with the same template nucleotide;

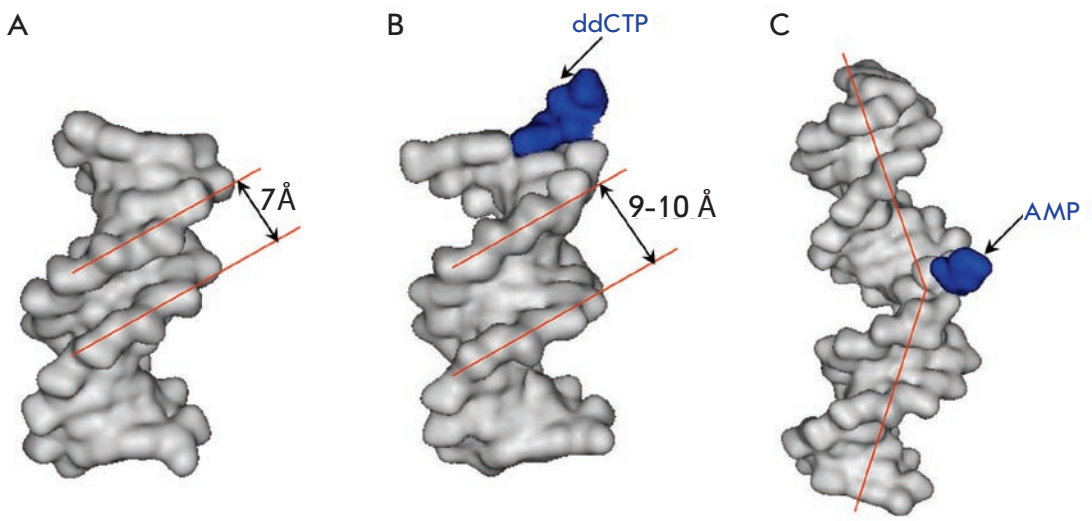


Fig. 5. Structure of a DNA-duplex in B-form (PDB 1BNA 12 bp) [53] (A), a hybrid form of DNA-duplex in the *Taq* DNA-polymerase active site (PDB 3KTQ 12–13 bp) [51] (B), and *Chlorella virus* DNA-ligase (PDB 2Q2T 21 bp) [57] (C).

– asparagine (Asn583) and lysine (Lys540), which form bonds with the 3rd , and 4th and 5th 3'-terminal nucleotides of the elongating strand, respectively [51, 61, 62] (*Fig.* 7).

Thus, the length of the DNA-complex in which the minor groove is involved in the formation of hydrogen bonds is the same as the DNA-fragment that converts to the A-form. Notably, the nucleotides which are near the enzymatic conversion site not only take part in the bonds in the minor groove, but also participate in Van-der-Waals interactions with several amino acid residues (histidine, arginine, tyrosine), which promote the fixation of the DNA-strand in its A-form [43, 47, 51]. The DNA outside this region, i.e. farther than 4-5 bp from the elongation site, is virtually all in the B-form, which makes protein-nucleic interactions possible only through the carbohydrate-phosphate backbone, making these interactions nonsequence-specific [43, 51, 52]. Interactions in this region of the enzyme-substrate complex are mainly of electrostatic and Van-der-Waals nature. A-family polymerases form various contacts with 5-8 3'-terminal base pairs of the DNA duplex [43, 51, 52, 61], and more than 40 conserved amino acids take part in these interactions [63–65] (*Fig.* 7, *A*, *B*).

The crystal structures of the DNA-ligase/dsDNA-substrate complex, which imitates the reactive state, were obtained and characterized only recently and only for a few enzymes. Before that, researchers knew only the lengths of the DNA-ligase binding sites on the substrate, which were identified by various foot-printing methods. Studies showed that the sizes of the regions which are covered by the enzyme on both sides of the single strand break are uneven. The enzyme binds 7–12 bp on the P-component side and 3–9 bp on the OH-component side [66, 67]. The overall interactive region of the DNA-substrate with the ligase varies from 10 to 20 bp depending on the enzyme. For instance, the ATP-dependant T7 bacteriophage DNA-ligase (41 kDa, 359 amino acids (aa)) was shown to bind *Taq, Bst*, etc., 3–5 bp in the 3' → 5' direction from the nick and 7–9 bp in the opposite direction along the ligated strand, using foot-printing methods [66]. Enzymatic (exonuclease III) foot-printing showed that the *Chlorella virus* ligase (34 kDa, 298 aa) can cover 19–21 bp of the DNA-substrate, of which 11–12 bp are on the 5'-phosphate donor side, and 8–9 bp are on the other side of the nick [67]. Studies [13, 68] show that T4 DNA-ligase (55 kDa, 487 aa) exhibits 11–12 bp and 6–7 bp and 5 bp regions, respectively (values are presented as in the previous example).

Currently, there is an opportunity to systematize the interactions of DNA-ligases with substrates and to determine which amino acid residues are similar in functions in enzymes extracted from various sources. It is known that the ligase/DNA-substrate complex is formed by a network of bonds which coordinate the 5'-terminal phosphate residue of the P-component, involve the minor groove of the DNA-duplex near the single-strand break, as well as the carbohydrate-phosphate backbone of each of the DNA-substrate's strands [47, 56, 57, 69, 70]. Figures 8, A and B show a map of the tight interactions which take place between the substrate and the DNA-ligases of the *Chlorella* virus and *E.coli*. It was proved that the bonds with the DNA-substrate involve all the domains of the enzyme. The major parts of these point intermolecular interactions are hydrogen bonds, which occur between the amino acid residues and the phosphate moieties

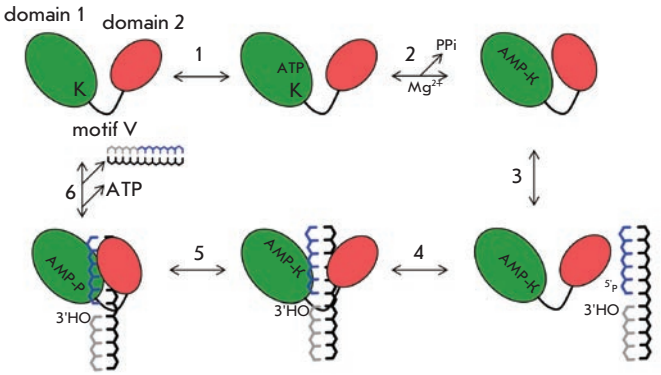


Fig. 6. Schematic representation of the conformational changes in DNA-ligases during the enzymatic ligation of a DNA-substrate with a single-strand break. K – conserved lysine residue, which is adenylated at the first step of the enzymatic reaction.

of the DNA. X-ray diffraction data indicate that these interactions involve approximately 8 to 5–6 bp from the P- and OH-components of the substrate, respectively [56, 57]. Bonds between the heterocyclic base moieties, which are exposed into the minor groove, and the amino acid residues of the enzyme involve only two nucleotide pairs on each side of the single-strand break [56, 57]. That is the precise length of the helical region of the duplex that experiences a B → A transition during the formation of a complex with the enzyme [57]. The same region of the DNA-substrate is involved in the formation of Van-der-Waals interactions with ligases. For a whole range of DNA-ligases, these bonds are formed due to the intercalation of arginine and/or phenylalanine residues between the carbohydrate residues exposed in the minor groove of the DNA-duplex [56, 57]. In the case of eukaryotic and NAD^+-dependent enzymes, DBD and HhH domains play a very significant role in the formation of bonds in the minor groove [56, 57, 69, 70]. Moreover, a bend in the DNA-substrate in the enzyme's active site has been demonstrated for a number of DNA-ligases (*Fig. 5, C*). In the case of the *E.coli* DNA-ligase, it has been shown that the HhH domain forms bonds with the phosphodiester backbone at four positions, thus stabilizing the bend of the main DNA-axis (~10°) near the nick [57] (*Fig. 8, B*). The authors note that the HhH motif, which is formed by five α-helical sub-motifs, has been found in many DNA-binding proteins [57, 71, 72].

FACTORS INFLUENCING THE EFFECTIVE PROCESSING OF THE DNA-SUBSTRATE DURING THE ENZYMATIC REACTION

There are many hypotheses on the factors affecting the sensitivity of DNA-dependent enzymes towards noncanonic base pairs in the part of the DNA-substrate that is recognized by the enzyme. These factors determine the selective activity of the enzymes including DNA-ligases and DNA-polymerases. Several of these factors will be discussed further.

The presence of canonic Watson-Crick hydrogen bonds near the processed region of the substrate and/or their stability, as well as the overall stability of the substrate complex, were long thought to be the criteria determining enzymatic catalysis in model systems based on oligonucleotides. The mechanism which helps achieve the selective conversion of

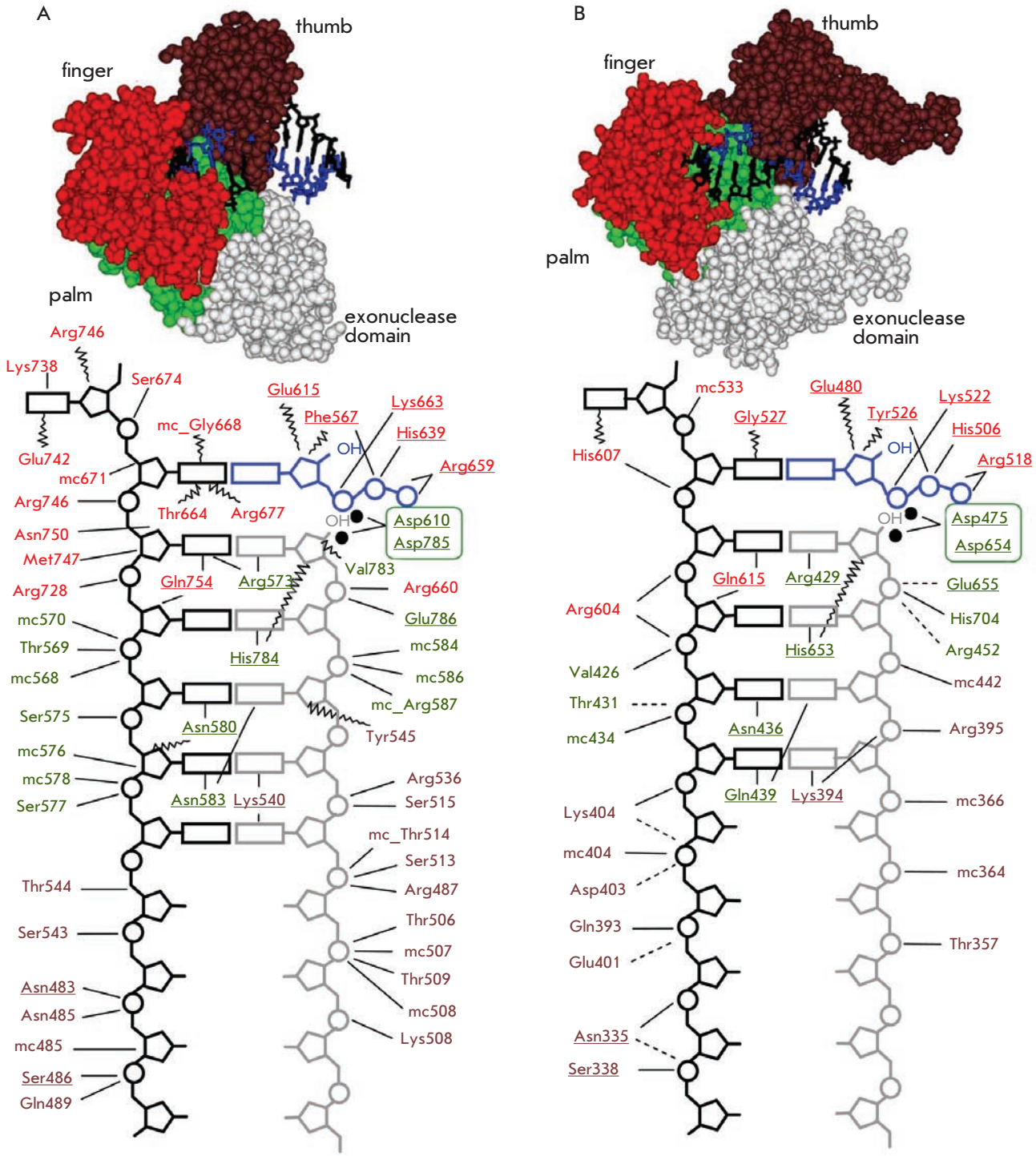


Fig. 7. Schematic representation of the network of protein-nucleic interactions, which are formed between the DNA-substrate, dNTP and *Taq* DNA-polymerase [51, 61] (A) or T7 DNA-polymerase [49] (B). Direct contact is depicted as solid lines, water-molecule mediated contact is depicted as dotted lines, Van-der-Waals interactions as wavy lines. Amino acids belonging to different domains of the enzyme are depicted in different colors. Highly conservative residues in the A-family polymerases are underlined. Interactions formed by the atoms of the main chain of the polypeptide have the "mc" prefix. Metal ions are depicted as black circles. The upper panel depicts the spatial structures of the respective enzyme-substrate complexes obtained using PDB structures, 3KTQ [51] (A) and 1T7P [49] (B)

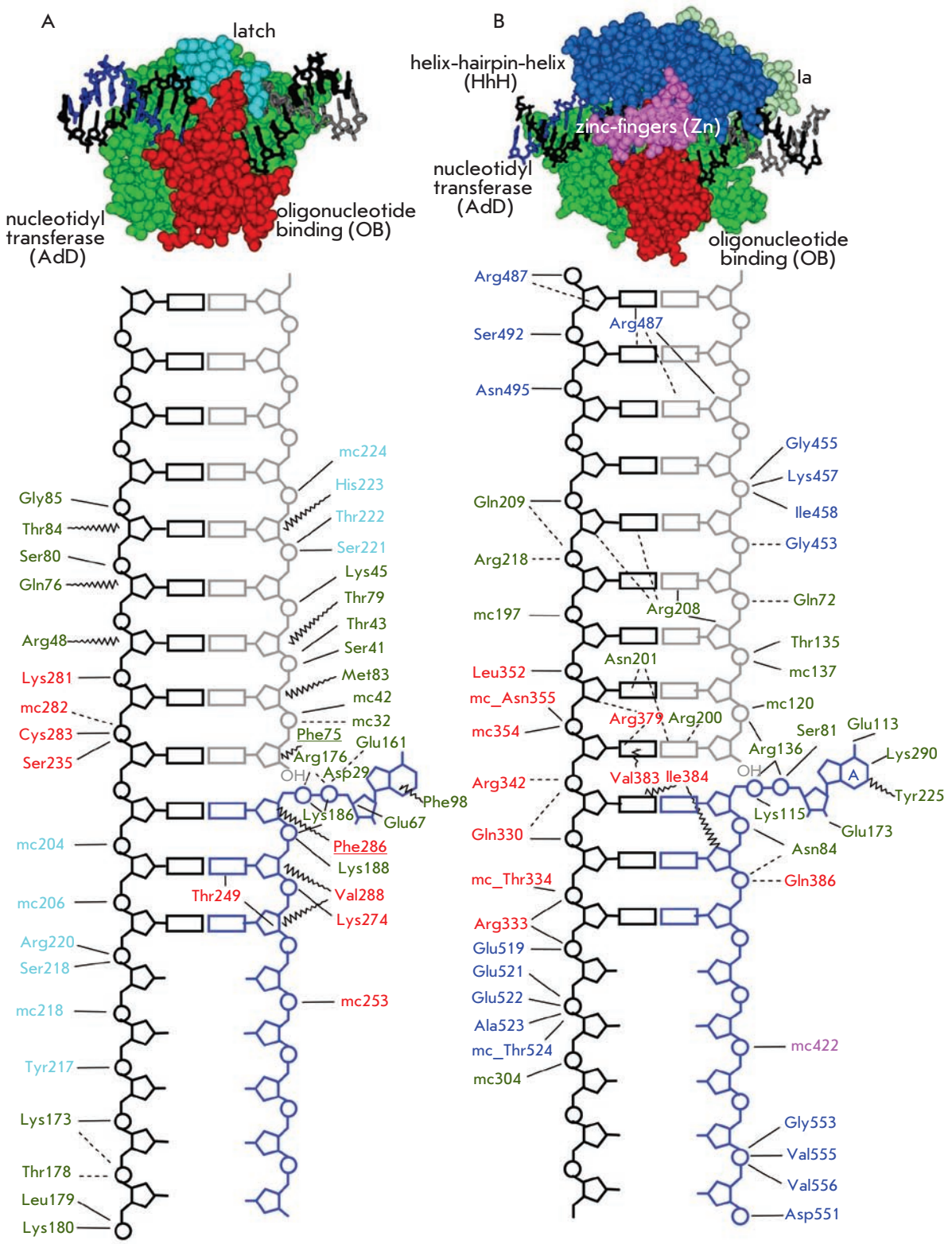


Fig. 8. Schematic representation of the network of protein-nucleic interactions, which are formed in the reactive complex between the adenylated DNA-substrate and the *Chlorella virus* DNA-ligase [57] (A) or the *E.coli* DNA-ligase [56, 69] (B). Direct contact is depicted as solid lines, water-molecule mediated contact is depicted as dotted lines, Van-der-Waals interactions as wavy lines. Amino acids belonging to different domains of the enzyme are depicted in different colors. Interactions formed by the main chain of the polypeptide have the "mc" prefix. The upper panel depicts the spatial structures of the respective enzyme-substrate complexes, obtained using PDB structures 1FVI [51] (A) and 2OWO [56] (B)

substrates is still not fully understood. More and more facts indicate that the contacts between the enzyme and the substrate not only bind and adjust the latter in the active site, but also help identify substrates with mismatches or any other disruptions of the regular DNA structure.

One of the criteria which allow enzymes to identify mismatches in the DNA may be the disrupted structure of the DNA helix caused by a mismatched base pair. This hypothesis is confirmed by experiments with 5-fluorouracil, which forms a pair with guanine (*Fig. 9*). Such a modified pair is "swinging," which means that depending on the pH of the solution it can be in a paired state, similar to the Watson-Crick C/G pair, or assume a different state (*Fig. 9*) [73]. The presence of such a stable, but structurally noncanonic base pair in the recognition site considerably lowers the reaction yield for *Tth* and T4 DNA-ligases as compared to the 5-fluorouracil/guanine pair, which is similar in geometry to a normal complementary pair [73]. The noncanonic nucleotide pairs uracil/guanine and uracil/hypoxanthine (*Fig. 9*) also lower the effectiveness of ligation of DNA-complexes, which have such pairs in the single-strand break site, by *Tth* ligase. It has been demonstrated that slowing of this enzymatic reaction takes place at the stage of DNA-substrate adenylation [73].

Another aspect of the selectivity mechanisms of the enzymatic process is the formation of hydrogen bonds between the enzyme and the minor groove of the substrate DNA. It so happens that the hydrogen bond acceptors of heterocyclic bases, which face onto the minor groove, all have a typical layout in case of correct pairing (bearing in mind the different roles of the processed and template strands of DNA-substrate) and an atypical one for noncomplementary pairs [59] (*Fig. 10, A*). Such a topological trait can promote the identification of mismatches and perfect pairs. The importance of such bonds is indirectly confirmed by the fact that human β DNA-polymerase and HIV-1 RT (reverse transcriptase) form only a single bond in the minor groove of the DNA-helix and are characterized by indiscriminate elongation of DNA-duplexes with mismatches, as opposed to A-family polymerases, which form multiple contacts [60, 74].

The role of enzyme-substrate bonds in the minor groove was studied using both nucleotide analogs in the DNA and mutant enzymes. It is clear from the structures of the enzyme-substrate complexes presented earlier that the base pairs in the enzymatic conversion site are always involved in hydrogen bonds with amino acid residues. In order to asses the importance of these contacts, a single nucleotide in this pair is replaced by a nonnatural analogue, such as 3-deazoguanine (guanine analog) [75, 76], 2,4-difluorotoluol (thymidine analog) [73, 77], or 4-methylbenzimidazol (adenosine analog) [77] (*Fig. 10, b*). The presence of such modified nucleotides, which are similar to their natural analogs, but do not have hydrogen bond acceptors in the minor groove, is one of the causes behind the termination of the enzymatic reaction catalyzed both by DNA-polymerases (Klenow fragment [75, 77], *Taq*, T7, HIV RT, polymerases α and β [77]) and DNA-ligases (*Tth* and T4) [73]. The presence of the modified analog significantly lowers the effectiveness of DNA-polymerase catalysis only when the analog is present in the enzymatic conversion site [77]. Introduction of the analog nucleotides into both strands of the substrate shows that for the tested DNA-polymerases hydrogen bond formation in the minor groove is required only on the primer strand side for most polymerases, and that it is needed in the template component only for HIV RT [77].

Investigations of the role of minor groove interactions from the point of view of the enzyme's structure were conducted using mutant forms of Klenow fragment of the *E.coli* DNA-polymerase I [76, 78, 79]. Mutants R668A and Q849A have amino acids involved in the formation of hydrogen bonds in the wild-type enzyme substituted. These two amino acids form bonds with the heterocyclic bases of the 3'-terminal nucleotide pair of the elongated and template strands, respectively. These functional amino acid residues are replaced by alanine, which does not have hydrogen bond donors in its side chain. The R668A substitution causes reduced effectiveness of the enzyme-substrate interaction with a perfect complex and has a very little altering impact on the processing of a 3'-mismatched substrate [76, 78, 79]. The Q849A substitution does not affect the identification of the DNA-substrate by the enzyme [78]. Thus, the study of mutant enzyme forms of DNA-polymerases confirms the importance of bonds in the minor groove as a factor that determines effective processing of the substrate. In this case, as well as in the case of use of modified nucleotides, the formation of hydrogen bonds in the minor groove of the DNA molecule was most important in the elongated strand.

There is data that suggest that the effectiveness of the enzymatic reaction does not depend on the nature of the side-chains facing the major groove of the substrate DNA, nor does it depend on the moieties at the 6th position of the heterocyclic base. Two such nucleotide analogs are used; 2-aminopurine and purine. Both of them can form a pair with uracil (*Fig. 10, C*) [73]. The presence of modified bases in the −1 and +1 positions from the nick causes no significant decrease in the effectiveness of a reaction catalyzed by *Tth* or T4 DNA-ligases [73].

In some cases, the effectiveness of the enzymatic reaction can be less dependent on the bonds in the minor groove and be affected by other interactions. These results were obtained in the studies of mutant forms of A-family DNA-polymerases

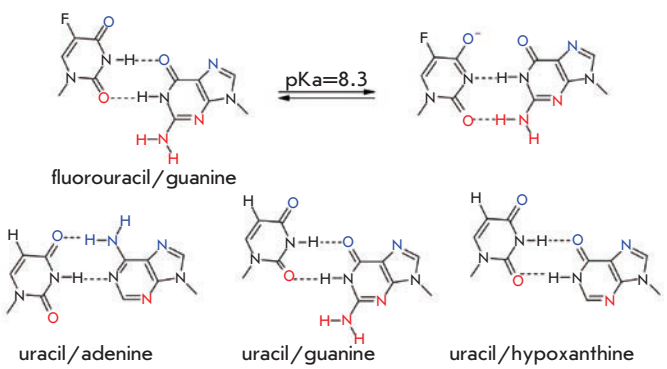


Fig. 9. Modified non-canonical base pairs. Blue and red colors depict atoms which are donors/acceptors of hydrogen bonds and face the major and minor groove, respectively.

(*Taq*, Klenow fragment) at the conserved Gln-Val-His (QVH) motif, which is a part of the C motif [43, 80]. It is known that this motif interacts with the deoxyribose of the 3'-terminal nucleotide of the primer. The histidine residue can then form a hydrogen bond via the minor groove of the duplex. Mutant forms of DNA-polymerases exhibited increased discrimination of substrates bearing mismatches (some of them were as far as 2–4 nucleotides from the enzymatic conversion site [80]), as compared to the wild-type enzymes. The most selective mutants for the Klenow fragment turned out to be PLQ, LVG, LVL and ILL, IVF, CLV for the *Taq* polymerase. Of all the obtained mutants, only PLQ had histidine substituted for glutamine, thus preventing the imidazole ring of the former acting as an electron donor. In most cases, the histidine residue was replaced by amino acids with nonpolar side-chains (leucine, valine, glycine, and phenylalanine) [80]. This suggests that in this case the hydrophobic interactions which stabilize the A-form of the duplex near the site of conversion plays a more important role than hydrogen bonds.

The Klenow fragment mutants N675A, R835L, R836A, R841A and N845A have lower selectivity [78]. The exact reason for the effect of these mutations on the selective elongation of the substrate is unclear. The authors suggest that N845 is involved in identifying the "correct" shape of the 3'-terminal base pair. Residues R835, R836 and R841 can interact with the single-strand region of the template strand, thus stabilizing the curve, which can be observed in this DNA fragment. The N675 amino acid interacts with the template at the position where the DNA changes from B- to A-form and can thus be implicated in the stabilization of this conformational disruption.

Substitutions in the amino acid sequence of DNA-ligases can also cause changes in the effectiveness of substrate ligation. Thus, the following mutants of the *E.coli* DNA-ligase with substituted conserved amino-acids in the OB domain (R379A, V383A, I384A and R333A-T334A) were shown to exhibit decreased ligation efficiency, which was no more than 10% of the original activity of the wild-type enzyme [69]. This altered ligation efficiency determined by the functions of the involved amino acid residues is due to the effects on the formation of hydrogen bonds in the minor groove on the template strand's side of the OH-component (amino acid R379), on the interaction with the carbohydrate backbone of the template strand (R333 and T334), on the formation of a Vander-Waals interaction network with the base pairs in close proximity to the single-strand break (two pairs in the OH-component and one in the P-component), and on the stabilization of the DNA-substrate in A-form (V383 and I384) [69]. DNA-ligases from the *Thermus* family (TAK16D and AK16D) were found to have mutants which exhibited increased discrimination of 3'-terminal single mismatches. The mutations D286E, G287A, V289I, and K291R were in the AdD domain [81]; and T599A, in the BRCT domain [82], respectively. In case of the *Tth* ligase, it was found that the use of K294R and K294P mutants led to increased selectivity in the reaction [30]. The causes of these interesting results are unknown. It is however evident that these amino acids are involved at different steps of the enzymatic reaction, and that the increased selectivity is most probably a complex feature involving several steps in the ligation process.

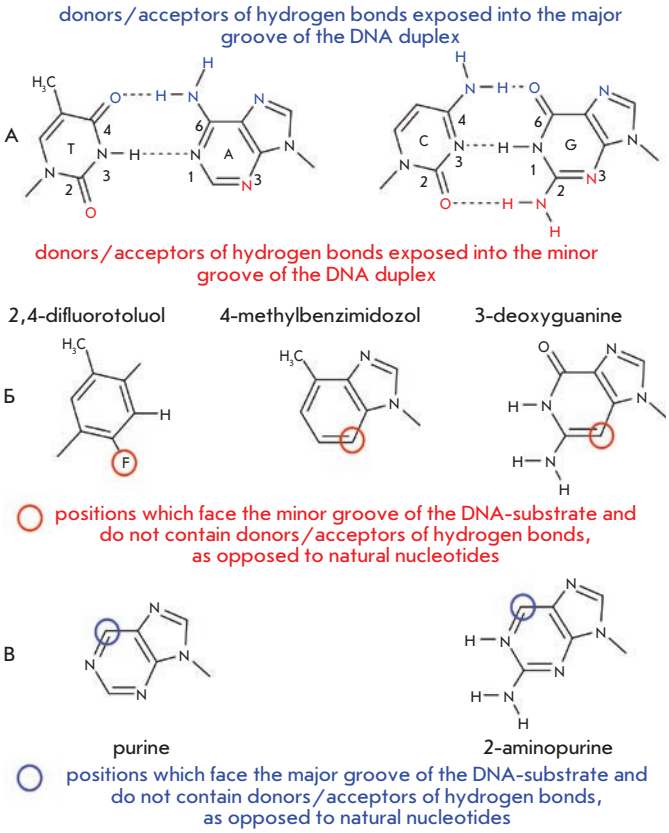


Fig. 10. Layout of the hydrogen bond donors/acceptors in the minor and major grooves for the Watson-Crick base pairs (A). Modified bases, which do not have hydrogen bond donors/acceptors in the appropriate positions in the minor (B) or major (C) grooves. Blue and red colors depict the atoms which are donors/acceptors of hydrogen bonds and face the major and minor groove, respectively.

Thus, the selectivity of enzymatic reactions is due to the formation of "correct" bonds in the enzyme-substrate complex. Most probably, the overall effect of these point interactions in the complex creates the basis for discriminating imperfect regions in the dsDNA-substrate.

EFFECT OF STRUCTURAL DISRUPTIONS OF THE DNA SUBSTRATE ON THE SELECTIVITY OF ITS ENZYMATIC CONVERSION

In the previous section, we reviewed the structure of DNA-dependent enzymes and the complexes they form with the substrate in order to identify the factors affecting the selectivity of enzymatic conversion. This section will review the structural traits of the DNA-complex which can increase the selectivity of the enzymatic reaction. One of the simplest ways to increase enzymatic ligation effectiveness is to use a method based on "modified" probes, which consist of tandems of short oligonucleotides [83–85]. The presence among the ligated components of mini-probes of penta- and even tetranucleotides makes these composite complexes less effective as substrates, and their enzymatic selectivity appears to be high [83, 85]. If a tetranucleotide is used as the central part of a three-part tandem, the discrimination factor for any type of

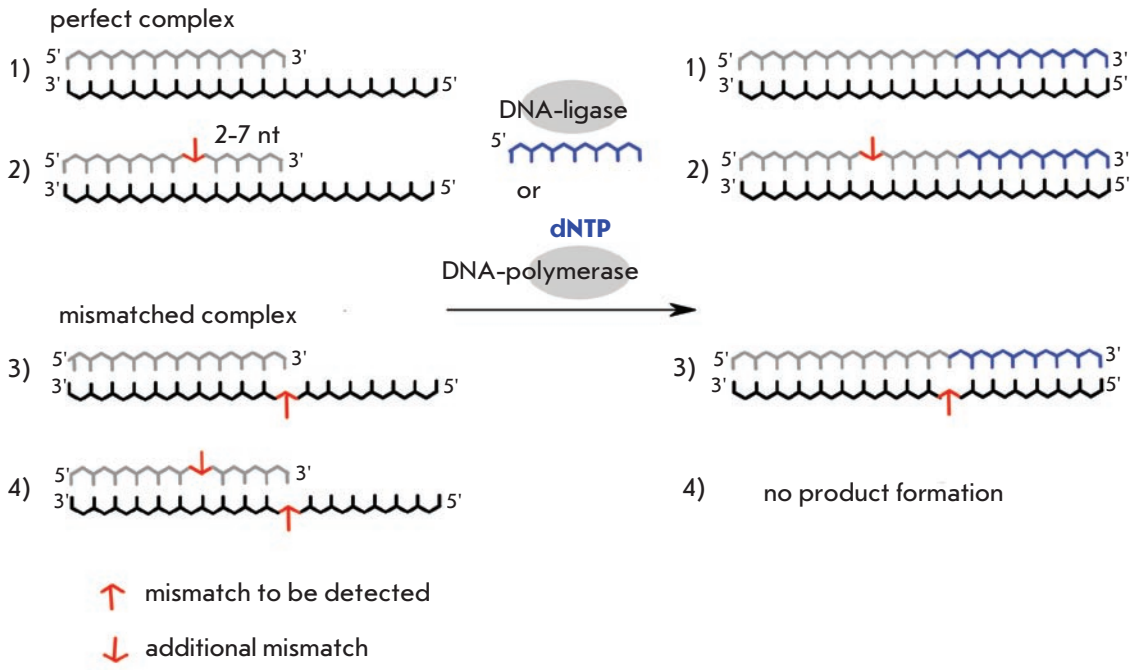


Fig. 11. Schematic representation of the process of detection of a non-complementary base in the DNA-substrate during its enzymatic conversion, using an intentional additional mismatch.

mismatch in the region of the substrate complex is more than 300 when using the mesophilic T4 DNA-ligase [85]. Such high selectivity of the enzyme is unattainable if the DNA-duplex is formed by oligonucleotides, which are long enough to provide optimal conditions for enzyme binding on the molecule (see [37] for an instance).

INTRODUCTION OF AN ADDITIONAL SINGLE NUCLEOTIDE MISMATCH

Another way to increase the selectivity of enzyme-dependent reactions not involving the use of modified nucleotide analogs is based on the use of DNA-substrates with an intentionally added mismatch next to the polymorphic site to be analyzed. The effectiveness of such an approach was demonstrated for *Taq* DNA-polymerase [14, 86–90] and *Tth* DNA-ligase reactions [30]. This method involves placing the studied mismatch on the 3'-terminus of the elongated oligonucleotide or the ligated OH-component and the additional mismatch in proximity to the 3'-terminus, specifically in the 2nd, 3rd or 4th positions [14, 30, 88–90], and in some cases in the 5th or 7th [86]. In these cases, the "perfect" complex has a single planned noncomplementary pair, while the complex with a mismatch contains two disruptions (*Fig. 11*).

Comparison of the effectiveness of full-size elongation or ligation product accumulation shows that a single noncomplementary pair does not cause a significant decrease in DNA-substrate conversion, while a double mismatch can lower the end-product yield by a factor of 100 or more [14, 30, 88]. The only exception is seen in case of the 3'-terminal mismatch 3'-T/**N**, when the introduction of a second mismatch only lowers the product yield 5- to 10-fold, and the presence of two neighboring 3'-T/**N** mismatches only lowers the yield 2- to 5-fold, which is not a significant decrease compared to a perfectly paired complex [14]. The effect of the position of the intentional mismatch on the decrease of the false-positive signal during olignucleotide probe processing was also studied [14, 30, 86, 88, 90]. An additional mismatch at any of the above-mentioned positions caused increased discrimination efficiency of the original mismatch. However, an intentional mismatch at the 2nd position did cause a significant decrease in the enzymatic reaction efficiency in several cases. Thus, a *Tth* DNA-ligase reaction exhibited a 175-fold yield reduction even with a complementary pair on the 3'-terminus of the oligonucleotide [30]. On the other hand, a noncomplementary base in the 4th position did not significantly increase selectivity [86, 90]. Of all of the examined positions for introducing an additional mismatch in order to increase the discrimination efficiency of a target mismatch in the enzymatic conversion site in elongation and ligation reactions, the 3rd position from the 3'-terminus proved to be the most effective [30, 88, 90]. In this case, the end-yield of the product which has a mismatched pair on the 3'-terminus was no more than 5 % [90]. The introduction of an additional mismatch was also effective in increasing the discrimination of mismatch during ligation. The ratio between the initial rates of complex conversion of a substrate with a complementary pair at the 3'-terminus and mismatched pair increased by a factor of 4 [30].

Thus, an intentional mismatch introduced into the DNA-substrate structure in addition to the original single nucleotide substitution causes increased enzyme-dependent reaction selectivity. The observed patterns are probably due to the fact that two closely spaced nucleotide mismatches cause a much larger disruption of the DNA structure at the enzyme recognition site and thus a more significant change of stability and structure of the DNA-helix. This causes a double mismatch to have a much stronger impact on the effectiveness of the enzymatic reaction. However, introduction of additional single nucleotide mutations is probably not a universal method for increasing selectivity, since the nature of the introduced "disruptions" is sequence-specific.

OLIGONUCLEOTIDE MODIFICATIONS THAT INCREASE THE SELECTIVITY OF DNA-DEPENDENT ENZYMES

Currently, oligonucleotides carrying modified bases or with an altered carbohydrate-phosphate backbone occupy a distinct niche in DNA hybridization probes design. Some modifications (PNA peptydylnucleic acids [91], LNA and ENA "locked" nucleic acids [92, 93]) increase the stability of the modified complexes, which can be used to increase the accuracy of DNA analysis at the level of hybrid complex formation. Other modifications (N4-alkylcytosine [94], 5-methyl- and 5-(1-propargyl)uracyl [95]) can equalize the hybridizational characteristics of complexes with a different nucleotide content, which is important during a parallel analysis of different DNA sequences. It is worth noting that not all oligonucleotide modifications are compatible with DNA-dependent enzymes, since the introduction of these modifications can disrupt the protein-nucleic interactions which are needed for effective enzymatic catalysis. Nevertheless, introduction of certain nucleotide analogs could become a method for increasing the selectivity of enzymes towards mismatches in modified DNA-duplexes (*Table 3*).

MODIFICATION OF HETEROCYCLIC BASES

One of the modifications used is a synthetic analog of a deoxyribonucleotide, which bears a universal 3-nitropyrolle base. This base is named universal because it can form pairs with all the natural bases thanks to its small size, which is comparable to that of the natural bases, and the ability it retains to take part in stacking interactions. The effect of this analog on the selectivity of the *Tth* DNA-ligase [30] was studied by introducing it into the 3rd position from the pair to be analyzed, which was placed on the 3'-terminus of the OH-component. The choice of the position was based on data on increased selectivity upon introduction of an additional mismatch, which was found to be optimal in the -3 position from the enzymatic conversion site. The presence of the nucleotide analog caused a 9-fold selectivity increase of the *Tth* DNA-ligase, which is 2.5-fold more than the increased selectivity effect seen upon the introduction of an additional mismatch based on canonic bases. *Taq* polymerase also exhibited decreased formation of PCR products during the use of a single mismatch and a primer with a 3-nitropyrolle, as compared with a normal primer [96]. The unpredictable binding of oligonucleotides bearing such a modification with the DNA-template is a disadvantage of this approach.

Another nucleotide analog, which can increase mismatch discrimination in *Tth* DNA-ligase reactions, is a 4-nitroimidazole deoxyribonucleotide. When introduced into the probe, it can form a pair with the guanine base, which is less stable than the native C/G-pair [97]. The use of probes with at least 2 such modified analogs lowers the ratio between the ligation products of the mismatched and "perfect" substrates by 15% (average value) as compared to the use of native oligonucleotides [97].

The effect of the above-mentioned modifications is based on the same principle as the introduction of an additional mismatch close to the polymorphism to be detected. The addition of modifications causes destabilization of the DNA-duplex, which can lower the efficiency of the enzymatic conversion only if an imperfect pair is present in the duplex structure. On the other hand, other studies demonstrate that modifications such as 7-deazaguanine and inosine lower the stability of duplexes, but do not increase the selectivity of *Tth* DNA-ligase when introduced into a DNA-substrate with a mismatch [97]. This is probably due to the specific steric complications in which these modifications are involved.

Overall, even though the analog nucleotides modified at their heterocyclic bases can form hydrogen bonds with the native nucleotides, it is evident that these bonds are different from Watson-Crick bonds. This may be one of the reasons for the disruption of the DNA-helix. The use of these analogs in the hybridization analysis of oligonucleotides with a modified carbohydrate-phosphate backbone may prove to be more effective, since this approach does not involve the part of the nucleotide which forms complementary interactions with the NA-template.

MODIFICATIONS OF THE CARBOHYDRATE RESIDUE

Such derivative oligonucleotides involve oligomers, which contain modified bicyclic RNA-like monomers with 2'-O, 4'-C methylene and 2'-O, 4'-C ethylene links, LNA (Locked Nucleic Acid) [98–102], and ENA (Ethylene Nucleic Acid) [103], respectively.

LNA- and ENA-containing oligonucleotides exhibit an increased affinity to the complementary NA-template, and this can be used for detecting mismatches in a PCR reaction, since the complex forms with higher specificity [93]. Primers with a single LNA-modification at the exact 3'-terminus or in the last but one position, or with an ENA-analog in the third position do not lower the effectiveness of elongation of modified primers by *Taq* DNA-polymerase if the DNA-substrate is perfect, but they facilitate discrimination of 3'-terminal mismatches if the forming DNA-complex is imperfect [98–103]. The introduction of modifications into the 3'-terminal region of the oligonucleotide lowers the yield of the PCR reaction in the presence of "difficult to detect" mismatches (C/A, T/G, G/T) by at least 50% as compared to the use of a native primer [98, 99, 103]. The reason for such altered selectivity of oligonucleotides with bicyclic analogs may be due to the altered behavior of the DNA-substrate in the active site of the enzyme. The presence of a locked "link" causes the fixation of the nucleotide's ribose in the 3'-endoconformation [104], which is characteristic of nucleotides in an A-form dsDNA-helix. Also, the modified nucleotide itself shows less conformational flexibility. Thus, LNA- and ENA-modifications facilitate the locking of the DNA-substrate in A-form, which has a positive impact on the formation of protein-nucleic interactions in the minor groove of the DNA-substrate.

Another type of modification of the carbohydrate residue which increases the selectivity of DNA-polymerase activity is the use of C4'-alkylated thymine nucleotides $\mathbf{T_R}$ [105–108]. These modifications, which cause the formation of atypical groups in the minor groove of the DNA-complex, were first tested for effectiveness of the elongation of an oligonucleotide modified at the 3'-terminal position. Such reactions depend on the size of the alkyl residue and the type of DNA-polymerase used. *Thermococcus litoralis* (*Vent*) archaeal polymerase was found to elongate the modified oligonucleotide even when a large vinyl residue was introduced, while the archaeal *Pyrococcus furiosus* (*Pfu*) polymerase could only elongate a prim-

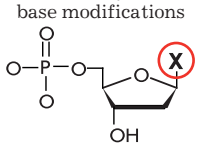
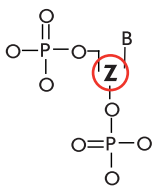
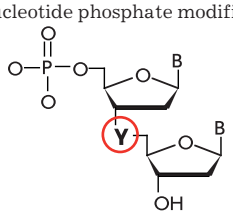
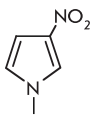
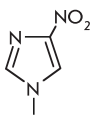
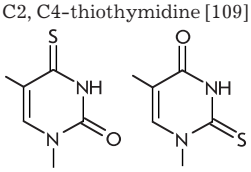
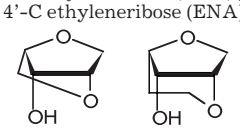
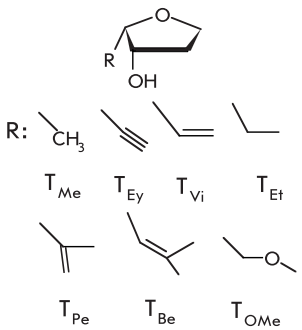
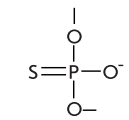
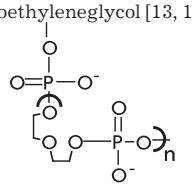
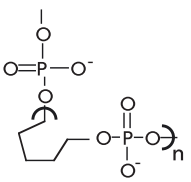
er that bore only the smallest available methyl side chain. *Taq* polymerase could not elongate modified oligonucleotides. The authors suggest that the inability to elongate a primer with a large 3'-terminal nucleotide was due to steric restrictions and could be explained for each individual polymerase by analyzing the size and shape of its active site [107]. C4'-alkyldeoxyribothymidine increases mismatch discrimination both when it is one of the mismatched nucleotides, but also if it is the second or third pair from the primer terminus [105]. Probes with a modified monomer exhibit increased mismatch discrimination, which rises in direct proportion to the size of the side-chain at the C4'-position of deoxyribose. The selectivity of the *Vent* polymerase catalyzed primer elongation increases in the following sequence: $\mathbf{T} < \mathbf{T}_{Me} < \mathbf{T}_{Ey} < \mathbf{T}_{Vi}$ (See chemical structures in *Table. 3*).

The ΔC_t value which equals the difference in the number of the threshold cycle during PCR comparison is 8.5 cycles for the formation of a perfect and 3'-terminally mismatched DNA-complex when using a modified (C4'-ethyl) primer. This value is close to zero when using a nonmodified primer [107]. This increased selectivity of the PCR reaction is evident even when the mismatches are removed up to 4 bp from the conversion site [106]. The authors note that introduction of such non-polar side-chains into oligonucleotide primers does not destabilize the DNA-complexes, and the PCR characteristics of these duplexes are not affected by buffering conditions or the nucleotide composition of the formed duplexes. Thus, the observed increased selectivity of the DNA-polymerase is caused by a disruption of the network of enzyme/substrate interactions due to the exposure of C4'-side-chains into the minor groove.

The authors took their research further and decided to use combinations of modifications. The 3'-terminus of the oligonucleotide primer was modified with a thymidine analog bearing a 4'-C-methoxymethylene residue in its deoxyribose, as well as a thiol group in the C2 or C4 position of the thymidine base [109]. Reactions with the *Vent* DNA-polymerase and primers with a single modification exhibited increased discrimination only with the use of a carbohydrate-substituted oligonucleotide ($\mathbf{T}_{OMe}$, $\Delta C_t = 9$). The presence of a thiol group in the C2 position of the base ($^{2S}\mathbf{T}$) caused only a slight increase in PCR selectivity ($^{2S}\mathbf{T}$, $\Delta C_t = 3$), and it did not change it at all at the C4 position ($^{4S}\mathbf{T}$, $\Delta C_t \approx 0$). However, the simultaneous presence of the methoxymethylene residue and modification of the heterocyclic base caused a dramatic increase in the discriminative ability of the DNA-polymerase. The ΔC_t values for dually modified primers $^{2S}\mathbf{T}_{OMe}$ and $^{4S}\mathbf{T}_{OMe}$ were 12 and 19 cycles, respectively.

Thus, it was demonstrated that combined use of various types of modifications can yield a high level of DNA-substrate mismatch discrimination by enzymes. Most probably, the DNA-complexes exhibit such characteristics because of the steric difficulties that occur in the case of a side-chain in the ribose residue, and in the case of an added thiol group, both

Table 3. Oligonucleotide modifications that promote the selective activity of DNA-dependent enzymes

Heterocyclic base modifications	Carbohydrate backbone modifications	Internucleotide phosphate modifications
		
3-nitropyroll [30, 96]  4-nitroimidazole [97]  C2, C4-thiothymidine [109] 	2'-O, 4'-C methyleneribose (LNA) [98–102], 2'-O, 4'-C ethyleneribose (ENA) [103]  C4'-alkylribose [105–109] 	thiophosphate [110–112]  oligoethyleneglycol [13, 113]  n = 1, 2, 3 oligomethylenediol [13, 113]  n = 1, 2

of which have their most dramatic effect when placed in the opposite strand from the nucleotide analog of the noncomplementary base. On the other hand, the 3'-terminal nucleotide primer is highly dependent on the formation of hydrophobic bonds during the formation of an enzyme-substrate complex (discussed in the previous section). Nonpolar side-chains could probably facilitate such contacts.

MODIFICATIONS OF THE INTERNUCLEOTIDE PHOSPHODIESTER RESIDUE

Increased selectivity of DNA-polymerases was demonstrated during the use of oligonucleotide primers modified at the internucleotide phosphodiester residue [110–112]. Substitution of the native phosphate groups, located between the first and the second nucleosides at the 3'-terminus of the primer, with thiophosphates increased the 3'-terminal mismatch discrimination efficiency of *Vent* and *Pfu* DNA-polymerases [110–112]. Such modifications did not alter the stability of lengthy DNA-complexes, but their presence increased the discrimination of nucleotide mismatches, single or multiplex, and even those located at a distance from the enzymatic conversion site. The DNA-polymerases showed no detectable elongation of the modified oligonucleotide, even when the mismatches were located up to 8 nucleotides from the 3'-terminus of the primer [111]. Notably, according to the data presented above, DNA-polymerases form tight interactions with the carbohydrate-phosphate backbone of the DNA helix up to the above-mentioned position in the primer strand. However, the authors also noted that for the conditions of the enzymatic reaction to be as stringent as possible, reactions using phosphothioate analog oligonucleotides had to be performed with DNA-polymerases that possesed exonuclease proof-reading activity and the conditions for allele-specific PCR had to be adjusted [112].

The use of oligonucleotide probes bearing nonnucleotide insertions into the carbohydrate-phosphate backbone also exhibited increased single mismatch discrimination efficiency of DNA-substrates during enzymatic ligation using T4 phage DNA-ligase, and during elongation by *Taq* DNA-polymerase as compared to the use of native DNA primers [13, 113]. The presence of insertions based on phosphodiesters of oligoethyleneglycol and oligomethylene diols inside the enzyme-binding site on the DNA-substrate or at its border caused a significant increase in the selectivity of the modified probe conversion. Enzymes could discriminate mismatches that were located at a distance from the enzymatic conversion site if the complexes bore this nonnucleotide loop. The mismatch discrimination factors for reactions with mismatches located six nucleotides away from the conversion site and an oligonucletide probe with an insertion at the 6th position amounted to up to 8 for ligation and 12 for elongation. The use of a native probe in such a complex did not provide efficient discrimination of a single mismatch in the duplex, and the mismatch discrimination factor was 2.9 and 1.2 for ligation and elongation, respectively [13].

The authors note that the presence of a nonnucleotide loop in the substrate complex is a sort of disruption of the substrate structure, which lowers the efficiency of enzymatic catalysis in any case, but the presence of an additional disruption, as in case of a mismatch, can considerably increase the probability of its discrimination and thus explain the observed increase in the selectivity of allele-specific enzymatic reactions.

Thus, introduction of "disruptive" elements into the structure of the DNA-substrate of enzymatic reactions can sometimes help achieve the desired accuracy in mismatch discrimination. Artificial modifications of the DNA-complex structure can increase the selectivity of DNA-ligases and DNA-polymerases, although a lowered efficiency of enzymatic conversion is also observed. In the future, modifications that affect the fidelity of the enzymatic reaction at the level of stabilization of the correct conformation of the reactive complex and facilitate the formation of vital enzyme-substrate interactions may prove to be the most effective way to increase selectivity.

CONCLUSION

The data reviewed in this paper prove that the problem of achieving high selectivity in the enzymatic conversion of oligonucleotide probes during nucleotide polymorphism analysis in DNA is an issue depending on multiple factors. It is safe to assume that a universal analysis scheme which allows an unequivocal discrimination of any nucleotide variation in DNA and which uses the discussed analytic approaches has yet to be devised. The choice of a DNA analysis scheme requires a complex design of the components of the analytical procedure, which factors in the "two sides of the same medal." Firstly, it is the specifics of substrate complex recognition by the DNA-processing enzyme, and secondly, the structural characteristics of the DNA-substrate which is formed by a molecular probe, based on an oligonucleotide or its derivative. This review summarizes the most relevant facts that characterize the peculiarities of nucleotide polymorphism analysis of DNA using DNA-ligases and DNA-polymerases. The data presented reveal the fundamental principles of selective oligonucleotide probe conversion during enzymatic DNA-analysis and also point out the most promising recent developments in this field of research. Our analysis of the available data shows that, despite the large amount of studies reviewed in this paper, the problem of achieving selectivity in probe conversion remains unresolved and undoubtedly requires further research. ●

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Regulation of immunogen processing: signal sequences and their application for the new generation of DNA-vaccines

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ABSTRACT **Immunization with naked genes (DNA-immunization) is a perspective modern approach to prophylactic as well as therapeutic vaccination against pathogens, as well as cancer and allergy. A panel of DNA immunogens has been developed, some are already in the clinical trials. However, the immunogenicity of DNA vaccines, specifically of those applied to humans, needs a considerable improvement. There are several approaches to increase DNA vaccine immunogenicity. One approach implies the modifications of the encoded immunogen that change its processing and presentation, and thus the overall pattern of anti-immunogen response. For this, eukaryotic expression vectors are constructed that encode the chimeric proteins composed of the immunogen and specialized targeting or signal sequences. The review describes a number of signals that if fused to immunogen, target it into the predefined subcellular compartments. The review gives examples of their application for DNA-immunization.**

KEYWORDS **DNA-vaccines, MHC-I, MHC-II, antigen presentation.**

ABBREVIATIONS **ER - endoplasmic reticulum, Ub - ubiquitin, HCV - human hepatitis C virus, ODC - Ornithine decarboxylase, RT - HIV-1 reverse transcriptase, CRT - Ca^{2+}-binding protein calreticulin, HVP-16 - human papilloma virus 16, LAMP-1 - lysosome-associated protein 1, sarsN - nucleocapsid SARS coronavirus protein, LCMV - lymphocytic choriomeningitis virus, MHC I - major histocompatibility complex class I, MHC II - major histocompatibility complex class II**

INTRODUCTION

One of the most promising vaccine types of today are DNA-vaccines. In its simplest form, a DNA-vaccine is a plasmid containing a gene of the pathogenic protein and the elements needed to transcribe this gene in mammalian cells. This DNA is introduced into mammalian cells during immunization. It is then transcribed, and the encoded antigen is synthesized initiating an immune response (Fig. 1). Unlike protein-based vaccines, DNA-vaccines based on microbial genes and tumor antigens have the advantage of synthesizing the specific antigen in the host's organism, where it is processed correctly to induce an immune response of the desired specificity. This approach is promising because of the simplicity and low cost of the production and transportation of DNA-vaccines as compared to the traditional vaccines. Moreover, gene engineering allows an easy modification of DNA-immunogens; new antigens can be designed with properties predicted by *in silico* studies. The use of DNA-vaccines causes some anxiety because of the possibility that the genetic material gets inserted into the host genome (insertional mutagenesis). However, the probability of this event is extremely low. It is in the range of around 1–7 insertions per 150, 000 nuclei, which is lower than the rate of the natural insertion mutation by a factor of 1,000 [1].

DNA-vaccines are used to induce a protective immune response against various infections in small animals (rodents) and in larger species [2–4]. Series of trials of prophylactic and therapeutic DNA-vaccines against various human pathogens including HIV-1 and HCV have been performed [5–7]. However, the immunogenicity of genetic vaccines needs improvement, especially for human applications [8–10]. Various approaches are being used in order to increase the efficiency of DNA-vaccines [11–13]. They include the development of novel methods of DNA-vaccine administration (electroporation has become increasingly popular in the recent years); supplementing vaccine formulation with cytokines and/or chemokines or their genes [14] ; optimizing plasmid vectors by selecting more effective gene promoters and regulatory elements [15] ; and modulating plasmid CpG content [14]. The DNA-immunogen is also modified: the coding sequence of immunogen is often changed to increase the expression [16]. One of the most

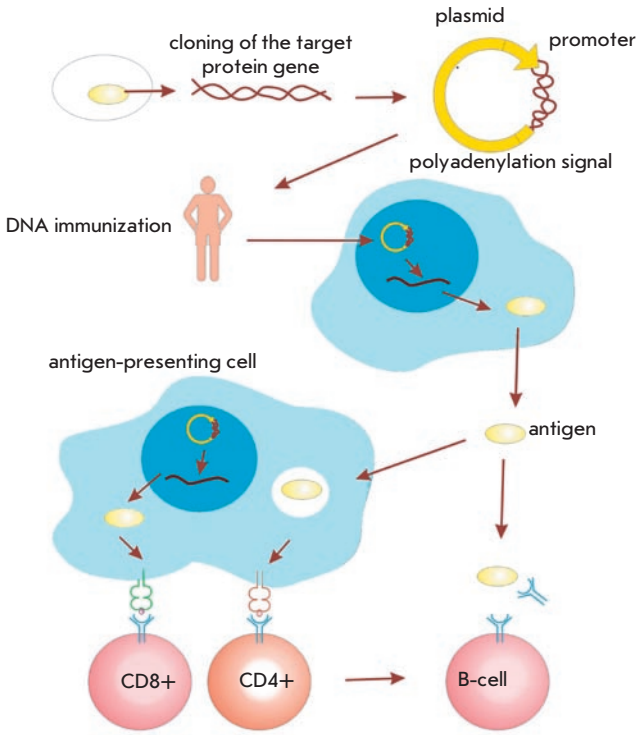

Fig. 1. DNA-immunization

promising approaches for modifying the immunogen is to alter its processing and presentation pathway [17]. Such re-direction can be achieved by "labeling" the protein with specialized signal sequences.

This review focuses on the signals directing protein into various cellular compartments and their use for DNA-vaccine design. In order to be recognized by the immune system, an antigen must be processed and presented on the surface of a cell by the molecules of the major histocompatibility complex (MHC). There are two main classes of these molecules: MHC class I (MHC-I) and MHC class II (MHC-II). In order to bind to the molecules of either class, the protein encoded by DNA-immunogen must go through antigen processing in the specialized cell compartments (Table 1). Endogenous proteins are degraded in the proteasome and are presented in a complex with MHC-I on the cell surface, where they can be recognized by the receptors of cytotoxic CD8+ T-cells (CTL), which then initiate a cytotoxic immune response [18].

Exogenous proteins are hydrolyzed by proteases in the lysosome, antigen fragments generated by processing are bound to MHC-II molecules and recognized by the CD4-receptors of T-helper cells, which facilitate cellular as well as humoral responses [19]. Thus, it is evident that processing pathway of the immunogen determines the type of immune response it induces.

PRESENTATION OF A DNA-ENCODED ANTIGEN VIA THE MHC-I PATHWAY

As has been mentioned earlier, processing of an antigen via the MHC-I pathway results in a CTL-response. This process involves several steps (Fig. 2). The protein is synthesized in the cell and then broken into small peptides in the proteasome, after which these fragments are taken up by the transporter-proteins associated with antigen processing (TAP). These proteins guide the peptides into the endoplasmic reticulum (ER), where they can bind to MHC-I molecules [18].

The peptide-MHC-I complex is transported to the cell surface to be recognized by CD8+ T-cells (CTL), manifesting the initiation of cellular response. This is why increasing the amount of the protein that is transported into the proteasome or ER should in principle increase its presentation via MHC-I pathway, hence, the availability on the cell surface resulting in an enhancement of the cellular response.

PROTEASOME-MEDIATED MECHANISM

To be degraded by the proteasome, proteins must bear a specific signal – a chain of ubiquitin molecules (Ub), small polypeptides consisting of 76 amino acid residues. The eukaryotic cell has a specialized group of enzymes that recognize protein substrates and covalently attach polyubiquitin to these substrates. This enzymatic group is called the ubiquitin-conjugating system [20].

This system recognizes various proteasome degradation signals. Signals can represent a specific amino acid sequence, a specific pattern of protein phosphorylation, or alterations in the protein structure, often missfolding. Several degradation signals with a characteristic amino acid sequence have been described. The first signal discovered was the N-degron. This is a specific first amino acid residue in the protein that serves as a substrate for the complex of cellular enzymes responsible for labeling the protein with a polyubiquitine chain [21]. Amino acids differ in their capacity to be recognized by the ubiquitinating enzymes (Table 2). There are also other degradation signals, such as the PEST-sequence [22] and the Destruction Box [23], often found in the short-lived cellular proteins (Table 2).The nature of the first amino acid residue in the protein is, thus, one of the main protein features determining its accumulation.

Table 1. Antigen processing pathways and types of immune responses

Antigen localization	Main processing compartment	Antigen presenting complex	Recognition cells of the immune system	Stimulated immune response
Inside the cell	Proteasome	MHC-I	CD8+	Cytotoxic
Outside the cell	Lysosome	MHC-II	CD4+	Cellular, humoral

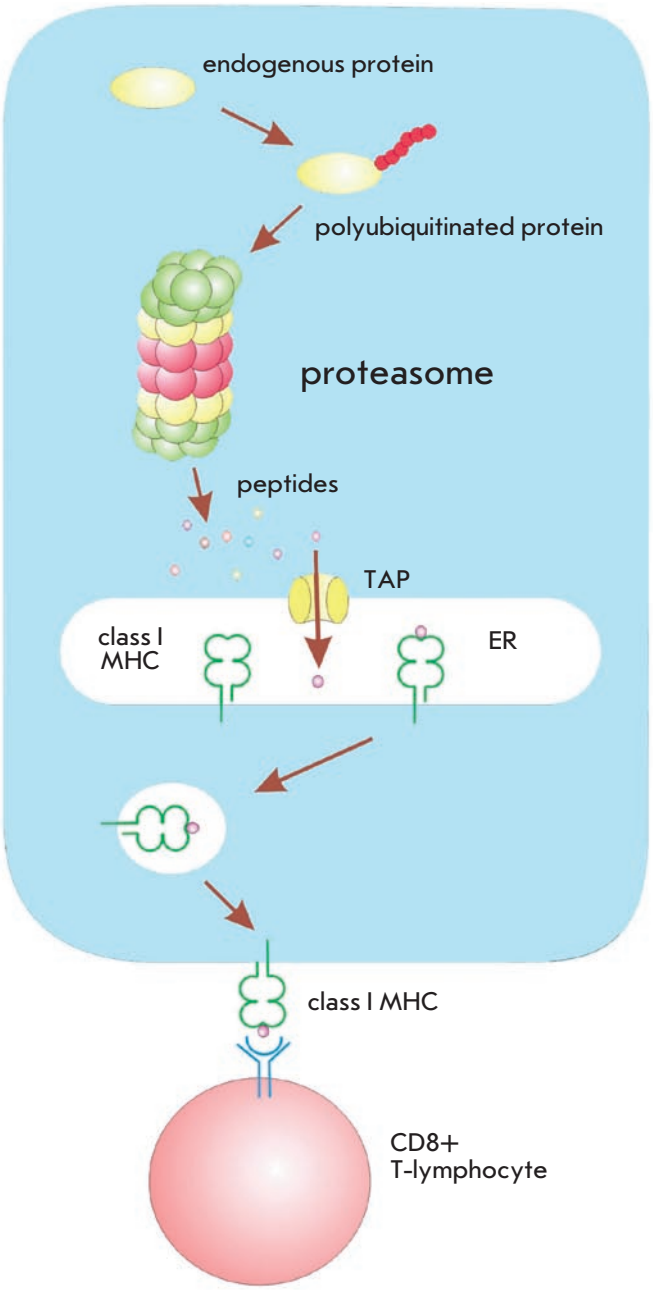

Fig. 2. Antigen processing during presentation via the MHC-I pathway

UBIQUITIN-DEPENDENT MECHANISM

Main focus of the researchers has been on the antigen (re)targeting into proteasome via the ubiquitin-dependent mechanism, thought to provide for antigen processing and presentation in complex with MHC-I and an enhanced antigen-specific CTL-response [25–28]. This (re)direction can be achieved by cloning an Ub-encoding sequence onto the 5′-terminus of the target gene and adding a destabilizing N-terminal residue after the Ub, which makes an antigen a better proteasome substrate. In the cell, Ub is cleaved off in a posttranslational modification of the chimeric protein catalyzed by the C-terminal ubiquitin hydrolase, thus exposing the N-degron. The HIV-1 nef was modified in this way to generate Ub-Arg-Nef, which led to the improved immunogenicity of nef in mice [29]. Fusion with ubiquitin was used for immunization with HIV-1 gene expression libraries. All of the ORFs (open reading frames) of HIV-1 encoded by 32 plasmids were modified by the addition of an ubiquitin encoding sequence. After a single immunization using gene gun, this library stimulated a strong T-cell response against all 32 antigens. This response was registered as an enhanced CTL-activity, IFN-γ+ production by CD8+ T-cells and HLA-tetramer binding [30]. Addition of ubiquitin to the N-terminus of a synthetic protein consisting of HIV CTL-epitopes also resulted in the increase of immunogenicity of this prototype DNA-vaccine [31].

An incorrect protein folding can also act as the proteasome degradation signal. This was used to increase the immunogenicity of the influenza virus proteins M1 and NS1. Unstable variants of M1 and NS1 were constructed by disrupting their alpha-helical regions via introduction of short (foreign) amino acid sequences. Immunization by the genes of the destructured M1 and NS1 resulted in a much stronger CTL-responses than that induced by the original genes [32].

However, for some viral proteins, fusion with the proteasome-targeting signals did not result in an increased degradation [27, 33]. The HIV-1 Gag protein modified by ubiquitination and by a destabilizing N-terminal arginine residue (Ub-R-Gag) showed only a slight increase in the degradation rate. Effective destabilization of this protein required an insertion of the additional exposed lysine residue eK (Ub-R-eK-Gag). The Ub-R-eK-Gag chimera was effectively directed into the proteasome, which increased the presentation of MHC-I-antigen peptide complexes on the cell surface. However, this did not significantly increase the anti-Gag CTL-response in mouse immunization [34]. Also, no enhancement in immunogenic performance was observed after a similar modification of DNA-immunogen expressing nucleocapsid (core) protein of Hepatitis C virus (HCV). HCV core genes carrying cleavable as well as uncleavable ubiquitin residues with N-stabilizing or N-destabilizing amino acid residues were equally poor immunogens [35]. Other (viral) models were described for which an increase in the proteasomal degradation did not result in an increased protective immunity [34].

UBIQUITIN-INDEPENDENT MECHANISM

Notably, some proteins do not require ubiquitin for degradation [36]. The first such protein to be discovered was ornithine decarboxylase (ODC) [37]. Its degradation is ATP- and antizyme-protein-dependent. The C-terminus of the antizyme binds to the N-terminal region of ODC, directing it to the proteasome while the antizyme is released. In addition to the antizyme-binding site on the N-terminus, ODC contains also a C-terminal PEST-signal [38]. Experiments with deletion mutants of ODC have shown that the minimal signal required for the rapid degradation of ODC in the proteasome is a 37-residue C-terminal stretch of amino acids [39]. It was demonstrated that this region is required for binding of ODC to the proteasome.

The ubiquitin-conjugating system is a multi-stage mechanism with a complex regulation. The use of protein degrada-

tion signals, which direct proteins to the proteasome via an ubiquitin-independent mechanism, circumvents the effects of a multitude of factors and thus provides a more straight-forward way of proteasome targeting. Fusion of HIV-1 reverse transcriptase with the minimal proteasome-targeting signals of ODC represented by two short amino acid sequences at the ODC C-terminus, led to an accelerated degradation and an increased immunogenicity of the chimeric gene in mice as compared to the original gene [40]. This modification was also successful when applied to the weakly immunogenic reverse transcriptase of drug-resistant HIV-1 [41] helping to enhance both cellular and antibody immune response against the mutant enzyme form [42].

ER-MEDIATED MECHANISM

Processing of an antigen via the MHC-I pathway involves the endoplasmic reticulum (ER). That is why increasing the antigen's affinity towards ER can improve the immunogenic performance of the antigen. The Ca2+-binding protein calreticulin (CRT) is abundant in ER, where it is associated with the components involved in the presentation of the antigen via the MHC-I pathway [43- 45]. Fusion of calreticulin to the tumor antigens was used to improve the T-cell immune response against tumor cells. A DNA-vaccine that encoded a fusion of CRT with E7 protein of human papilloma virus 16 (HPV-16) was created. Mice immunized by this DNA-immunogen exhibited a significant increase in the population of the E7-specific CD8+ T-cells and in their lytic activity against E7-expressing tumors [46, 47]. A fusion-protein of CRT with another HPV-16 protein E6 also improved the antigen-specific CD8+ T-cell immune response in mice [48].

Altered localization of E7 HPV-16 protein associated with an increased affinity for ER was also achieved by a different strategy that involved antigen fusion with the extracellular domain of the Fms-like tyrosine kinase 3 ligand (FL) [49]. The E7 gene fusion introduced subcutaneously by the gene gun technique led to a considerably increased capacity of E7 to activate specific CD8+ T-cells compared to the unmodified E7 gene. *In vitro* studies showed that 293 cell line transfected with FL-E7 DNA presented E7 antigen in complex with MHC class I molecules more effectively than cells transfected with the original E7 gene. The FL-E7 chimera potently activated CD8+ T-cells; anti-tumor effect was dependent on the CD4+T-cells [49]. Another successful case of enhancing the immune response by manipulating with the ER-signals was reported for the envelope protein E2 of hepatitis C virus. The effect was achieved by duplication of the ER-localization sequence, which promoted the accumulation and subsequent release of E2 from the endoplasmic reticulum [50].

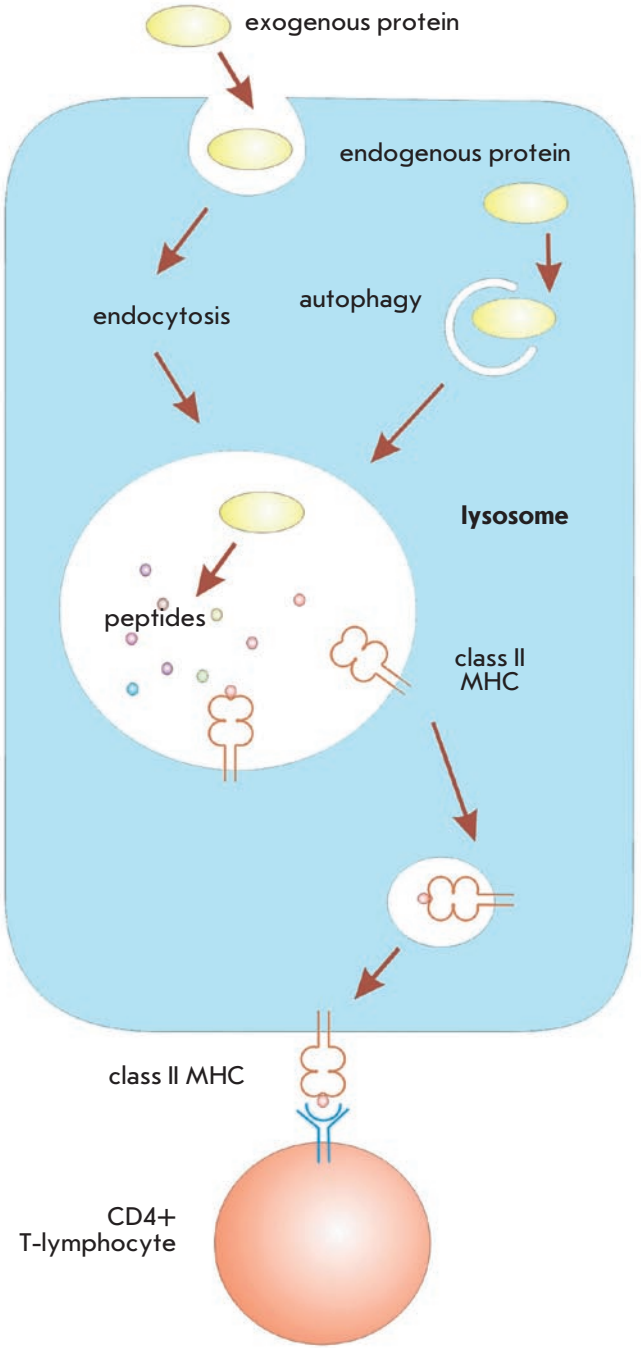

Fig. 3. Antigen processing during presentation via the MHC-II pathway

PRESENTATION OF A DNA-ENCODED ANTIGEN VIA THE MHC-II PATHWAY

Peptides to be bound to MHC-II are mainly of the exogenous origin and are captured by endocytosis to be directed into the lysosomes (Fig. 3). However, it has been demonstrated that some intracellular proteins can be presented by MHC-II as a result of autophagy [51]. Such proteins are transported into the lysosome via the chaperone-mediated transfer carried out by a transport-protein; by the engulfment of cytoplasm by the lysosome membrane; or by the formation of double-membrane autophagosomes [51-55]. After transporting to the lysosomes, the antigen is cleaved by the acidic proteases and the resulting peptides are loaded onto the MHC-II molecules and brought to the cell surface. On the cell surface, these complexes are recognized by the CD4+ T-cell receptors [56]. This leads to the stimulation of cellular (Th1-type) and humoral (Th2-type) immunity.

Traditional therapeutic vaccinations with soluble protein antigens aim to recruit CD4+ T-cells. The relatively weak response of CD4+ T-cells is one of the weak points of DNA-vaccines of today. Clinical trials have also shown that the current generation of DNA-vaccines cannot induce strong antibody response [57]. Therefore, targeting of antigen presentation into the MHC-II pathway in order to activate CD4+ T-cells seems especially advantageous. Such targeting can be achieved artificially by supplementing immunogen with the lysosome localization signals.

The DNA-antigen can be specifically (re)directed into lysosomes using protein sorting signals. Such intracellular sorting signals can be found in the cytoplasm-terminal regions of the transmembrane and lysosome-associated proteins [24, 58, 59]. Most of them are short amino acid sequences and can be divided into the tyrosine- and dileucine-bearing sequences [24, 60]. Tyrosine-bearing signals have a consensus motif NPXY or YXXØ (where X is any amino acid, and Ø is an amino acid with a large hydrophobic side-chain). The consensus sequence of the dileucine signals is (DE)XXXL(LI) or DXXLL. These signals are recognized by the adaptor protein AP or by related complexes and are then directed into the trans-Golgi, to the plasmatic membrane, and further into the endosomes. There are other motifs, such as a cluster of acidic amino acids and the NPFSD sequence [58]. A number of cellular proteins are directed into lysosomes due to the presence of a phosphorylated mannose residue attached to the consensus sequence NX(ST) [61–63]. There are also signals that seem to direct cytoplasmic proteins into the lysosome via an autophagosome mechanism [64].

LYSOSOME-MEDIATED MECHANISM

It has been shown that tyrosine- and dileucine signals can effectively direct heterologous proteins into the lysosome [19, 65, 66]. The most actively used signals targeting to MHC-II presentation are those of the lysosome-associated membrane protein 1(LAMP-1) [67]; invariant chain (Ii) [65], and the AP3-binding motif of the lysosome protein LIMP II [66].

LAMP-1 SIGNAL

Sorting signal of the lysosome-associated protein 1 (LAMP-1) targets the antigen to processing via the MHC-II presentation pathway and enhances its presentation to CD4+ T-cells, as has been shown in *in vitro* experiments. Mouse immunization experiments have demonstrated that LAMP-1gene chimeras induced stronger lymphoproliferative activity, CTL-activity and higher antibody titers as compared to the non-modified DNA-immunogens. An increase in the Th2-type immune response of CD4+ T-cells in response to the LAMP-1 fusions was shown after immunization with DNA encoding LAMP-1 fusions of HIV-1 gp160- and p55gag [68, 69]. A LAMP/gag DNA-vaccine stimulated prolonged B-, CD4+ and CD8+ T-cell responses, while an immune response caused by the injection of a non-modified Gag gene rapidly receded [70]. Another successfully redirected cytoplasmic protein was the nucleocapsid protein of the coronavirus SARS (sarsN). Immunization of mice with DNA encoding the LAMP-1-sarsN chimera led to a balanced specific IFN-γ and IL-4 production and strong CTL-response against sarsN [66]. Also, fusion of HIV-1 reverse transcriptase with LAMP-1 improved the immunogenicity of a prototype DNA-vaccine against drug-resistant virus. A strong immune response of the mixed Th1/Th2-type was raised both against the wild and drug-resistant HIV-1 reverse transcriptases, circumventing tolerance of the immune system towards this conserved retroviral antigen [71].

Fusion with LAMP-1 increased the immunogenicity of DNA immunogens encoding flavivirus envelope proteins. In a candidate vaccine against Dengue virus type 2 based on the DNA encoding the premembrane (preM) and envelope (E), the transmembrane and cytoplasmic domains of E were replaced by similar domains of LAMP-1 [72]. LAMP-1/preM-E chimera exhibited a characteristic granular cytoplasmic staining that indicated co-localization with the endogenous LAMP-1, MHC-II, and H2-M proteins that was not observed in the case of the non-modified antigen. Mice immunized with the gene of the LAMP-1/preM-E chimera exhibited a much higher level of neutralizing antibodies than the controls that received the parental preM-E gene. A similar prototype DNA-vaccine was designed against the West Nile virus. In this case, the premembrane and envelope (WN preM-E) coding sequences were fused to the sequences encoding the transmembrane and cytoplasmic domains of LAMP-1 [73]. Mice immunized by the gene of the WN LAMP-1/preM-E chimera responded by a long-lasting production of high titers of neutralizing antibodies, while DNA encoding the original antigen induced a short-termed low-titer antibody response. Altogether, these results provide a basis for creating a panel of effective DNA-vaccines against flaviviruses.

Introduction of the HPV-16 E7 protein gene, fused to the sequence encoding LAMP-1, also increased the Th2-type immune response [74]. Introduction of a secretory variant of E7/LAMP-1 in the form of a DNA-chimeric recombinant virus induced a strong anti-tumor immune response, which pre-

Table 2. Signals for proteasome degradation with specific amino acid sequences

Name of signal	Amino acid sequence
N-degron	N-terminal amino acid (recognizing enzyme): Destabilizing – F, L, W, Y, I, R, K, H, A, S, T, G (E3 -ligase); N,Q (N- N,Q (N-terminal hydrolase); D,E,C (Arg-t-RNA-transferase) Stabilizing – M, S, G, V (no recognizing enzymes)
PEST- sequence	Sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
Destruction box	R-A/T-A-L-G-X-I/V-G/T-N

vented the formation of tumors and reduced the size of the ones already present [75].

There are, however, few unsuccessful cases of applying this modification to the cytoplasmic proteins, as in the case of the nucleocapsid protein of HCV and of p53 [35, 76]. A plasmid was constructed expressing a chimeric fusion protein of HCV nuclecapsid protein with signal- and C-terminal LAMP-1 sequences. Immunization of mice with this construct did not led to any detectable antibody response or cell proliferation and induced only weak CTL-activity [35]. Thus, direction into the lysosome by fusing the immunogen to LAMP-1 does not necessarily ensure an enhancement of the Th-2 type immune response.

INVARIABLE CHAIN SIGNAL

MHC-II molecules require transportation into the lysosome compartment. This transport involves the invariant chain of MHC class II molecules (Ii) [77]. Two sorting signals were found in the cytoplasmic domain of Ii [65, 77, 78]. It was shown that endogenously synthesized proteins, normally not presented via the MHC-II pathway, can be effectively presented by MHC-II if fused to Ii [79]. Numerous experiments in the animal models demonstrated that fusion of the recombinant antigens to Ii can enhance, broaden, and prolong the protective immune response to the resulting chimeric DNA-vaccines. *In vitro* and *in vivo* experiments had shown that immunogens based on an adenovirus expressing fusion of Ii with the glycoprotein of lymphocytic choriomeningitis virus (LCMV) had an increased ability to stimulate LCMV-specific CD4+ and CD8+ T-cells. Moreover, mice that had been immunized by this plasmid only once were resistant to the infection by the lethal LCMV dose [80].

This approach is also effective for immunization of larger species. A DNA-construct was made encoding the major surface protein 1a of *Anaplasma marginale* fused with the lysosome-targeting motif of the bovine Ii, which directed the chimera into the lysosome compartment [81]. A single dose of this plasmid effectively stimulated an immune response seen as a potent proliferation of IFN-γ+/CD4+ T-cells and production of specific IgG. A single injection of this construct induced antigen specific memory cells, which formed the basis for an accelerated response to repeated doses of the antigen [81].

DIRECTION INTO AUTOPHAGOSOMES

The precise mechanisms behind autophagy are yet unknown despite the intensive ongoing studies. With regard to antigen retargeting, it was shown that fusion of the autophagosome-associated protein Atg8/LC3 with influenza virus matrix protein 1 leads to a considerable increase in the MHC class II presentation and in the M1-specific response of CD4+ T-cells [82]. This confirms that autophagy constantly and effectively directs cytoplasmic proteins into presentation via MHC class II pathway, where they can be used to stimulate a T-helper response.

SECRETORY DIRECTION

An effective approach to the induction of a T-helper immune response is targeting of proteins for secretion into the extracellular environment. Fusion of HIV-1 Gag and E proteins to the secretory chemokine MCP3 directed these viral proteins into the secretory pathway. Chimeric genes induced an effective production of anti-HIV-1 antibodies in the macaques. Macaques immunized with the chimeras and infected with a pathogenic SIVmac251 had lower viral loads than the infected naïve animals [83]. This is an example of the conversion of an endogenous antigen into the exogenous, for further capture by endocytosis and transfer to the lysosomes. This pathway, as well as the endogenous lysosome (re)targeting, enhances antigen presentation in complex with the MHC class II molecules and can considerably increase the immunogenicity of DNA-vaccines.

CONCLUSIONS

Today, the mankind faces an acute problem of creating vaccines against such hazardous diseases as hepatitis C, the immunodeficiency caused by HIV-1, and cancer. DNA-vaccine technology opens a wide range of possibilities for creating effective vaccines, one such is through immunogen (re)targeting. In this paper, we have reviewed a number of signal sequences that can be introduced into the immunogens to direct them into a predetermined processing and presentation pathway, usually different from the one they would naturally take. Direction of an antigen into the MHC-I presentation pathway would enhance the cytotoxic T-cell response, while direction onto the MHC-II pathway would activate T-helper cells and stimulate both cellular and humoral responses. On the overall, the use of signal sequences to control and guide immunogen presentation can increase the immunogenic potential of the existing DNA-immunogens and help to create new effective prophylactic and therapeutic vaccines for diverse human applications. ●

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Ecological Basis for Rational Phage Therapy

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ABSTRACT **Understanding the mutual interactions of bacterial and phage populations in the environment of a human or animal body is essential in any attempt to influence these complex processes, particularly for rational phage therapy. Current knowledge on the impact of naturally occurring bacteriophages on the populations of their host bacteria, and their role in the homeostasis maintenance of a macro host, is still sketchy. The existing data suggest that different mechanisms stabilize phage-bacteria coexistence in different animal species or different body sites. The defining set of parameters governing phage infection includes specific physical, chemical, and biological conditions, such as pH, nutrient densities, host prevalence, relation to mucosa and other surfaces, the presence of phage inhibiting substances, etc. Phage therapy is also an ecological process that always implies three components that form a complex pattern of interactions: populations of the pathogen, the bacteriophages used as antibacterial agents, and the macroorganism. We present a review of contemporary data on natural bacteriophages occuring in human- and animal- body associated microbial communities, and analyze ecological and physiological considerations that determine the success of phage therapy in mammals.**

KEYWORDS **bacteriophages, phage therapy, human body microbiota, animal body microbiota, bacteriophage ecology**

ABBREVIATIONS **GIT – gastro-intestinal tract, PFU – plaque-forming unit, which corresponds to a one viable bacteriphage particle, if the efficiency of infection in these conditions and in this strain is close to 1; CFU – colony-forming unit, which corresponds to a one viable bacterial cell.**

INTRODUCTION

The use of bacteriophages as antibacterial agents was first suggested by one of the scientists who discovered bacteriophages. F. D'Errele was the first to use a phage preparation to treat a severe case of child dysentery in 1919, only two years after the publication of his first article, which reported the discovery of bacterial viruses [1, 2]. The therapeutic use of phages in Western medicine spread widely in the late 30s–early 40s, but it was subsequently almost completely forgotten because of highly unstable results, that was due to insufficient development of phage biology, phage production methods, the storage and use of drugs, and on the other hand, to the triumph of antibiotic treatment of bacterial infections, which seemed a simpler and better solution to the clinical problems in that field [2]. Nevertheless, the development and use of therapeutic phage preparations never stopped in the Soviet Union, as well as in Poland and the Czech Republic. The vast experience accumulated in those countries indicates that phage-based drugs are effective and safe. Today, mass-production of such antibacterial drugs continues in Russia and Georgia (where F. D'Errele personally took part in the establishment of the Bacteriophage, Microbiology and Virology Institute (now named after G. Eliava) in the 1930s). In Poland therapeutic phage preparations are produced and used in a specialized center at the Institute of Immunology and Experimental Therapy in Wroclaw.

The continuing increase in occurence of drug-resistant strains of pathogenic microorganisms and the dramatic slump in the development and marketing of novel antibacterial agents have spurred renewed interest towards phage therapy in the last 20 years in both Russian and Western medicine [3, 4]. Several reviews on issues related to phage therapy have recently been published [2, 5, 6, 7].

Modern phage therapy mostly uses bacteriophages, which belong to the Caudovirales order ("tailed phages"). About 96% of all known phages belong to this group [8]. Tailed phages are divided into 3 families:

1. Podoviridae, which have a short noncontractile tail;
2. Siphoviridae, which have a long noncontractile tail; and
3. Myoviridae, which have a contractile tail.

RNA-bearing phages from the Leviviridae family, as well as filamentous phages bearing a single-strand DNA belonging to Inoviridae family, are also of ecological importance. Leviviral virions have small (~26 nm) icosahedral particles, which encapsulate a single-strand RNA genome. The filamentous particles of inoviruses are molecules of circular single-strand DNA, which is covered by low-molecular-weight envelope proteins.

Members of the other prokaryote virus families (currently there are 14 families defined by ICTV) are rarely encountered and probably do not play a major role in the microecology of symbiotic populations in mammals. More details on the classification of bacteriophages and the biology of particular groups of these viruses can be found in [8] and [9].

Despite the fact that virulent phages (those which cannot integrate their genome into the bacterial genome and cannot form lysogenic strains) cause the death of all infected cells and, reproducing exponentially under certain conditions and

kill a large number of sensitive bacteria, these viruses stably coexist with their hosts in practically all known natural ecosystems [10]. This coexistence has continued throughout whole history of the life on the Earth [11], and the interactions between bacteria and phages at the level of the population are fairly complex and multifaceted; thus, the simplistic interpretation of phages as the "natural enemies" of bacteria is inaccurate.

From the microbiologist's point of view, an animal or human organism is a complex microcosm that includes several interconnected ecosystems located in various organs and regions of the body [12] and is inhabited by more or less dense populations of microorganisms, including bacteriophages [13].

The role of bacteriophages in the microflora of the body is considered to be "significant," but in reality, knowledge on the subject is scarce. The presence of phages in normal body microflora was demonstrated by the discoverer of phages, Felix D'Errele (see above). He discovered enterobacterial phages in human and animal faeces [1]. Nevertheless, neither qualitative nor quantitative methods for measuring the interactions of phage and bacterial populations in symbiotic microbial systems have been developed for even a single animal species. The understanding of the peculiarities of ecologico-physiological interactions in the tri-partite system of "bacteria-phages-macroorganism" must form the theoretical groundwork for controlling this system, including the application of exogenous phage preparations which will eliminate unwanted bacterial populations.

This review analyzes the current ideas on the ecology of bacterial viruses in human and animal microbial systems, the mechanisms of direct interaction between endogenous and exogenously applied phage particles and between these phages and the cells and organs of a macroorganism. Also we briefly discusse the key theoretical principles underlying the therapeutic use of phage preparations.

BACTERIOPHAGES IN THE NORMAL MICROFLORA OF A BODY

The quantity and variety of phages in animal microflora. The bulk of articles on this subject study bacteriophages inhabiting the digestive tract of mammals (see further), including the colon (and feces), the rumen (for ruminants) and forestomac (for marsupials) microflora [14], where phages are the most abundant free viral particles [13– 26]. Interestingly, most of the RNA-bearing viruses in human feces are plant viruses particles, which are ingested with food [27]. The presence of free viral particles in the lungs or on the skin of animals has yet to be established. Yet several pathological conditions, such as mucoviscidosis, are characterized by the simultaneous presence of phages and bacteria, including *Pseudomonas aeruginosa* [28]. Surprisingly, free virus particles have not been detected in the mouth [29].

Since the symbiotic populations of the human and animal gastro -intestinal tracts (GIT) include several hundred species (and thus up to a 1,000 strains) of bacteria and archaea, each of which is a potential target for phage infection, the majority of phages that inhabit the organism cannot be studied using culturing methods. However, the use of direct methods of analysis, such as extraction and purification of non cultutable viral populations from different regions of the GIT and their analysis by light and electron microscopy, electrophoretic separation and metagenomics, has allowed researchers to assess the representation and variety of phages in microbial populations. A detailed analysis of these studies is beyond the scope of this article; they are comprehensively reviewed in [13]. Notably, the concentration of free virus particles in the colon region and the rumen of ruminant animals is estimated at about 10^8–10^{11} particles per milliliter. The number of distinct morphological types is estimated to range from tens to hundreds; and the number of distinct genotypes, from several hundred to 1,200 [13]. The overall number of phages and the ratio between the different species can change considerably during the course of time. Issues of whether certain types of phages are associated with certain animal species, and the biological geography of phages associated with animals and humans still remain obscure.

Culturable bacteriophages in the normal microflora of animals and humans.

Bacteriophages usually have a very narrow range of host specificity; the infectivity of each phage is usually limited by a specific range of bacterial strains, which belong to a single or several closely related species of bacteria. However, in certain cases phages can infect bacteria from different species and even different genera. F. D'Errelle (1921) first suggested that the isolation of *Yersinia pestis* phages from rat fecal matter 3 months after the end of a plague epidemic can be attributed to the growth of these viruses in bacteria of different species (in our view, it can be also explained by the persistence of *Yersinia* in rats). A recent study reported the persistence of vibriophages in oysters, in absence of the host during the winter period [30]; the authors suggested that vibriophages can use alternative host(s). Thus, the multi-species or multi-genus specificity of certain phages can be of ecological significance in the symbiotic microflora of animals. Nevertheless, even closely related strains of the same species can differ in their sensitivity to phages. This means that the effect of a phage infection on different populations of various species or strains can vary significantly. Cultural methods are currently the only approach for studying the ecological interactions between phages and their hosts at the strain level.

There is a large number of studies which report the isolation and characterization of certain bacteriophages, which are obtained from humans or animals. Bacteriophages of *Streptococcus bovis, S. durans, Prevotella bryantii, Bifidobacterium ruminale* were extracted from the rumen of various species of ruminant animals [14, 31]. Fecal matter from humans and various animals was used to isolate phages of *E. coli*, *Salmonella*, *Bacteroides*, *Klebsiella* and other bacteria [32–43]. In most cases, the phages were isolated using laboratory strains of the appropriate species, or using wild isolates obtained and characterized in advance during the course of a large body of studies which used phages as indicators of the fecal pollution in water.

The presence and titers of DNA coliphages vary considerably between different animal species and even between individual animals, which is consistent with the fact that a noncultivatable viral community is highly variable [44, 45]. However, there are no reports on the specific association of any types or groups of DNA coliphages with a particular spe-

cies of animal. In contrast to this, the presence of F-specific RNA (F-RNA) coliphages (*Leviviridae* family) in animal fecal matter exhibits a certain species-specificity. These bacteriophages can be divided into 4 genotypic groups, which can also be distinguished serologically. The occurrence of specific serotypes can vary considerably between different species of animals: horse fecal matter, for instance, rarely has any F-RNA phages, while more than 70 % of chicken droppings samples show high titers of these viruses (10^5–10^7 PFU×g^{-1}). Only 10–20 % of human fecal matter samples contain F-RNA phages, but groups II and III are most often present in these samples (> 80 % from the overall number of isolates), as opposed to other animals, in which groups I and IV are found with about the same frequency [33, 39, 42, 46]. Currently, there are no satisfactory explanations of this type of association.

The detection of a possible association between several genetic subgroups in the known genera of tailed bacteriophages and their macro-host species may be complicated by the fact that simple and productive methods for the selective extraction of genetically related phages from wild samples have not yet been developed. Nearly selective isolation of phages related to T-even phages from the fecal matter of a patient suffering from pediatric diarrhea in Bangladesh was observed when using *E. coli* K803 strain as a test culture of [47]. Inoculation of a lawn of the enteropathogenic *E. coli* O127:K63 strain led to the isolation of a completely different set of phages, all of which were members of the *Siphoviridae* family.

STUDY OF ENTEROBACTERIAL INDIGENOUS PHAGES

Furuse *et al.* [36] discovered that the amount of titers of coliphages in the fecal matter of healthy people is usually low and that the pool of free viral particles is usually formed by temperate phages. These data indicate that phage particles in the intestines of healthy people are produced mainly via spontaneous induction of lysogenic bacteria. Thus, the reproduction of phages in lytic cycle is of limited importance to the ecology of coliform bacteria in the human intestine. As opposed to healthy people, some patients suffering from internal diseases or leukemia exhibited an elevated number of titers of coliphages, and a major portion of isolated bacteriophages are virulent phages [46]. In several patients, the growth of coliphage titers correlated with the severity of the patient's condition.

The data presented in [36] are in agreement with the fact that attempts to isolate coliphages from dog fecal matter by using indigenous strains of *E. coli* had no success [48]. Using more than 500 indigenous strains of *E.coli* as hosts, 6 samples of fecal matter from domestic dogs were screened for coliphages, but none were found. Only one of the samples yielded phages, which were active on the laboratory *E. coli* strain C. On the other hand, 16 dogs kept in an animal kennel were found to have various titers of coliphages from 0 to 10^7 PFU×g^{-1}. The authors suggest that the absence of phages in domestic dogs was due to their isolation from their kin and the "too clean" living conditions. Recontamination by fecal microorganisms is understandably limited for humans as well, which can partly account for the above-cited results [36].

The results of our recent study, which used horses as a model [15, 49], are in contradiction with the data in [46]. The celluloseolytic microbial community in the horse colon is highly complex and can include more than 500 species of bacteria, archaea, and also fungi and protozoa. In contrast to the rumen community, the microbial biomass of the colon is not subjected to further enzymatic hydrolysis and is excreted with the fecal matter in practically unchanged conditions. The physico-chemical conditions in the equine colon seem to be more stable than that of most other animals, since the average time of cellulose rich food (grass) digestion is 72 hours, which is considerably more than the time windows between eating or defecation events [50].

In order to study the ecological interactions between coliphages and their hosts under these conditions, we monitored the dynamics of coliphage and coliform bacteria titers in 4 animals taking samples every 48 hours. Phage titer was measured using the laboratory *E. coli* test-culture C600. Study [36] analyzed 9 series of samples obtained from 19 healthy people at two week intervals and did not detect any significant temporal changes of coliphage titers or variety. In horses we observed significant changes in the phage titer (2–4 logarithmic units for different animals during the course of the 16-day observation period). However the titers of coliform bacteria were much more stable and fluctuated only slightly around 5×10^5 CFU×g^{-1}. The difference between coliphage dynamics in humans and in horses may be an indication of a more significant role played by phage infection in the ecology of the latter's enterebacterial ecology.

We did not detect any correlation between the titers of coliform bacteria and coliphages in our animal group. This result may be due to the extremely high variety of *E. coli* strains and other bacteria which make up the coliform pool in horse intestines [51]. In order to differentiate between closely related isolates, which can however differ in their phage sensitivities, we employed a simple scheme of PCR-fingerprinting [49]. Using this approach, we demonstrated that the pool of coliform bacteria present in the samples of horse fecal matter includes up to 1,500 individual strains (estimated using the nonparametric Chao1 criterion). We also estimated the share of coliform strains which were sensitive to a certain phage isolate from the same sample. On average, about 1-8% of the strains turned out to be sensitive. The variety of bacteriophages which display activity towards a specific indigenous strain of *E. coli* obtained from the same sample is usually very low and is limited to 1-2 genotypes distinguishable by restriction analysis. The variety of phages which could be detected by plating on the laboratory *E. coli* strain C600 was also limited; only 1–3 types in each of the samples studied. It seems that this results reflect the severe competition of the viruses for the available host cells.

EFFECTS OF VIRAL INFECTION ON THE BACTERIAL POPULATIONS OF THE GIT

Microbial biomass can account for up to 54% of the hydrated mass of human feces. [52]. Coliform bacteria (> 80 % of which are usually represented by *E. coli* strains) are usually present in human and other animal feces in titers of about 10^5–10^8 CFU×g^{-1} [39]. *Streptococcus bovis* is present in the ruminal fluid of sheep in concentrations of 10^6–10^7 CFU×ml^{-1} [14, 53]. These population densities are higher than needed for the exponential reproduction of phages (10^4 cells×ml^{-1} for most

phage-host systems) [54]. Thus, some mechanisms that stabilize the co-existence of phages and bacteria must exist.

We assume that coliphage infection is a selective factor which retains a high level of intraspecies variety of coliform bacteria in horse intestine limiting the chance for the few best competitors strains to outgrow the others. On the other hand, the high variety severely limits the availability of host cells for each specific type of coliphage, thus stabilizing the whole system. The high degree of variety and the reticular organization of the system make direct differential monitoring of individual strains and their associated phage populations very difficult, thus preventing direct validation of this hypothesis. However, we discovered recently that the intraspecies variety of coliform bacteria experienced a severe reduction after peroral application of enrofloxacin for a prolonged period of time, due to a severe leg wound to a horse. The overall titer of coliforms remained at normal level (the dominant strains were resistant to the antibiotic). Currently, we are developing the idea of using such animals with an artificial decrease in the intraspecies variety of symbiotic coliforms as a model for studying bacteriophage ecology, since it will allow us to perform direct experimental tests to prove our hypothesis. Another approach to this problem is analyzing the formation of a normal microflora in newborn horse foal.

The observed structure of the intestinal coliform bacteria and coliphage community is in agreement with the results of mathematical and experimental modeling of communities consisting of bacteria and their phages living under conditions allowing the co-evolution of both components [55–58]. The population of hosts exhibited an observable split into a multitude of genetic lines with varying phage sensitivities. In turn, the phages were selected for widening of their host variety, albeit at the price of absorption efficiency. Thus the initial two-part system became much more complex [57]. Interaction with bacteriophages can also facilitate the selection of bacterial strains bearing mutations of their mismatched base reparation genes, or other mutations that destabilize the genome [59, 60]. Such hypermutable strains have an advantage only in rapidly changing conditions, where the possibility of obtaining new traits counterbalances the accumulation of deleterious mutations [61]. The presence of bacteriophages which are able to evolve *in situ* seems to be one of the types of such a variable selective pressure. The presence of a large number of hypervariable strains in natural enterobacterial populations has been demonstrated [62]. Thus, phage infection can facilitate the increase in phenotypic variety not only by direct selection of resistant strains, but also by supporting the hypermutable strains in the population.

Bacteriophage infection can play a certain role during the events that triggered in the horse GIT when it is overloaded with carbohydrates. A hypothesis has been advanced that certain toxins produced by the altered microflora found their way into the bloodstream and somehow, either directly or indirectly, caused lowered adhesion of the hoof epithelium to the basal membrane. It was recently shown [63] that a rapid increase in the number of streptococcus of the *S. bovis / equinus* group in the colon takes place 8–16 hours after the administering of oligofructane. However, the number of these bacteria dropped very rapidly afterwards. One of the possible explanations is a massive infection of the streptococci by the appropriate phages, similarly to the phage control mechanism during cyanobacterial water blooming [10].

The significant selective influence of natural phages was demonstrated on a population of *Campylobacter jejuni* in chickens. The amount of *C. jejuni* phages in the chicken's appendix was negatively correlated to the degree of colonization by bacteria (an average of 10^5 CFU×g^{-1} in phage-containing samples, as opposed to 10^7 CFU×g^{-1} in samples where no phages were found) [64]. It later turned out that the presence of phages selects variants of *C. jejuni* bearing extensive rearrangements of the genome [65], which occur because of horizontal transfer or as a result of intragenomic inversions with breakpoints in two Mu-like prophages integrated into bacterial DNA [66]. In both cases, the variants which were sensitive to the phages had a considerable advantage in the colonization of broiler chickens. In the absence of phages, the population rapidly reverted to the sensitive phenotype. The authors suggested that the genomic rearrangements in *C. jejuni* occurring in the GIT of the birds played the role of an adaptive mechanism that allowed the bacteria to survive the periods of phage activity and the consequent competition for resources.

THE EFFECT OF ARTIFICIALLY INTRODUCED PHAGES ON GIT BACTERIA

Effective elimination of pathogenic bacteria from the GIT using phage preparations has been demonstrated in multiple experiments on the therapeutic use of phages [2, 7]. The therapeutic effect of the phages can be limited to a decrease in the pathogen's population down to a point when the immune system can effectively control its reproduction. In some cases, even such low doses of phage as 10^2 PFU could prevent the development of an infection by artificial introduction of pathogenic *E. coli* [67]. It is noteworthy that these low dosages were much less effective if they were administered after inoculation with the pathogen. This is an indication that a population of pathogenic bacteria is much more susceptible to phage attack before it has colonized certain protected regions of the intestine.

The impact of an artificially introduced phage cocktail was studied on resident and introduced populations of *E. coli* in mouse intestines [68]. The mice used in these experiments stably excreted *E. coli* at titers of about 10^6 CFU×g^{-1} of feces. This value varied slightly during the course of time, but the presence of natural coliphages was undetectable on the indicator *E. coli* K803 strain, which was sensitive to all the phages used in later experiments. The mice were treated with a phage cocktail (titer 10^7 PFU×ml^{-1}) through their drinking water. These phages were summarily active against practically 100% of the resident strains, which were isolated from the animals before the experiment. Nevertheless, the impact of the externally introduced phages on the titers of coliform bacteria was insignificant, and the phages were incapable of sustained propagation by infection of the resident bacteria. The *E. coli* isolates obtained during the experiment exhibited the same sensitivity pattern to the components of the cocktail, which ruled out a shift of the resident population towards the prevalence of resistant variants. However, the phages did effectively lower the amount of a sensitive *E. coli* strain that was introduced into gnotobiotic mice a week

prior to the administering of the phage cocktail. This effect was accompanied by active replication of the phages in the intestine. However, the surviving portion of the population remained sensitive to the phages, that indicates the existence of certain niches in the mouse intestine where the bacteria are either physically or physiologically shielded from the phage infection. Since practically all of the resident population of normal mice is protected, these "shelters" can accommodate almost all the *E.coli* population in the mouse intestine by *E. coli*. Interestingly, mice obtained from the same animal care facility a year after the experiments had practically no *E. coli* in their intestines and were colonized by other species of enterobacteria [Brüssow, personal communication]. This observation is in agreement with [69], which found that less than 20% of the 48 mice studied in the project bore resident *E. coli* in their GIT, while coliphages were present in minimal titers in those cases. The author suggested that one of the main reasons blocking the reproduction of coliphages in the GIT of mice is the absence of a sufficient population of hosts.

The production of phages in the intestine is also affected by the physico-chemical factors in the environment. Bile salts and carbohydrates, for instance, can have an inhibitory effect on the absorption of many coliphages [70]. This effect is negated by the expression of the Ag43 protein, which is a bacterial surface adhesin that promotes cell aggregation, for instance during the formation of biofilms. Ag43 synthesis is controlled via the phase-variation mechanism; thus, a phage infection can sometimes screen for cells that have a reduced ability to form biofilms. Notably, some phages of *Bacteroides* exhibited an opposite reaction to the addition of bile salts to the culture media, growing more effectively [71].

Growth in a biofilm on the surface of a mucous membrane or on food particles can play a major role in containing viral infection of bacteria in the GIT. Some data [72] seem to indicate that *E. coli* populations in the mouse's intestine are in great part made up of cells in a state of starvation, which are physiologically ill-suited for phage reproduction. The actively reproducing share of the population may be located only in microcolonies on the mucous membrane of the intestine [73]. However, both of these studies used mice treated with streptomycin and artificially inoculated by a single *E. coli* BJ4 strain with a known phenotype. Moreover, the fact that the digestion processes of mice can vary significantly from those of larger animals is material. The metabolic rate of mice is much higher, and the food travels through the intestine much more quickly than in horses or humans. Furthermore, the ratio between the mucous membrane's surface and the volume of the intestine is considerably higher, which facilitates a much more rapid uptake of nutrients. Thus, the issue of to what degree can mouse-obtained data be extrapolated onto larger animals remains to be settled.

REGIONS OF THE BODY EXHIBITING LOWERED PHAGE INFLUENCE ON THE MICROFLORA

Current data suggest that phages can, at least in some animals, successfully overcome the physico-chemical barriers that prevent infection and effectively reproduce using resident hosts, thus constraining their populations. Taking into account the openness of the GIT ecosystem in terms of matter exchange with the outside environment, the significant role of bacteriophages comes as no surprise. However, the situation can be significantly different in other ecosystems of the organism, which are also densely populated by microbiota. Hitch *et al.* [29], for instance, could not isolate from the human oral cavity any phages that proved active towards indigenous bacteria, which made them conclude that the bacteria in that system are not significantly affected by phage infection. A satisfactory explanation has yet to be given to account for this fact.

Some species of bacteria are abundant in the microbial system of ruminant animals. *S. bovis* can be present in concentrations of 10^6–10^7 CFU×ml^{-1} [14, 53]; moreover, the whole *S. bovis* population of a sheep's rumen can belong to the same phagotype [53]. The authors observed a rapid shift from the dominant strain to a different one, with a different phagotype, but these processes were not coupled with the appearance of any bacteriophage able to lyse any of the above-mentioned strains in the rumen fluid [53]. Nevertheless, the presence of *S. bovis* phages in the rumen in titers of 10^1–5×10^4 PFU×ml^{-1} variable in different individual animals was later demonstrated [74].

The ecology of ruminal streptophages was thoroughly studied by Tarakanov and coauthors in the 1970s-1980s. Sadly, most of the results published by these authors are contained in hard-to-access journals and government reports, which is why we reference a book [14] that has a detailed review of these studies. The authors detected *S. bovis* phages in the rumens of cows and sheep in titers of 10^1–10^4 PFU×ml^{-1}. The results of carefully controlled field experiments showed that the peroral introduction of an *S. bovis* phage preparation (streptophagin) into cows led to an increase in the phage's concentration by a factor of 10^4-10^5 , accompanied by a decrease in the amount of amilolytic bacteria, a decrease in the amilolytic and increase in the cellulolytic activity of the ruminal contents. These changes led to improvement of yield and quality of meat and milk produced by treated animals. This was confirmed by detailed and well-documented research [14]. After the discontinuation of streptophagin, the phage titers returned to their initial values fairly quickly and the amount of amylolitic bacteria increased. These results were an indication that a preparation of a single type of phage could have a considerable impact on a large portion of the *S. bovis* population in rumens. This is in agreement with research [53], which demonstrates the low intraspecies variety of *S. bovis* in sheep rumens. On the other hand, it is obvious that an unknown factor prevents the effective reproduction of streptophages in the resident bacterial population in the system.

It was shown that tannic acid can inhibit phage reproduction in physiological concentrations by causing the precipitation of phage particles [75]. Tarakanov [14] has demonstrated that the ruminal fluid of cows and acetic acid at physiological concentrations can both inactivate *S. bovis* bacteriophages. Notably, the sensitivity of different phages varied considerably and was dependent on the concentration of the inactivating agent used. Inhibition of phage activity in the rumen by various chemicals can play a major role in the control of the phage lysis of resident cells. This model is in agreement with the conclusion in study [76], which declares that the

sometimes-observed mass lysis of bacterial biomass in the rumen is most probably a result of autolytic processes as opposed to phage infection.

The female and animal vaginas are also an ecological niche extensively colonized by bacteria. The bulk of the human vaginal microflora is made up of lactobacilli. Species and strains of these bacteria vary among individuals [77], but normally each individual woman has 1–2 dominant strains [78]. The colonization density is relatively high and is about 10^6–10^7 CFU per vaginal swab. Thus, it can be assumed that this population may be susceptible to mass lysis by bacteriophages. Yet all attempts to detect free phages in vaginal excretions have been unsuccessful [79], even though lysogenic lactobacilli strains are often found in the vaginal microflora [79, 80].

Even though the vagina is less susceptible to bacterial and viral exchange with the environment, excluding the exchange associated with sexual intercourse, studies of vaginal swabs of clinically healthy women often show an unexpected drop in the population of lactobacilli. In most cases, the normal flora regenerates on its own, but some individuals develop the so-called anaerobic bacterial vaginosis, a disease during which the lactobacilli are replaced by anaerobic bacteria, such as *Gardnerella vaginalis*, *Prevotella, Porphyromonas, Atapobium* and *Mobiluncus species*. Epidemiologically, the disease seems to be a sexually transmitted disease [81, 82], even though the etiological agent has yet to be identified. In [81], A. Blackwell suggested that the reason for this disruption in lactobacilli flora, which in some cases leads to vaginosis, is a phage infection. We hypothesize that this can be the case when the vagina is infected by lactobacilli lysogenic with respect to a phage active in the resident strain.

Lysis of host cells by moderate phages is usually pretty infrequent: 10^{-1}–10^{-2} [83–85]. Thus, the reproduction of these viruses in an ecological niche densely populated by the sensitive host strain usually happens via the lytic scenario, causing the death of the majority of resident cells. The latter may then be replaced by the descendants of the lysogenic strain which initially released the phage, and by newly lysogenized bacteria or by other bacterial species. Such a scenario was modeled mathematically and experimentally [83] on a model population of *E. coli*. Bearing in mind that the frequency of a spontaneous induction of most lysogenic strains of vaginal lactobacilli is less than 10^{-8} PFU×cell^{-1} [79] in lab cultures, any factor that increases the induction frequency can increase the chance of an "ecological catastrophe" in the vaginal microflora. This can explain the strong epidemiological correlation between smoking and the risk of bacterial vaginosis [81], since some substances of tobacco smoke can cause induction of prophages in lactobacilli [86]. However, the results in study [80] contradict Blackwell's hypothesis [81]. The authors showed that lactobacilli prophages often lose their ability to induce and to reproduce in cells, seemingly because they are selected in the presence of hydrogen peroxide, which is produced by many bacterial strains and is an activating agent for SOS-inducible prophages. A detailed study of the temporal dynamics of lactobacilli strains in the vagina and of the lysogeny of these populations may provide the key to understanding the phage nature of vaginosis cases.

PHAGE–CELL INTERACTIONS, ECOLOGICALLY SIGNIFICANT EFFECTS

In many cases, isolates of enterobacteria (and probably other groups of microorganisms) obtained from the wild are partially resistant to bacteriophages, which coexist with them. This resistance can manifest through a lowered inoculation efficiency of a specific phage, slow absorption, or a decreased yield of the phage, which may be due to the effects of the restriction-modification system [59, 87], and also of a whole range of specific resistance systems developed by bacteria to counter some groups of phages [59, 88, 89]. Moreover, prokaryotes often have CRISPR-loci (clusters of regularly interspersed palindromic repeats) [90], which contain short sequences extracted from the bacteriophage genome, plasmids, or other alien DNA by an as-of-now-unknown mechanism. The genes associated with CRISPR encode for proteins that form enzymatic complexes that can attack RNA or DNA molecules that include sequences identical to the so-called CRISPR spacers [91, 92]. Such systems allow bacteria of numerous species to rapidly develop resistance to a specific phage strain, due to a decreased efficiency of infection. This resistance can however be circumvented by point mutations of the attacked sequences in question [28]. Quantative data that characterize the frequency and ecological significance of the systems that provide resistance to phages in microbial communities by affecting the intracellular development of the virus is still missing. This issue deserves special attention and study, since the mechanisms that cause abortive infection turn a portion of the cells that can absorb the phage into ecological traps, which accelerate the rate of decay of free phage particles considerably [94].

In a recent study by our group, only 1-4% of the coliform bacteria strains isolated from horse feces samples exhibited sensitivity after cultivation on phage agar prepared with phages extracted from the same series of samples from the same animal [49, Tarasyan, unpublished data]. However, these phages showed a considerably decreased inoculation efficiency (10^{-2}–10^{-4}) on lawns of some isolates [Tarasyan *et al*, manuscript in preparation]. In some cases, this effect is due to the ineffective absorption of the phage and can be circumvented by mutational or recombinational rearrangements of the phage's adsorption apparatus. Interestingly, even closely related bacteriophages extracted from the same animal can have slightly different host ranges [49]. This seems to indicate the high *in situ* adaptation rate of the absorption apparatus. Thus, the level of isolation existing in the phage-host system can be severely underestimated. As a result, the populations of each distinct genetic line of phage exist in conditions of strictly limited numbers of available host cells. In some cases, the effective concentration of phages and their respective sensitive cells drop to such a low levels that one can expect the extinction of the phage population. For instance, the concentration of bacteria in a horse feces sample in our example mentioned above was 10^3–10^4 CFU×ml^{-1} [49, Tarasyan *et al*., unpublished data], which is below the minimal value needed for effective phage reproduction [54]. The concentration of the corresponding virulent phage was approximately 10^2–10^5 PFU×ml^{-1}, and this strain of phage, which can be identified by its restriction profile, persisted in the intestine of this animal for more than 2 years [our unpublished data].

The mechanisms that stabilize the long-term coexistence of this virus and its hosts in the intestinal ecosystem remain unclear. Obviously, any adaptation of the bacteriophage that leads to an increase in the range of potential hosts will provide an immediate selective advantage.

It is thought that some phages can carry "secondary" adhesion proteins which are not directly connected with the apparatus triggering the DNA ejection . Many of these putative secondary adhesins contain immunoglobulin-like domains which are exposed on the surface of the heads, contractible covers of the tail, or on the ends of the collar filbers of the phages [95]. Some phages can carry domains on their collar filaments, which are similar to adhesins not related to immunoglobulins [96]. Data on whether such structures can affect the growth of phages in laboratory conditions is still missing, but in an ecological situation such as the one prevailing in an animal intestine, their role can be considerable. First of all, it can be achieved by increasing the probability of reversible absorption after a phage particle has met with a sensitive host-cell. The increased absorption rate is not beneficial in some ecological situations. When phages grow on a dense population of hosts immobilized on a viscous agar medium (for instance when cultured using the double-layer method), mutants that have a lower adsorption constant have a considerable advantage and form plaques of larger size and with a higher number of virus particles [97]. This is due to the increased rate of the viral diffusion into the viscous media filled with host cells, and into the surrounding liquid. The authors suggest that similar advantages for slowly absorbed phages can be important during development in biofilms and during the spread to new regions. The mechanisms that lead to the reversible loss of secondary adhesins (for instance some of the tail fibrils of many phages) can thus have a significant effect on the adaptation of phages to growth on bacterial biofilms or on plankton cells [97]. Notably, secondary fibrillar adhesins can limit the diffusion of viscous media in several ways, including through an increase of the effective radius of the virus particles [97]. From this, it follows that preparations of phages obtained by cultivation in liquid or viscous media can be enriched with genetic variants optimal for the given mode of growth of the host population. The phages that form larger plaques may be less effective during growth in liquid media, since they exhibit a decreased level of absorption. These ideas should be taken into account when developing therapeutic phage preparations.

The importance of lysogeny in the ecological interactions between phages and bacteria in symbiotic microbial communities of animals requires further study. Some data [36] suggest that the induction of lysogenic bacteria is the main source of free coliphage particles in the intestine of healthy humans. However, most moderate coliphages isolated from the free phage particles pool of a sample of human or animal feces were members of the lambdoid phage group, while phages obtained by induction of lysogenic strains were mostly of the P2-like group [98]. In our recent study, all the phages extracted from horse feces and placed into one of the two groups by analyzing particle morphology and sequencing random portions of the genome turned out to be professional-virulent phages [49, our unpublished data]. It is impossible to tell at this point whether this discrepancy in the results is indicative of a difference in the microbial systems of animals or is an artifact of the research method.

Coliphages extracted from feces can often from metastable associations, which can be cultivated in laboratory conditions for several passages. We have termed these pseudolysogenic associations [A. Zimin, personal communication; our unpublished data]. The mechanisms which stabilize such associations are not always understood [99]. The most probable explanation is that some portion of the cells in a colony is partially shielded from phages by polysaccharide material or due to the physiological condition of the cell. In some cases, phages and bacteria can diverge during propagation on a agar medium, turning the pseudolysogenic association into a complex multicomponent community [our unpublished data]. Additional research is needed to understand whether pseudolysogenic associations of virulent phages and their hosts play a significant role in the *in vivo* ecology of coliphages or whether this is only a laboratory phenomenon.

THE PHARMACOKINETICS OF PHAGE PREPARATIONS

Penetration of bacteriophages into the internal environment of the organism.

It is known that bacteriophages can penetrate into the bloodstream from the intestine and other regions of the body, which are inhabited by microorganisms, and then be transported by the blood flow [100, 101]. It seems that the presence of a certain number of natural bacteriophages in the blood is normal (term "physiological viremia" was suggested for this phenomeneon) [101]. A recent study of the pharmacokinetics of three different phages on chickens [102] showed that peroral introduction of phage preparations led to the appearance of only one of the three phages in the liver and spleen in small quantities. This phage was also present in the lungs, however it could be accidently inhalted by the birds since the phage preparation was instilled by a syringe into the mouth. So, this begs the conclusion that this way of administering the suspension does not lead to effective translocation of phages, despite the fact that this study used relatively high doses of phages (10^6, 10^7 and 10^8 PFU per individual). Phage administration was slightly more effective in aerosol form through the lungs. However, earlier studies [103–106] have demonstrated multiple cases of the effective introduction of therapeutic phages into the blood using rectal and peroral administration. Peroral use of the "Sextaphage" preparation (FSUE «Microgen», Russia) yielded detectable bacteriophages in the blood an hour after administration [105]. Similar results were obtained when using this preparation as a rectal suppository [105, 106]. These studies do not report the phage dosages, listing only the volume of the preparation. They also do not report the phage titers in the blood, and there is no data on the differences between the penetrations of the various phages from the complex preparation. Notably, the rate of phage translocation from the GIT to the blood stream (similarly to bacteria) can vary significantly depending on the physiological conditions. An inflammation considerably increases the translocation of bacteria (and probably phages) from the intestine [12]. It is not yet known which specific phage traits affect their translocation through the mucosa. Probably, the transport is receptor-dependent and is executed as an active process

by specialized cells of the immune system (M-cells, goblet cells) and possibly by epithelial cells of the intestine and other components of the GIT. Several studies have attempted to identify the amino acid sequences that are involved in the binding with the appropriate receptors. These studies used the phage display approach [107–110]. Libraries of random peptides exposed on the surface of the filamentous M13 phage (which normally exhibits ineffective translocation through the mucous membranes of the GIT) were introduced into the GIT of experimental animals, after which the phages were extracted from the blood and internal organs or from the appropriate tissues from the GIT itself, and the peptides that were exposed on their surface were sequenced. The results showed that peptides YPRLLTP [108] and CSKSSDYQC [110] facilitate phage transport, peptides LETTCASLCYPS and VPPHPMTYSCQY [109] enhance the binding to M-cells and the to rat Payer patch tissue, and the LTHPQDSPPASA peptide stimulates the binding of the phage particles to the mucous membrane of the mouse intestine, which was damaged as a result of a strong systemic inflammatory reaction [107]. As opposed to the cited results, study [111] identified no significant effect of 7-amino acid peptides on the transport of the M13 phage from the mouse intestine into the bloodstream. It is quite possible that this result is due to the specifics of the experimental system used by the authors, or that 7 amino acid residues are not of sufficient length for the effective binding with appropriate cell receptors on the intestinal mucous membrane of mice. Currently, there is data in the literature on attempts to select therapeutically promising phages (mostly so-called tailed phages) for their ability to effectively travel into the bloodstream from the GIT. The success in selecting the phages that can circulate in the blood for prolonged periods of time (see further) gives hope that this procedure can be effective in some cases. Notably, numerous results indicate the high efficiency of phage translocation into the blood during rectal administration of the preparation [103, 105]. This phenomenon is being exploited in therapeutic practice now. There are phage preparations on the Russian market that are sold as rectal suppositories (see above); however, the mechanisms of phage translocation from the rectum and the factors that influence this process deserve a more thorough analysis.

Intramuscular or intraperitoneal administration of phages leads to much more effective translocation into the blood and organs than peroral methods. Thus, data from [102] shows that 3 hours after intramuscular injection of 10^8 PFU of three types of phages into chickens, their concentrations in the spleen was 2×10^2– 3×10^4 PFU×ml^{-1} and 6×10^2 – 10^4 PFU×ml^{-1} in the liver. During the following hours, the phage concentration dropped rapidly, and after 24 hours the phages were practically eliminated. Of note were the interesting differences in the effectiveness of infiltration of bird organs by different types of phages injected at the same doses. Intraperitoneal injection of 10^{11} PFU of phages (4 phages were tested) into mice resulted in a blood concentration of 5×10^8–2×10^{10} PFU×ml^{-1} two hours after injection, which indicates the effective infiltration of the inner environment of the organism [112]. One of the earlier (but also one of the most rigorously performed) studies of the phage pharmacokinetics [113] showed the effective infiltration of *Shigella dysenteriae* bacteriophages through the mouse's hematoencephalic barrier. Intraperitoneal injection of ultrafiltration-purified lysates with phages led to an almost immediate appearance of phages in the blood. Infiltration of healthy animal's brain tissues was recorded only in a few individuals on a small scale. On the other hand, mice infected with *S. dysenteriae* (injected intracerebrally with a dose exceeding DL95 %) showed a rapid increase of the phage titer of up to 10^7–10^9 PFU×g^{-1} in the brain tissue. During the following monitoring period, the phage titer in the brain decreased slower than that in the blood. The survival rate of mice that received 10^7–10^9 PFU of the phage was 72 % as opposed to 4 % in the control group, which received a heat inactivated phage preparation.

THE DISTRIBUTION OF PHAGE PARTICLES IN THE ORGANISM AND THEIR ELIMINATION

Early research in the 1920s-1930s [2] demonstrated that bacteriophages are eliminated from the bloodstream very rapidly. Mice that received intraperitoneal doses of phage [112] had very quick increases in blood phage titer (up to 10^{10} PFU×ml^{-1}), after which the titer decreased approximately by a factor of 10^3 after 12 hours and continued to decrease in a soft curve. The authors approximated this two-phase curve using the following equation: $C = 3.525\times10^9\times e^{-0,753t} + 2.35\times10^7\times e^{-0,0997t}$, where C is the concentration expressed in PFU×ml^{-1}; t is the time, in hours; while the numeric parameters varied for the different types of phages. Some bacteriophages can be absorbed onto erythrocytes and leucocytes in the bloodstream [93, 114]. Thus, it is unclear if such sequestered phages can infect the targeted bacteria during phagotherapy. Phage particles are most actively absorbed by the liver, where they quickly decay [102, 115]. Phages survive slightly longer in the spleen [2, 102, 116]. It seems that the reticuloendothelial system plays a major role in this process as opposed to the circulating phagocytes, since mice with cyclophosphamide-induced neutropenia exhibit only limited inhibition of phage elimination [112].

Phage particles are subjected to effective elimination by the reticulo-endothelial system. However, it is possible to select phage strains with altered surface proteins, which exhibit a significantly increased half-life in the bloodstream (one amino acid substitution may suffice [117]), and such long-circulating mutants can remain in the bloodstream for up to more than a 1,000 times longer than the initial strain [117–119].

Excretion of phages with urine has been shown in many cases [104–106, Darbeeva, personal communication]. However, this mechanism does not seem to play a major role in the phage elimination process, since the concentration of viruses in urine is approximately 10^1–10^2 [106], and such excretion is insignificant. On the other hand, even this insignificant number of bacteriophages, which make their way into the urinoexcretory system, is sufficient to have a therapeutic effect in the treatment of urological infections by peroral administration of phage preparations [104]. It is safe to assume that inflammation-mediated damage to the basal membrane of the Malpighian tufts can lead to increased phage excretion. It is also possible that the phages find their way into urine not via filtration through the tufts, but also through transfer from the blood into the canal epithelium. The issue of phage

excretion with urine and the factors that regulate this process require further investigation.

The production of antibodies specific to bacteriophages in patients can block the positive effect of phage therapy. Moreover, healthy people and patients who have never undergone phage therapy also have a certain level of antiphage antibodes [120]. However, the available data shows that the presence of such antibodies has a weak correlation with the results of therapy [A. Gorski, personal communication, 2]. It is worth noting that not all of the antibodies which interact with the phage particle have a direct neutralizing effect. In order to have this effect, antibodies would have to interact with adhesins and, in the case of some phages, with other elements of the tail. The production of specific high-affinity immunoglobulins of the IgG class takes several weeks; that is why this problem is of little significance for short courses of phage therapy. Moreover, the immunogenic potential of various phages is very different, and some bacteriophages require the use of adjuvants and repeated immunizations in order to achieve a significant immune response. It is also worth noting that the amount of antigens in the therapeutic doses of purified bacteriophages is relatively low; for instance, 10^{10} particles of T4 phage contain only about 1 microgramm of protein (this calculation is based on the assumption that the mass of DNA and protein is approximately the same in tailed phages [121]).

IMMUNOBIOLOGICAL ACTIVITY OF PHAGE PARTICLES

The direct effect of certain phage particles on the immune systems of humans and animals has been demonstrated recently by a research group led by prof. Gorski. It was shown that the viral particles of T-even bacteriophages lower the amount of the reactive oxygen species produced by neutrophils, which were induced by bacteria or endotoxin, and suppress the activation of T-cells, thus facilitating transplant tolerance. These phages also had a degree of antitumor effect [122–124]. The use of bacteriophage preparations in the treatment of various infections associated with *Staphylococci* and *E. coli* was often accompanied with a significant drop in the amount of serum C-reactive protein, which is an important marker of inflammation. It seems that such a decrease cannot be explained only by the antibacterial effect of the phages and must be connected to the immunomodulating activity of the phage particles [5].

It was suggested that the binding of T4 particles to eukaryotic cells is mediated by the capsid apex protein gp24 (gene product), which contains the KGD amino acid motif related to the RGD motif recognized by β3 integrins [101]. This binding can partially account for the immunosupressing and antitumor effect of the T4 phage *in vivo* and *in vitro* [125]. A mutant of the T4 phage, which has no gp hoc capsid decorating protein, has an increased immunobiological activity [125], which is possibly due to the partial masking of the β3 integrin-binding motif by these proteins. Some phages have surface proteins that are structurally similar to the molecules involved in the signaling cascades of immunocompetent cells. A whole range of structural proteins of various phages have immunoglobin-like [95] or collagen-like domains [126]. Study [24] noted the considerable increase in the concentration of phage particles on an ulcerated intestinal mucous membrane and hypothesized that the immunologic activity of phage particles can play some role in the pathological process in the case of Crohn's disease. On the other hand, it is thought that natural phage populations can take part in the interaction between the lymphoid tissues of the intestine and the microflora; for instance in the suppression of the inflammatory effect caused by massive contact with an antigen [101]. However, as of today, there is no experimental data that characterize the interaction of the natural viral community with the immune system.

DYNAMICS OF PHAGE--BACTERIA INTERACTION DURING PHAGE THERAPY

Physicians and microbiologists, who are not well informed on the theoretical basics of bacteriophage ecology and phage therapy, often hold the misguided opinion that the appearance of even a limited number of phage particles specific towards the target strain of pathogen in the area of the body colonized by these bacteria will trigger rapid phage reproduction, which will continue until the whole bacterial population is destroyed. These views are even expressed in some methodological literature (which we do not reference for ethical reasons). However, the coexistence of phages and bacteria in the same ecotope is an often-seen phenomenon in nature [10], and it has been around since before the separation of eukaryota, bacteria, and archaea [11]. Moreover, the interaction between bacteria and their viruses is not always antagonistic on the population level. Furthermore, the activity of bacteriophages can lead, under certain circumstances, to increased variety and overall metabolic activity of natural bacterial communities [10].

Currently, the standard procedure in phage therapy is to use only virulent phages; meaning those that can exclusievly reproduce in the course of a lytic cycle. During such a course of events, each infected cell is lysed after a certain period of time, called the latency period, releasing 50-200 and sometimes more phage particles. This results in an increase in phage particle concentration. In natural systems, however, the viral reproduction process is constantly countered by viral particle destruction and their elimination from the studied system. In the case of phage therapy, the major factors are phage consumption by the reticulo-endothelial system, their sequestration through association with erythrocytes, tissue cells or the extrarcellular matrix, their destruction as a result of adsorption on dead or specifically resistant cells (see above), as well as excretion from the organism with urine and feces. It is also worth noting that the reproduction of phages is usually localized in the infection center, while the spread of the phage's descendants occurs throughout the whole organism. If the bacteriophage's *in situ* reproduction rate is higher than the rate of the spread and (or) destruction, then the phage concentration will increase up to the depletion of the available host cells. This is called active phage therapy [127], and it is probably the working scenario during phage therapy of certain intestinal infections, including experimental ones. In these cases, one or several doses of phage are enough to cure the infection [2, 6, 7]. Cases where the concentration of phages must be maintained in order to suppress the growth of bacteria (see further) and large quantities of phages must be introduced artificially, such as during the treatment of chronic diseases, are termed passive phage therapy [127]. Many real-

world cases are combinations of these two scenarios.

Let us discuss the factors affecting the kinetics of the interaction of the bacterial population with the phages during therapy. The limiting step in the lytic cycle of the bacteriophage is the adsorption of the virus' particle on the surface of a bacterium, which usually results in the injection into the cell of the viral nucleic acid (for therapeutic phages, this is genomic DNA). The absorption process is mediated by highly specific recognition of the appropriate receptors on the cell's surface by phage adhesins [128]. These receptors are usually liposaccharides, surface proteins, teichoic acids, the components of the bacterial capsule and other surface structural elements of bacteria. This recognition is a passive physical-chemical process, which is dependent on the collision frequency of phage particles and cells during Brownian motion [129]. This process is described by a kinetic equation of the second order, which means that its rate depends on the concentrations of the bacteria and the phage. Specifically, $\ln(P/P_0) = e^{-kCt}$, where P is the amount of phage particles adsorbed up to the time point "t"; C is the concentration of bacterial cells, which does not change with the passage of time t; and k is an absorption constant ($ml \times min^{-1}$). All the differences in the phage diffusion, number of receptors on the cell's surface, inhibition of absorption by the physico-chemical factors of the medium, etc. are all factored into the value of "k," which is measured empirically for each case [129]. Under laboratory conditions, most tailed phages in liquid media have a k-value of about $(1.5-2.5)\times10^{-9}$ $ml \times min^{-1}$.

It was discovered empirically that different phage-host systems have threshold values of host cell concentration, which are needed for "triggering reproduction" of the virus [54] (usually about 10^4 $CFU \times ml^{-1}$). In real natural ecosystems (including animal organisms), the host concentration threshold needed to trigger phage reproduction (MT) can be calculated as the concentration at which the number of phages absorbed during the time of the elimination of half the phages ($t_{1/2}$) minus the length of the latency period (t_{lat}) is enough to provide offspring numbering half of the initial population. Therefore, it is a cell concentration (C), for which $P_{(t1/2-tlat)} = 0.5P_0/Y$, where Y is the yield of the phage per infected cell in these conditions (the death of some infected cells before the release of phage progeny must be factored into this value). When C exceeds the threshold MT value, phage production will exceed its elimination and, thus, the phage concentration in the system will increase.

There is also a threshold value of phage concentration C_{phage}, which inhibits the growth of the bacterial population if exceeded [127]. For this value of C_{phage} (IT), half of the bacteria will become infected during the time of an average generation (under the condition that the phage concentration during this time remains constant). The growth of rapidly reproducing bacterial cultures is inhibited under laboratory conditions at phage concentrations of about 10^7 [127, 129] $PFU \times ml^{-1}$. This value has no practical importance for laboratory use, since *in vitro* C >> MT, and the rate of phage particle destruction can be ignored; thus viruses accumulate very rapidly, causing practically complete lysis of the bacterial culture. It is obvious that the *in vivo* use of phage preparations will create other ratios between these processes, which is why populations of pathogenic bacteria are rarely destroyed completely during phage therapy. Thus, it makes more sense to speak of reducing the numbers of the pathogen to levels at which the infection can be controlled by the immune system [127].

In real-world phage therapy, most bacterial populations colonize only specific niches in the organism, and a major part of the bacteria are in physiological states, which do not allow reproduction of phages, for instance in biofilm form [130]. Cells that inhabit a biofilm are in a condition different from that in a suspended culture [131], and the cell's sensitivity to phages is lower [119, 132]. This is partially due to the limited diffusion in the matrix surrounding the bacteria in biofilms. Neverthelss, bacteriophages can infect cells that inhabit biofilms [132, 133]; some viruses encode enzymes which can hydrolyze the matrix polysaccharides. Moreover, the appropriate genes can be introduced into the phage genome by genetic engineering [134]. Notably, phage infection causes disruption in the structure of the biofilm, making the surviving cells more available to the immune system. Bacteriophages seem able to infect persister cells [135], which are one of the major causes of failure in antibacterial chemotherapy of such infections [136]. The spatial heterogeneity of a biofilm's structure [131], and many other microenvironments in the human and animal organisms, can, as it seems, limit spread of phage infection. Dead and stationary cells also restrict the phage expansion, since they can absorb phage particles. Thus, an active process at the micro-level is coupled with a passive process at the level of the whole organism. This effect can help explain the success in phage therapy of a range of infections; for instance pseudomonal osteomyelitis [M. Kutetaladze, personal communication], which cannot be effectively treated with antibiotics not because the pathogen is resistant, but because of the specific localization of the infection. Even though the infiltration of phages into the center of infection is limited, the possibility of local infective chain reactions is probably the major cause of success in the therapy. Moreover, stationary cells with reduced physiological activity can be infected, even though they do not allow the phage to reproduce. The infection will then be delayed until these cells are activated [135, 136]. Thus, the combination of active and passive phage therapy is the most probable scenario during the treatment of stable chronic infections. Such treatment requires prolonged courses of therapy, which can lead to patients being cured [5, 6]. Such cases require maintaining the phage concentration in the infection center for a prolonged time by administering new doses. The treatment of wound infections can be performed by using drainages with a constant flow of the phage preparation into the wound, or by using preparations that release phage particles slowly, such as the drug «Phagebioderm», designed in the G. Eliava Tbilisi Institute for Bacteriophages, Microbiology and Virology [137].

It is obvious that phage-resistant mutant bacteria can emerge during phage therapy. However, it is important to note that we could not find any documented case of unsuccessful phage therapy caused by the emergence of phage-resistant mutants. This can be explained by the fact that such mutants often have altered cell surface structures which act as receptors for the appropriate phages. The physiological price of such resistance is usually reduced growth [10, 138], colonization ability [7, 65, 66, 139, see also section 3.3], and virulence [139].

CONCLUSION

The accumulating genomic and structural data [11] point to the fact that bacterial and viral coexistence has been continuing for more than 2 billion years. The "rules of the game" that have emerged during this time are such that the presence of phages in natural systems not only has no negative effect on the variety and overall activity of the bacterial community, but also increases them considerably [10]. As more and more becomes known about the biology and ecology of bacteriophages, it becomes more apparent that the seemingly simple idea of using the "natural enemies" of bacteria to fight infection, which was first tried in practice in 1919 by Felix D'Errelle and by prof. Victor-Anri Hutinel [2], is a clever trick that should allow the complete suppression of one player (the bacterial population) and thus result in the elimination of the other player (the bacteriophage population). In order to achieve this goal through a rational approach (as opposed to an empirical one), it is important to understand the mentioned "rules of the game" in the ecological systems that are part of the human and animal organisms.

The analysis of the specifics of phage ecology allows to understand the additional requirements for therapeutic phages (other than their host range and *in vitro* efficiency) that could help identify the directions phage therapy research ought to take in the 21st century. Among these promising directions, we can mention 1) improving the technology of therapeutic phage selection, including developing and standardizing quick moderate-virulent tests, creating systems for typing potential therapeutic phages in order to allocate them to one of the known groups, as well as developing a methodology for analyzing phage genomes in order to asses their use in phage therapy; 2) creating databases and collections of characterized therapeutic phages, which can be used to quickly formulate individualized phage cocktails adapted to the needs of an individual patient; 3) developing methods to control the translocation and spread of phages in the organism, including methods for providing long-term circulation of phages in the blood, and their infiltration into various tissues and organs, etc.; 4) developing methods for testing the infective potential on bacteria which are in various physiological conditions or which colonize various "shielded" ecological niches in the organism, such as resident bacterial populations in the intestine, bacteria inhabiting biofilms, etc., developing methods for modifying these phage's traits, including methods involving genetic engineering; 5) monitoring and controlling the immunobiological activity of phages and targeted use of the ability of phage particles to interact with immune system cells; 6) using certain bacteriophage proteins as therapeutic agents, such as phage lysines [140] and bacteriocins.

Moreover, further studies of the role of bacteriophages in the homeostasis of normal microflora and in the development of pathological phenomena may help identify new approaches to the therapeutic control of these processes. ●

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Calcium Signaling and Neurodegeneration

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ABSTRACT **Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and spinocerebellar ataxias (SCA) are very important both for fundamental science and for practical medicine. Despite extensive research into the causes of these diseases, clinical researchers have had very limited progress and, as of now, there is still no cure for any of these diseases. One of the main obstacles in the way of creating treatments for these disorders is the fact that their etiology and pathophysiology still remain unclear. This paper reviews results that support the so-called "calcium hypothesis of neurodegenerative diseases." The calcium hypothesis states that the atrophic and degenerative processes in the neurons of AD, PD, ALS, HD, and SCA patients are accompanied by alterations in calcium homeostasis. Moreover, the calcium hypothesis states that this deregulation of calcium signaling is one of the early-stage and key processes in the pathogenesis of these diseases. Based on the results we reviewed, we conclude that the calcium channels and other proteins involved in the neuronal calcium signaling system are potential drug targets for AD, PD, ALS, HD, and SCA therapy.**

KEYWORDS **Alzheimer's disease, Parkinson's disease (PD), amyotrophic lateral sclerosis, Huntington's disease, spinocerebellar ataxias, calcium channels, calcium signaling, mitochondria, transgenic mice, clinical trials, imaging, memantine, dimebon, riluzole.**

ABBREVIATIONS **endoplasmic reticulum (ER), mitochondrial Ca^{2+} uniporter (MCU), Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), spinocerbellar ataxias (SCA), huntingtin protein (Htt), N-methyl-D-aspartate receptors (NMDAR), medium spiny neurons (MSN), tetrabenazine (TBZ), heritable Alzheimer's disease (HAD), phosphatidyl serine (PtdS). $InsP_3R1$ - type 1 inositol (1,4,5)-trisphosphate receptor, mPTP mitochondrial permeability transition pore**

Calcium signaling in neurons connects membrane excitability with the biological function of the cell [1]. Since Ca^{2+} channels are located on the boundary between the "electrical" and the "signaling" worlds, they play a key role in various aspects of neuronal function. Ca^{2+} signaling is required for short-term and long-term synaptic plasticity. Because of its extreme importance, neurons use multiple mechanisms to control intracellular levels of Ca^{2+}, usually within local signaling microdomains.

NEURONAL Ca^{2+} SIGNALING

A variety of Ca^{2+} channels are involved in neuronal Ca^{2+} signaling: the voltage-dependant Ca^{2+} channels of the plasma membrane (VGCC), NMDA receptors, AMPA receptors, TRP channels, and depot-controlled channels. Release of Ca^{2+} from the intracellular ER depot is mediated by inositol-1,4,5-triphosphate receptors (InsP3R) and ryanodine receptors (RyanR). The SERCA pump in the ER, the Ca^{2+} pump of plasma membrane, and the Na^+/Ca^{2+} exchanger of the plasma membrane are involved in the accurate control of the Ca^{2+} level in the cytosol in a very narrow range. The mitochondria play a very important role in the formation of cytosolic Ca^{2+} signals. The mitochondrial Ca^{2+} uniporter (MCU) is an ion channel involved in the rapid and massive entrance of calcium into the mitochondria. A large number of Ca^{2+}-binding proteins are involved in maintaining a certain level of Ca^{2+} in the cytosol (such as calbindin-D28, calretinin, and parvalbumin) and inside the ER (such as calreticulin and calnexin) in neurons.

Since neurons are highly sensitive to changes in the intracellular Ca^{2+} concentration, they have a whole range of Ca^{2+}-dependent structures, including proteins that are involved in the fusion of synaptic vesicles with the presynaptic membrane (such as synaptotagmins), Ca^{2+}-dependant kinases and phosphatases (such as the Ca^{2+}/CaM kinase and the Ca^{2+}-dependant phosphatase calcineurin), Ca^{2+}-dependent signaling enzymes (such as Ca^{2+}-dependent adenylate cyclase and Ca^{2+}-dependent NO-synthase), and Ca^{2+}-dependent transcription factors (such as the cAMP-dependent element-binding protein, calcineurin B-controlled activated T-lymphocyte nucle-

Table 1. Neurodegenerative diseases (Bezprozvanny, 2009)

Disease	Affected neurons	Age of onset	Sporadic/ inherited	Genes	Drugs	Target	Effect
AD	Neurons of the cortex and hippocampus	>65	95% sporadic, 5% inherited	APP PSEN1 PSEN2	Namenda (Memantine)	Blocks NMDA-receptors, reduces toxicity	Moderately improves cognitive function
					Donepezil (Aricept), Galantamine (Razadyne), Rivastigmine (Exelon)	Inhibitors of acetylcholine esterase. Increase the amount of acetylcholine in the brain	Moderately improves cognitive function
PD	Dopaminergic neurons of the pars compacta of the substantia nigra	>65	95% sporadic, 5% inherited	Synucl LRRK2 Parkin PINK1 DJ-1	L-Dopa (Levodopa)	Increases the amount of dopamine in the neurons of the substantia nigra	Symptomatic relief
ALS	Motor neurons	40–60	95% sporadic, 5% inherited	SOD1	Riluzole (Rilutek)	Antiglutamate effect (activates the capture of glutamate, blocks the NMDAR receptor and Na-channels)	Increases life-span by a few months
HD	Medium spiny neurons of the striatum	40–50	100% inherited	Huntingtin	Tetrabenzine (Xenazine)	Antidopamine effect (inhibits VMAT, lowers the amount of excreted dopamine)	Reduces chorea
SCA	Various regions of the brain involved in motor control	40–50	100% inherited	Ataxins	None	None	None

ar factor, and Ca^{2+} binding downstream regulatory element modulator). Such a variety of Ca^{2+} dependent elements makes the fine Ca^{2+}-dependent regulation of a neural function on time-scales possible in the microsecond range (as is the case of the Ca^{2+}-dependent fusion of a synaptic vesicle with the presynaptic membrane), in second and minute ranges (as in the case of Ca^{2+}-dependent phosphorylation and dephosphorylation), and up to day and year ranges (for Ca^{2+}-dependent changes in neural gene expression). These Ca^{2+}-dependent processes lead to short- and long-term changes in neuronal excitability (by changing the activity of ion channels and the expression pattern) and changes in synaptic transduction (by modifying the synaptic machinery and forming or disjoining synaptic connections). Since neurons are extremely sensitive to changes in Ca^{2+} signaling, even fine defects and deregulation of Ca^{2+} signaling can cause destructive consequences [2].

CA^{2+} BLOCKERS AND A COMPLEX APPROACH FOR TREATING NEURODEGENERATIVE DISORDERS

Neurodenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and spinocerbellar ataxias (SCA), are a very important problem both for fundamental science and for practical medicine. Despite extensive research into the causes of these diseases, clinical researchers have had very limited progress and as of now there is still no cure for any of these diseases. Therapeutic drugs used for treating these disorders have only a limited effect, causing only temporary relief of the symptoms or slowing the disease's progression (Table 1). A significant advance in the study of these disorders was achieved with the discovery of mutations that cause the pathological processes. HD and SCA are genetic disorders, and the genes which cause these diseases were cloned around 15 years ago (Table 1). Most cases of HD, PD, and ALS are sporadic, but around 5% of the patients inherit the disorder. Most of the genes which are involved in the development of the heritable form of the disease have been cloned (Table 1). The study of the genes which cause the above-mentioned diseases allowed researchers to form a mechanistic hypothesis for the pathological process and create a mouse model for studying these pathologies. Most attempts at studying the above-mentioned pathologies are focused on identifying the main causes of diseases and developing approaches to affect these causes. For instance, the main cause of AD was thought to be the formation of amyloid. Because of this, the main research efforts are directed at preventing the accumulation of amyloid by blocking its production or facilitating its clearance from the brain. In case of HD, the main reason is the expression of a mutant form of the huntingtin (Htt) protein. This means that most experimental efforts are directed at lowering mutant Htt expression in the brain (such as by using interfering RNA or a antisense knockdown).

Despite impressive scientific results, these approaches are hard to use in clinical treatment. For instance, in case of AD, the clinical trials of the amyloid-binding drug trampiprosate (Alzhemed) and the γ-secretase inhibitor tarenflurbil (Flurizan) were both unsuccessful. Clinical trials of amyloid-binding monoclonal antibodies (Bapineuzumab) had a very limited or even a negligible effect. The clinical trials of approaches for treating HD have problems with devising an adequate approach for siRNA or antisense RNA delivery into the human brain. There is still no solution for this problem, and clinical trials cannot be performed. While focused attention on amyloid and mutant Htt is understandable, it is worth

mentioning that the collected data indicate that these are targets which are very difficult to affect and that the creation of successful therapy based on these approaches will take a long time and take up considerable resources. In addition to developing methods for treatment, we propose a treatment that can delay the age at which the symptoms of the disease are manifested and/or lower the degree of the disease manifestation. Further, we will focus attention on the concept that the proteins involved in the calcium signaling in neurons are promising targets for developing "disease-delaying" therapy for neurodegenerative pathologies. We surmise that the most promising approach for clinical treatment will be a combination of approaches developed for each disease (such as amyloid-directed therapy for AD and huntingtin-directed therapy for HD) and of Ca^{2+} blockers.

NEURONAL Ca^{2+} SIGNALING AND AGING

Our neurons are the same age as us. Thus, it is not surprising that the risk of neurodegenerative diseases increases with age (Table 1). Comparative studies of neurons from young and old rodents have shown that the neuronal Ca^{2+}-signaling system experiences changes during aging. These data have been extensively published in the scientific press [2]. Recently, an integral model of age-dependent changes in hypocampal Ca^{2+} signaling has been proposed [3]. One of the main features of aging neurons is an increase in the Ca^{2+} concentration via active Ca^{2+} release from the intracellular depot using InsP3R and RyanR, an increased release of Ca^{2+} through the L-type VGCC, an increase of the slow trace hyperpolarization caused by the activation of Ca^{2+}-dependent K+ channels, a lowered involvement of NMDAR-mediated Ca^{2+} entrance, and a lowered buffer capacity of the cytosol and activation of calcineurin and calpains. Such changes in the neuronal Ca^{2+} dynamics lead to increased sensitivity, to the induction of long-term depression, and to the increased threshold frequency required for long-term potentiation in aging neurons [4]. The importance of these changes was also discussed in connection with the age-dependent disorders of the memory function [4].

The mechanisms involved in age-dependent changes in neuronal Ca^{2+} signaling have still not been elucidated. One possible scenario is connected with the age-dependent defects of mitochondrial function caused by overall oxidative damage sustained by the mitochondria. The mitochondria of aged neurons are depolarized and less effective in the control of Ca^{2+} uptake [2]. Age-dependent changes in the transcription of Ca^{2+}-signaling proteins were also discovered [2]. Some of these changes were directly dependent on the aging process and some of them were compensatory, but the whole picture is in agreement with the presence of age-dependent changes in the neuronal Ca^{2+}-signaling system on various levels.

NEURONAL CA^{2+} SIGNALING AND HUNTINGTON'S DISEASE

Huntigton's disease (HD) is a genetic disorder which is caused by a single mutation: the expansion of the CAG (polyglutamine) repeat in the huntingtin (Htt) gene [5] (Table 1). Medium spiny neurons (MSN) in the striatum are cells that sustain the most damage during HD. Most researchers agree that the mutant protein Httexp experiences a "gain of its toxic function" [6]. The destabilization of neuronal Ca^{2+} signaling is one of the toxic functions of the Htt^{exp} protein. Studies of HD patient's brains and also model experiments with mice show that the brain experiences sequential changes in the expression levels of Ca^{2+}-signaling proteins [7]. We proposed the "calcium hypothesis for HD" [8]. There are several main pathways for the effect of Httexp on Ca^{2+} signaling in MSN (Fig. 1). Our laboratory has established that Httexp directly and specifically binds the C-terminus of InsP3R1 [9]. The association between Httexp and InsP3R1 was independently discovered by random screening [10]. Binding with Httexp increases the affinity of InsP3R1 for InsP3 [9]. The key role of InsP3R1 activation in Httexp neurotoxicity was confirmed experimentally in mouse MSN cultures, which were used to model HD [11, 12], and in genetic experiments on the Drosophila based HD model [10]. Recent studies show that the

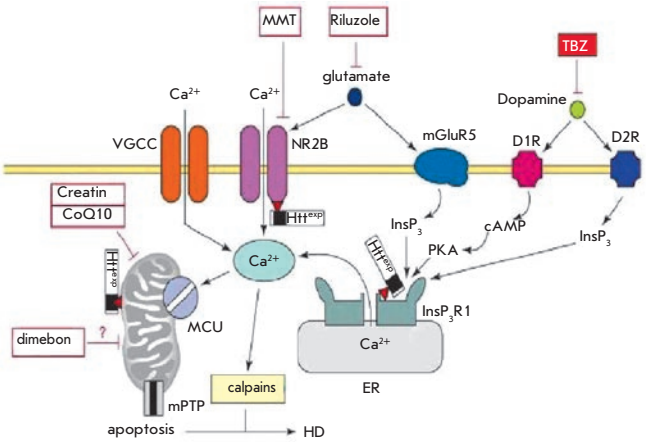

Fig. 1. A model of Ca^{2+} deregulation during HD (cited from (Tang et al., 2007)). In MSNs during HD, Httexp disrupts Ca^{2+} signaling by three synergistic mechanisms. Httexp increases the function of the NR2B-bearing NMDA receptor (probably by increasing its transport into the plasma membrane). Httexp tightly binds to the C-terminus of InsP3R1 and increases its affinity to InsP3.
The low level of glutamate secreted by the neurons of the corticostriatal projection causes an excessive influx of Ca^{2+} via the NMDA receptor and the release of Ca^{2+} from the ER via InsP3R1. The additional uptake of Ca^{2+} into MSNs is mediated by VGCC. Dopamine excreted by the dopaminergic neurons of the mesencephalon stimulates the type-1 (D1R) and type-2 (D2R) dopamine receptors, which are highly expressed in MSN. D1R is connected with an adenylate cyclase, and together they increase the cAMP level and activate the protein kinase A (PKA). PKA enhances the glutamate-induced Ca^{2+} signals by increasing the activity of the NMDA receptor and InsP3R1. D2R is directly involved in the production of InsP3 and the activation of InsP3R1. The excessive uptake of Ca^{2+} activates calpain, which cleaves Httexp and other substrates. The excess of Ca^{2+} in the cytosol leads to the capture of Ca^{2+} by the mitochondria via MCU, which in turn induces the opening of mPTP and apoptosis. The calcium regulation of mitochondria is also disrupted due to the direct interaction between Httexp and the mitochondria. The antidopamine drug tetrabenzine (TBZ) has been approved in the United States for the symptomatic treatment of HD. The NMDA receptor antagonist memantin (MMT), the soluble "mitochondrial agent" dimebon and "mitochondrial stabilizers" creatin and coenzyme Q10 (CoQ10) are all in clinical trials. The antiglutamate agent Riluzole has passed clinical trials, but it turned out to be ineffective for HD treatment [19]

viral delivery of a peptide that destabilizes the interaction between Httexp and InsP3R1 has a protective effect on the striatum MSN in the mouse HD model both in in vitro and in vivo conditions [13]. These data confirm the importance of increased InsP3R1 activity in HD pathogenesis. The expression of Httexp causes the increased activity of the NR 2B-bearing NMDA-receptor [14]. The increased flow through the NMDA-receptor is a consequence of the effect of Httexp on the transport of the NMDA-receptor to the plasma membrane [15]. Striatum MSNs expressing Httexp are sensitive to NMDAR-mediated toxicity. The pharmacological inhibition of the NMDA-receptor has a neuroprotective effect on a mouse MSN HD-model culture [11, 16]. Both memantin and riluzole had a neuroprotective effect on MSN cultures with HD. Memantin was more effective [17]. Memantin also had some positive effects in a small-scale experimental survey of this drug on HD patients [18], and it will soon be in the fourth phase of clinical trials for HD therapy (Table 2). Riluzole has completed the third stage of clinical trials on HD patients, but this study did not turn out to be successful [19] (Table 2).

In addition to InsP3R1 and to the NMDA-receptor, Httexp can also affect potential-dependent Ca^{2+} channels (VGCC). Huntingtin directly binds the α2/δ accessory subunit of VGCC [10] and the CaV2.2 pore-forming subunit of N-type VGCC [20]. The genetic removal of Dmca1D (pore-forming subunit of the L-type calcium channel in Drosophila) decreases the neurodegeneration of the photoreceptor in HD-model fruit flies [21]. An electrophysiological analysis of the striatum neurons of HD-model mice showed an initial increase of the VGCC channel density, which was followed by a decrease in their density [22]. Just as for other neurodegenerative disorders, Ca^{2+} toxicity mechanisms during HD are most often mediated by calpain activation and Ca^{2+} accumulation in the mitochondria (Fig. 1). The activation of calpains is observed during HD, and calapin-mediated cleavage of Httexp and the NMDA-receptor plays a key role in the pathology of this disease [23–25]. A large body of evidence indicates mitochondrial dysfunction during HD [26]. Mitochondria isolated from the HD patient lymphoblasts and from the brains of transgenic HD mice exhibited clear defects of the calcium system regulation [27]. The mitochondrial function was also disrupted in cell HD models [11, 12, 16, 28]. In addition to the effect on the mitochondria caused by the excessive concentration of Ca^{2+} in the cytosol, Httexp can also affect these organelles by directly binding with their outer membrane [27] (Fig. 1). It is worth noting that clinically adequate inhibitors of mitochondrial membrane permeability demonstrated a neuroprotective effect both on cellular HD models and on animal models of this disease [11, 28].

The first drug approved for HD treatment in the United States in 2008 was a dopamine tetrabenzine antagonist (TBZ) (Table 1). TBZ is a powerful inhibitor of the monoamine vesicular transporter, which causes the depletion of the dopamine contents of presynaptic vesicles. The clinical trials demonstrated that TBZ had a reliable suppressor effect on chorea symptoms in HD patients [29]. Our laboratory studied the effects of TBZ on the mouse HD model. It was shown that the effect of this drug lowered the deficit of motor coordination

Table 2. The most recent clinical trials of Ca2+ inhibitors and mitochondrial stabilizers as treatments for neurodegenerative disorders

Disorder	Drug	Target	Clinical trial stage	Clinical trial ID	Information was supplied by	Status/commentary
AD	Dimebon	mitochondrion (?)	Phase III	NCT00675623	Medivation	Completed, unsuccessful http:// www.alzforum.org/new/detail.asp?id=2387
	Ketasyn (AC-1202)	mitochondrion	Phase II	NCT00142805	NIA	Completed
	MEM-1003	L-type VGCC	Phase II	NCT00257673 NCT00257673 NCT00257673	Memory Pharmaceuticals	Completed
	EVT-101	NR 2B NMDA-receptor	Phase I	NCT00526968	Evotec Neurosciences	Completed, Phase II is planned
HD	Dimebon	mitochondrion (?)	Phase II	NCT00497159	Medivation	Completed, weak effect on cognitive function
	Creatine	mitochondrion	Phase III	NCT00712426	MGH	Forming test subject group
	Coenzyme Q10 (CoQ10)	mitochondrion	Phase III	NCT00608881	NINDS	Forming test subject group
	Memantine	NMDA-receptor	Phase IV	NCT00652457	UCSD	Forming test subject group
	Riluzole	antiglutamate	Phase III	NCT00277602	Sanofi-Aventis	Completed, unsuccessful ([19], 2007)

in the early stages of the disease and protected the striatum neurons from degeneration under in vivo conditions [30]. It was concluded that dopamine and glutamate have a synergetic activity in the formation of Ca^{2+} signals in the neurons of the striatum and that the effect of TBZ might be due to lowered Ca^{2+} signaling [30] (Fig. 1). These facts confirm that TBZ can not only be used as a drug for symptomatic treatment on late stages of the disease, but also as a drug for treating the disease presymptomatically. However, TBZ caused strong depression in some patients [29], which is why alternative dopamine antagonists should be researched, for instance, the dopamine-specific inhibitor of the vesicular monoamine transporter or blockers of D1 or D2 receptors.

NEURONAL CA^{2+} SIGNALING AND SPINOCERBELLAR ATAXIAS

Like in the case of HD, spinocerbellar ataxias (SCA) are autosomal dominant genetic disorders caused by the expansion of the polyglutamine sequence in ataxin proteins (Atx) [5]. There is a number of observations which indicate that disorders in the neuronal Ca^{2+} signaling can play a role in the pathogenesis of these diseases. Some of these data are presented further.

SCA1 leads to the degeneration of Purkinje cells of the cerebellum caused by the expansion of CAG repeats in the cytosol/nuclear protein ataxin-1 [5]. Purkinje cells of the cerebellum express very high levels of Ca^{2+}-signaling proteins and Ca^{2+}-binding proteins. A decrease in the Ca^{2+}-binding proteins in Purkinje cells was also observed in patients with early-stage SCA1 and in mouse models of this disease [31]. Crossing transgenic SCA1 mice with calbindin knockout mice led to an increased disease phenotype [31]. The transgenic CMA1 mouse model made it possible to observe the lowered expression of Ca^{2+}-signaling proteins such as InsP3R1, Ca^{2+}-channel TR PC3, and the ER pump SERCA2 during the early stages of the disease [32]. Albeit indirectly, these data confirm the fact that the disruption of the calcium signaling in Purkinje cells probably plays a key role in the etiology of SCA1.

During SCA2, the Purkinje cells of the cerebellum experience degeneration due to the expansion of CAG repeats in the cytosolic protein ataxin-2 [5]. The genetic connection between the polymorphism of the type-P/Q VGCC sequence and the age at which the first symptoms of SCA2 are manifested confirms the fact that Ca^{2+} signaling plays a very important role in the pathogenesis of this disease [33]. Our laboratory has discovered that the mutant form of ataxin-2 specifically binds and activates InsP3R1 similarly to the mutant form of Htt (article in print). We also demonstrated that inhibitors of Ca^{2+} signaling protected Purkinje cells from apoptosis during SCA2 under in vitro conditions and had a pronounced positive effect in experiments on transgenic mice (article in print).

During SCA3, neurons of the *substantia nigra* and the pontine nuclei experience degeneration as a result of the CAG repeat expansion in the ataxin-3 cytosolic protein [5]. Calpain-mediated cleavage of ataxin-3 plays an important role in the pathogenesis of SCA3 [34]. Recently we showed that the mutant form of ataxin-3 specifically binds and activates InsP3R1 similarly to how it binds the mutant form of the Htt protein [35]. It was further determined that the long-term feeding of CMA3 mice with a RyanR inhibitor and the Ca^{2+} stabilizer dantrolen facilitated the age-dependent deficit of motor coordination in these mice and prevents the loss of neurons in the *substantia nigra* and the pontine nuclei [35].

SCA6 causes Purkinje cells of the cerebellum to degenerate as a result of the expansion of CAG repeats in the C-terminus of the CaV2.1 (the pore-forming subunit of the P/Q-type Ca^{2+} channel) [5]. It was reported that this mutation increased the activity of the P/Q-type Ca^{2+} channel in an expression system [36]. However, most recent studies of SCA6 mice have shown that this pathology is also associated with the aggregation of CaV2.1 subunits and with the reduced density of the Ca^{2+} flow through the P/Q-type channels in dendrites [37]. Thus, the issue of the precise role of Ca^{2+}-signaling deregulation during SCA6 still remains unresolved. Anomalous neuronal Ca^{2+} signaling is not limited to ataxias with expanded polyglutamine repeats, but it can also play an important role in the ataxias of other types. The most recent genetic studies have shown that the cause of SCA15 is the loss of a fragment of the InsP3R1 gene [38].

NEURONAL CA^{2+} SIGNALING AND ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disorder which causes memory loss. In most cases AD appears sporadically and the first symptoms emerge in the elderly (after 60). A small fraction of cases (heritable AD (HAD)) are characterized by the early onset of symptoms and genetic inheritance.

NEURONAL CA^{2+} SIGNALING AND SPORADIC AD

Sporadic AD is "multitraget" disorder caused by the synergistic effect of several pathological factors. One of these factors is aging. The other factors are determined by the populations of neurons affected by the disease, in this case the cortical and hypocampal neurons. The main "disease-specific" factor during AD is probably the accumulation of amyloid. Since AD is a multitarget disease, the successful therapy must have a complex nature. The population of neurons which express high levels of Ca^{2+}-binding proteins remain mostly untouched by AD, while the populations of neurons which express these proteins at a low level experience extensive damage. A decreased level of Ca^{2+}-binding proteins is one of the most usual consequences of the natural aging process. Most likely one of the causes of an increased susceptibility of aged neurons to AD is the decreased buffer capacity of the neuronal cytosol for Ca^{2+}. Neurons of elderly patients who suffer from the sporadic form of AD exhibit an activation of Ca^{2+}-dependent proteases of the calpain family. Calpain activation takes place as a response to the increased levels of Ca^{2+} in the cytosol. Activated calpains cleave various proteins which are required for the normal functioning of the neuron, which results in neuronal dysfunction and apoptosis.

The mitochondria in neurons of AD patients experience severe damage. These organelles are partly depolarized, they exhibit lowered ability to bind Ca^{2+}, the disruption of the stoichiometry of the electron transfer chain, and the mutation of the mitochondrial DNA. Similar---but less visible---changes also take place in the mitochondria during the natural aging process. Damage to the mitochondria is probably caused by an oversaturation of this organelle by Ca^{2+}, which causes the formation of large quantities of active forms of oxygen which then cause extensive oxidative damage to the mitochondrial DNA. Thus, mitochondria seem to be the final step in the cal-

cium-signaling chain of this pathogenic cascade. However, it is still expected that "mitochondrial stabilizers" (such as coenzyme Q10 and creatine) should have some positive effect on these disorders. Drugs which are targeted at the mitochondrial permeability transition pore (mPTP) should be extremely useful as a "last line of defence" for the neuron delaying of the onset of neuronal dysfunction and cell death.

The aging process affects neuronal Ca^{2+} signaling and seems to be one of the factors involved in pathogenesis during sporadic AD. Thus, it is expected that blockers of Ca^{2+} signaling can have a positive effect on this disease. The NMDA-receptor antagonist Memantin has demonstrated a certain degree of clinical efficiency in AD treatment. The treatment of this disease requires the development and clinical trial of new Ca^{2+} signaling blockers by themselves and as part of a complex therapy in combination with mitochondrial stabilizers and with mPTP inhibitors.

NEURONAL SIGNALING AND HAD

The calcium hypothesis of AD pathogenesis. The central idea for explaining AD pathology is currently the amyloid hypothesis, which states that the main reason for neuron death and the decreased number of synapses during this disorder is the increased production of Aβ42 amyloid peptide (or the increased Aβ42/40 ratio) [39].

Experimental proof of the amyloid hypothesis is based on the following facts: (1) amyloid plaques are accumulated in the brains of AD patients; (2) the heritable form of AD (HAD) is caused by nonsense-mutations in the β-amyloid Aβ precursor protein (APP); and (3) HAD is also caused by nonsense mutations in presenilins, which form the catalytic subunit of γ-secretase, an enzyme that cleaves APP. Currently amyloid-directed therapy is the central strategy in developing drugs for AD therapy. Recent clinical trials have shown that targets other than amyloid need to be found in order to create an effective therapeutic solution for the treatment of AD [40]. A large mass of data indicates that the disruption of Ca^{2+} homeostasis in neurons plays a significant role in AD pathogenesis. The data in favor of the calcium hypothesis of AD development have been actively discussed in recent years [41]. This hypothesis is reviewed below. One of the key connections between AD pathogenesis and Ca^{2+} is based on data which state that Aβ oligomers can form Ca^{2+}-permeable channels in membranes [42]. The ability of Aβ oligomers to associate with membranes is enhanced if the membrane is treated by phosphatidylserine (PtdS) [43], which occurs naturally in cells experiencing a deficit of energy. Age-related changes in the mitochondria can increase the amount of surface PtdS in neurons and thus facilitate the Aβ-mediated formation of pores, the uptake of Ca^{2+}, and cell death (Fig. 2). In fact, neurons with decreased levels of cytosol ATP and increased levels of PtdS are especially sensitive to Aβ-mediated toxicity [44]. The ability of Aβ-oligomers to form Ca^{2+}-permeable channels in the neuron plasma membrane is in agreement with the results of the most recent experiments on in vivo measurements of intracellular Ca^{2+} concentrations on transgenic APP mice [45]. The authors of this study demonstrated that the quiescent-state Ca^{2+} levels in approximately 35% of neuronal axons located in close proximity to amyloid plaques were reliably higher than in control cells. The most likely explanation for this fact is that the local concentration of Aβ-oligomers in the regions adjacent to the plaque causes the formation of Ca^{2+}-permeable ion channels in the plasma membrane of neurons. Axons with increased Ca^{2+} levels lose their spikes and exhibit defective morphology [45]. The morphological changes in these axons can be alleviated by the activity of the calcineurin inhibitor FK-506 [45]. Based on this fact, we can hypothesize that calcineurin plays an important role in the

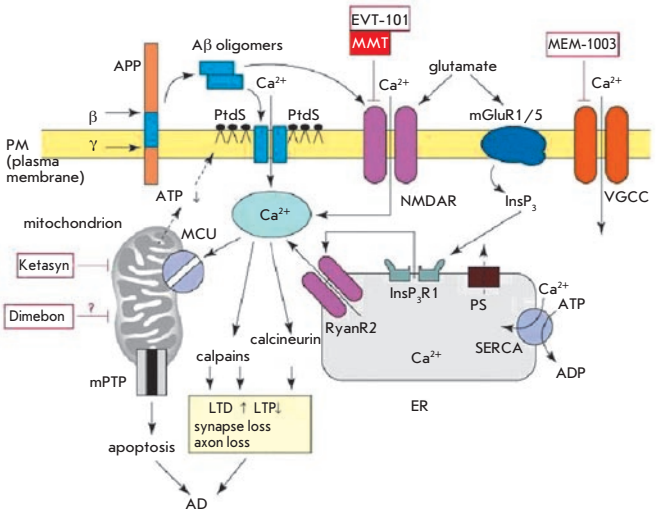

Fig. 2. Model of Ca^{2+} deregulation in AD (Bezprozvanny and Mattson, 2008). Sequential cleavage of the β-amyloid precursor-protein (β) by the γ-secretase (γ) leads to the formation of Aβ. Aβ forms oligomers, which can integrate into the plasma membrane (PM) and form pores permeable to Ca^{2+}-ions. The association of Aβ oligomers with the plasma membrane is facilitated by binding with the surface phosphatidylserine (PtdS); the aging process and Ca^{2+}-mediated damage of mitochondria cause a decrease in the ATP level and lead to the transfer of PtdS from the inner to the outer surface of the plasma membrane. The decrease of the ATP level and the loss of membrane integrity cause membrane depolarization, which in turn causes an increase in the uptake of Ca^{2+} through NMDA receptors and VGCC. Aβ oligomers can also directly influence the affinity of the NMDA, AMPA, and VGCC receptors. Glutamate activates mGluR1/5 receptors, increases the production of InsP3, and facilitates InsP3-mediated release of Ca^{2+} from the ER. Presenilins (PS) function as channels for Ca^{2+} drain from the ER and various mutations associated with HAD disrupt the Ca^{2+} drain function of presenilins. This causes the excessive accumulation of Ca^{2+} in the ER. An increase in Ca^{2+} levels in the ER enhances the release of Ca^{2+} via the type-I InsP3 (InsP3R1) and type-2 ryanodine receptor (RyanR2). PS can also directly modulate the activity of InsP3R, RyanR, and the SERCA pump. The increase in cytosolic Ca^{2+} concentration activates calcineurin and calpains, which in turn enhances long-term depression (LTD), suppresses long-term potentiation (LTP), and causes the modification of the neuronal cytoskeleton and the loss of synapses and axon atrophy. The excessive amount of Ca^{2+} in the mitochondria, which appears due to the activity of mitochondrial calcium uniporter (MCU), leads to the opening of the mitochondrial permeable transit pore (mPTP) and apoptosis. An inhibitor of the NMDA receptor called memantine (MMT) has been approved for AD treatment, and a NR2B-specific antagonist EVT-101 has also been developed. Currently, several other drugs are under clinical trials for the treatment of AD: a "CNS optimized" L-type VGCC inhibitor MEM-1003, the soluble "mitochondrial agent" Dimebon, and "mitochondrial antidepressant" Ketasyn

pathological response of neurons to an increase in the level of Ca^{2+}. Together with the direct effects on the permeability of the plasma membrane for Ca^{2+} ions, Aβ oligomers also affect the neuronal Ca^{2+} homeostasis via the modulation of the NMDA-receptor [46, 47] (Fig. 2), AMPA-receptor [48], and P/Q-type VGCC activity [49].

Another important relationship between Ca^{2+} signaling and AD was discovered from the fact that various mutations of presenilins found in HAD cases cause the deregulation of Ca^{2+} signaling. Initially, the connection between presenilins and Ca^{2+} signaling was discovered in a report that observed that fibroblasts from patients with HAD released abnormally high amounts of Ca^{2+} in response to the effect of InsP3 [50]. Similar data have been obtained in experiments on cells expressing mutant presenilins characterstic of AD [51], as well as on murine cortical neurons expressing mutant forms of presenilins characterstic of HAD [52, 53]. In order to explain these results, researchers hypothesized that mutant forms of presenilins affected the depot-controlled uptake of Ca^{2+} [54, 55], increase the activity and/or expression of intracellular Ca^{2+} ion-channels such as RyanR [53, 56, 57] and InsP3R [58, 59], or modulate the function of the Ca^{2+}pump SERCA in the ER [60]. Research done in our laboratory demonstrates that presenilins by themselves can work as channels for the draining of Ca^{2+} from the ER and that numerous mutations of presenilins associated with HAD lead to the overstocking of the ER with Ca^{2+} and the excessive release of Ca^{2+} from the ER [61, 62]. Despite some differences in the details of the proposed mechanisms, most of the reviewed studies are in agreement with the idea that various mutations of presenilins associated with HAD lead to the excessive release of Ca^{2+} from the ER via InsP3R and RyanR. There are several effects from releasing Ca^{2+} through the Aβ channels and excessively releasing Ca^{2+} from the ER which are especially toxic. As was said earlier, an increased level of cytosolic Ca^{2+} can activate calcineurin and cause atrophy [45] (Fig. 2). An excessively high level of Ca^{2+} also activates calpains, which destroy signaling enzymes involved in the processes of learning and memory [25, 63] (Fig. 2). Aged neurons become sensitive to the toxicity of cytosolic Ca^{2+} since aged cells have lower cytosolic buffering capacity.

In fact, an evident correlation has been found between the lowered expression of Ca-binding proteins in the region of the *dentate gyrus* of the hippocampus and the emergence of cognitive disorders associated with AD [64]. Abnormally high cytosolic Ca^{2+} signals can cause the excessive uptake of Ca^{2+} into the mitochondria and lead to cell apoptosis (Fig. 2). The known positive effects of nonsteroid anti-inflammatory drugs can be explained by their ability to lower the mitochondrial uptake of Ca^{2+} [65].

In conclusion we must note that a large mass of experimental data demonstrate excessive levels of Ca^{2+} in the neuronal cytosol as an effect of the Aβ oligomer accumulation or of the expression of mutant presenilins characteristic of HAD. Further proof of the connection between Ca^{2+} signaling and AD was obtained from a recent study which demonstrated that a mutation in the new Ca^{2+}-uptake channel CALHM1 can increase the risk of late-onset AD [66] (however, see also [67]). The proposed model (Fig. 2) offers a whole range of potential drug targets for AD therapy. The Aβ-based Ca^{2+} channels by themselves are very promising drug targets [68]. Thus, the US Food and Drug Administration has already approved memantin, which is a noncompetitive inhibitor of the NMDA-receptor as a therapeutic drug for AD (Table 1). There is the potential possibility of developing even more specific inhibitors for the NMDA-receptor, such as nitromemantins [69]. Recently, Evotec Inc has developed potential AD drugs based on a specific antagonism of NR2B receptors : EVT101 and EVT103 (Table 2). An L-type VGCC inhibitor MEM-1003 (Memory Pharmaceuticals) has successfully passed second stage clinical trials (Table 2). Other potential and mostly unstudied targets for AD therapy include intracellular Ca^{2+} channels (RyanR and InsP3R), the SERCA pump, calcineurin, and the mitochondrial Ca^{2+} regulation system.

The presented data constitute a new view on the therapy of neurodegenerative pathologies. Our proposed Ca^{2+} hypothesis creates a basis for the development of a new class of drugs.

Ca^{2+} SIGNALING: CURRENT PERSPECTIVES FOR THERAPEUTIC APPLICATIONS

Mitochondrial stabilizers and antidepressants. Ketasyn, Creatine, coenzyme Q10 (CoQ10), and MitoQ have all passed clinical trials for the therapy of AD and HD. Since mitochondria play a key role in the pathogenesis of these diseases [70], these clinical trials were expected to yield some positive results. However, mitochondria are involved in the pathological process at a relatively late stage, so the effect of these drugs can be expected to be limited. In fact, according to reports on this type of drugs, only modest therapeutic effects have been reported in the treatment of neurodegenerative disorders [70].

Dimebon. Dimebon (Medivation Inc) showed promising results (based on cognitive tests performed on patients) in the second phase of AD-therapy clinical trials [71]. Dimebon also passed the second stage clinical trials for HD therapy and demonstrated a weak effect on the brain activity of patients (Kieburtz at al, 2010 Arch Neurology, in press)

Dimebon is a well known antihistaminic drug used throughout the world and in Russia which, according to reports, had a neuroprotective effect when used in picomolar concentrations via a new effect on the mitochondria [72]. However, our studies on a culture of medium spiny neurons from the striatum showed a reliable neuroprotective effect of Dimebon only at 50 μM concentrations [73]. We concluded that the cognitive effect of Dimebon observed in clinical trials for the treatment of AD [71] was probably caused by the ability of this drug to inhibit α-adrenergic, histamine, and serotonine high affinity receptors [73]. In March 2010, the third stage clinical trials of Dimebon as a therapy for AD were completed and deemed to be a complete failure (http://www.alzforum.org/new/detail.asp?id=2387). Currently it is unclear whether Dimebon will be studied further as a treatment for AD and HD.

Antagonists of the NMDA-receptor. Memantine is noncompetitive antagonist of the NMDA-receptor which has been approved by the FDA for the treatment of AD. Memantine is also in a clinical trial for the treatment of HD. NR

2B-specific antagonists EVT101 and EVT103 (Evotec Inc) have been developed for the treatment of AD and they are expected to be tested in second stage clinical trials soon. The same drugs are also promising therapeutic compounds for the treatment of HD.

Riluzole. An antiglutamate agent which has been approved by the FDA for the treatment of ALS, Riluzole has also competed third-stage clinical trials for the treatment of HD; however, it did not exhibit any reliable positive effect on the motor measurements performed on patients [19].

Antagonists of L-type VGCC. An "CNS-optimized" inhibitor of L-type VGCC MEM-1003 (Memory Pharmaceuticals) showed a moderate positive effect in the second stage clinical trials on AD patients.

In conclusion, we must acknowledge that research in new directions of brain studies using modern molecular-biological and electrophysiological approaches will inevitably lead to an elucidation of the mechanisms behind highly effective informational flow and will also help discover approaches for treating neurodegeneration. ●

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A Novel High-resolving Method for Genomic PCR-fingerprinting of Enterobacteria

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ABSTRACT **We developed a novel PCR-fingerprinting system for differentiation of enterobacterial strains using a single oligonucleotide primer IS1tr that matches the inverted terminal repeats of the IS1 insertion element. Compared to widely used BOX-PCR and ribotyping methods, our system features higher resolution allowing differentiation of closely related isolates that appear identical in BOX-PCR and ribotyping but differ in their phage sensitivity. The IS1-profiling system is less sensitive to the quality of the material and equipment used. At the same time, BOX-PCR is more universal and suitable for bacterial strain grouping and reconstruction of the low-distance phylogeny. Thus, our system represents an important supplement to the existing set of tools for bacterial strain differentiation; it is particularly valuable for a detailed investigation of highly divergent and rapidly evolving natural bacterial populations and for studies on coliphage ecology. However, some isolates could not be reliably differentiated by IS1-PCR, because of the low number of bands in their patterns. For improvement of IS1-fingerprinting characteristics, we offer to modify the system by introducing the second primer TR8834 hybridizing to the sequence of a transposase gene that is widely spread in enterobacterial genomes.**

KEYWORDS **genomic fingerprinting, whole-cell PCR fingerprinting, insertion element, Enterobacterial diversity, strain differentiation.**

ABBREVIATIONS **IS – insertion sequence, ERIC – enterobacterial repetitive intergenic consensus, REP – repetitive extragenic palindromic sequence, dNTP – deoxyribonucleotide triphosphate, OTUs – operational taxonomic units.**

INTRODUCTION

Animal (including human) bodies are the ensembles of econiches populated by both various microorganisms and their viruses comprising the regular microflora. The animal (human) body is the main, if not sole, habitat for many microbial species [1]; however, atypical microorganisms may also be present [4]. The animal gut is one of most densely populated part of the body, and the host animal's health is directly associated with the composition and state of its resident intestinal microflora [2]. In some cases, *Escherichia coli* and related enterobacteria, the most common mammalian intestinal colonists, cause migratory diseases in animals [2].

At present, the role of conditionally pathogenic indigenous microorganisms in the infectious pathology of animals is regarded as essentially significant. Since many substantial physiological and biochemical features of microorganisms, such as phage sensitivity, antibiotic resistance, toxin production, and so on, are variable at strain level, there is a need for test-systems that allow reliable and constant differentiation of microorganisms according to their genetic background. Analysis of population events in highly dense microbial biocenoses, such as those existing in colon, is also important in fundamental ecological studies on symbiotic microbial associations. So, there is a need in simple and inexpensive molecular methods for bacterial strain differentiation that would be suitable for mass screening of isolates and offer high resolution and reproducibility.

Existing methods of typing microorganisms that are based on phage sensitivity and antibiotic resistance tests are characterized by their inherent considerable drawbacks. In particular, phage typing is highly time-consuming and material-intensive, because it requires the creation and maintenance of phage libraries for typing enormous amounts of indigenous strains and, hence, is hardly appropriate for mass screening of isolates [5, 6, 11, 12, 16]. Antibiotic-resistant genes are often localized in plasmids that can be easily gained or lost in response to environmental changes, which raises the question of the stability of some "classical" phenotypic traits of different strains and of the dependence of resistance factors on environmental conditions [11].

Molecular differentiation of microbial strains is carried out today using universal DNA fingerprinting systems, such as ribotyping and repetitive element-PCR with primers corresponding to conserved repetitive (REP), extragenous (BOX), and intragenic (ERIC) elements of genomic DNA [11, 16]. BOX-fingerprinting with the primer BOXA1R complementary to the nucleotide sequence of boxA locus, as well as ERIC-PCR, is used for identifying sources of water pollution and for classification of *E. coli* isolates in wastewater and in horse, neat, and canine feces as well [5, 12]. The relatively high (70%) GC level in primers used for BOX- and ERIC-PCR [10, 11] allows them to hybridize and initiate DNA synthesis with partially complementary nucleotide templates at the annealing temperature used (52°C). This nonspecific annealing highly depends on temperature; so a slight deviation from the amplification parameters determined by the accuracy of the thermal cycler used can fundamentally influence the amplification results. Increasing the annealing temperature allows to achieve better accuracy, but in this case BOX- and ERIC-PCR lose their omnitude and require a specialized primer set for each bacterial genus [10]. This peculiarity complicates a comparison of the BOX and ERIC patterns obtained by different researchers in different series of experiments.

The ribotyping of *E. coli* indigenous strains is based on combining the strains into groups (ribotypes) sharing the homology of 16S rRNA gene sequences, the universal genome markers [4]. Several modifications of this method include systems with restriction enzyme profiling of the 16S rRNA gene PCR products or those with sequencing of the PCR products. The genes encoding rRNAs are highly conserved within any of the bacterial species, thus making virtually impossible intraspecific differentiation. The resolution power of this method is not enough for the tasks mentioned above, often cannot provide information on the taxonomic position of the studied microorganism below the specific rank, and is inconvenient in terms of outlay for analysis, number of stages, and interpretation of obtained data [5].

In this work, we intended to develop a reliable and easy-to-use universal molecular method for express-differentiation of enterobacteria on the basis of PCR-amplification of their genomic DNA sequences and to test the method on isolates from natural animal gut microflora.

MATERIALS AND METHODS

Isolation of coliform strains. Horse feces were sampled immediately after defecation into sterile plastic containers and stored at -70°C before use. Coliform bacteria were isolated as follows: a sample of 15-20 g wet weight was thawed at room temperature for 30 min and suspended in four volumes of physiological saline. Following shaking for 20 min at room temperature, the suspension dilutions 1:100 and 1:1,000 were seeded onto Petri dishes with LTA agar selective for enterobacteria: 20 g of Bacto-Triptose (Difco, USA), 5 g of lactose, 5 g of NaCl, 2.75 g of K_2HPO_4 (anhydrous), 2.75 g of KH_2PO_4 (anhydrous), 0.1 g of SDS, and distilled water up to 1,000 ml, pH 6.8.

The colonies grown on LTA agar (20 colonies from each of the three different samples) were streaked by sterile toothpicks onto the dishes with LB agar: 10 g of Tryptone (Amresco, Spain), 5 g of yeast extract (Difco, USA), 5 g of NaCl, 15 g of Bacto-Agar (Difco, USA), and distilled water up to 1,000 ml.

Preparation of PCR templates. A small portion of a single bacterial column was transferred with the bacteriological loop into a sterile Eppendorf tube containing 100 μl of deionized water, heated at +95°C for 20 min by using an Eppendorf Thermostat 5320 heating block, vortexed, and centrifuged for 1 min at 13,000 rpm on an Eppendorf 5414 benchtop microcentrifuge. Supernatant was used as template.

IS1-fingerprinting. We previously constructed the IS1tr primer (Golomidova *et al.*, 2007): 5'-ATCAGTAAGTTGGA(G/A)(T/G)CATTACC-3' that anneals to inverted terminal repeats of the insertion element IS1. The PCR reaction mixture (20 μL total volume) contained 67-mM Tris-HCl, pH 8.3, 17-mM $(NH_4)_2SO_4$, 0.001% Tween-20, 2.5-mM $MgCl_2$, 25-pM of the IS1tr primer, 0.2 mM of dNTP, 1.25 U of *Taq*-polymerase (Sigma), and 1 μL of the template under study. The reaction was conducted using either a Mini Personal Thermal Cycler (BIO-RAD) or previous generation cyclers Thercyc (DNA-Technology, Russia) and Perkin-Elmer Cetus (Perkin-Elmer).

The amplification protocol was as follows: denaturation for 30 s at 94°C; 30 cycles of denaturation for 15 s at 94°C, annealing for 30 s at 56°C, and elongation for 45 s at 72°C; and final elongation for 2 min at 72°C.

The PCR products were analyzed by electrophoresis in 1% agarose gel.

We also constructed several other primers for improvement of strain differentiation (see the section "Results and Discussion"): IS2tr (5'-CAGATGTCTGGARATWYAGGGG-3'), IS3tr-L (5'-CCATATTACGTGGGTAGGATCA-3'), IS3tr-R (5'-CCACTATTGCTGGGTAAGATCA-3'), IS4tr (5'-TSCTTAACTGACTGGCATTA-3'), IS5tr (5'-SSRCTTRTTCGCACCTTCC-3'), IS30tr (5'-TGTTGCRTTGACMRATTGAATCTACA-3'), TR8D (5'-ATGCACGTCATACTCTTTTTT-3'), TR8R (5'-AAGAGTATGACGTGCATCCTA-3'), and TR8834 (5'-ATCGGCGATGCGTTGACGAAT-3').

Rep-PCR. BOX-fingerprinting was carried out according to the authors' protocol [15]. The BOX primer A1R (5'-CTACGGCAAGGCGACGCTGACG−3') was used instead of the IS1tr primer in the reaction mixture above. However, the amplification protocol was essentially different. The reaction began with denaturation for 2 min at 95°C, followed by 30 cycles of denaturation for 3 s at 94°C and 30 s at 92°C, annealing for 1 min at 50°C, and elongation for 8 min at 65°C; and the final elongation for 8 min at 65°C. The overall program took about seven hours. PCR products were detected according to the standard protocol (see above).

Ribotyping of *E. coli* autostrains. The genes encoding 16S rRNA were amplified using the primers 27F (5'-AGAGTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [4] that are universal for eubacteria. Endonuclease restriction profiling was fulfilled using *Hind*III and *Hae*III restrictases (Fermentas, Lithuania). The restriction products were analyzed by electrophoresis in 2% agarose gel.

Phage sensitivity of coliform isolates was estimated according to the Gratia bilayer method on a LB medium. The upper layer was LB containing 0.6% Bacto-Agar.

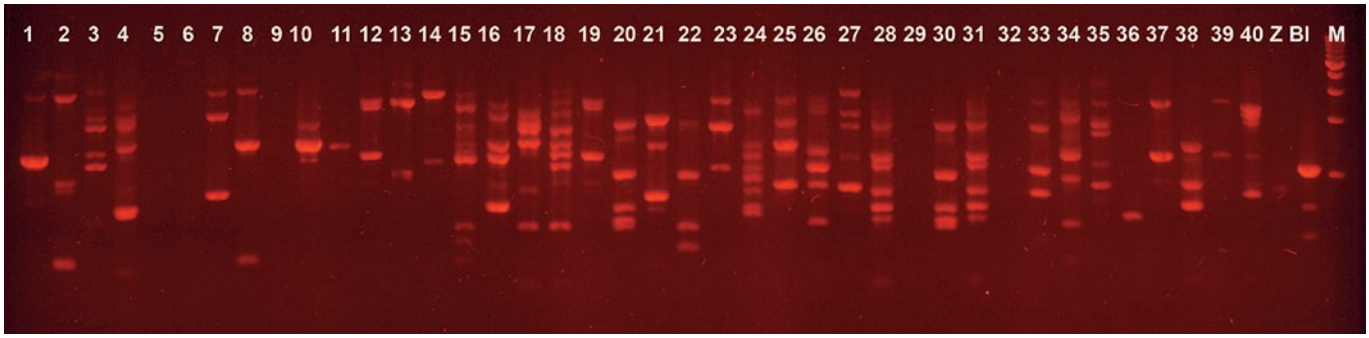

Fig. 1. IS1-fingerprinting of indigenous coliform strains. Lanes 1-20 – strains isolated from sample of horse feces No 1 Lanes 21-22 – strains isolated from sample of horse feces No 2 Z-: *E.coli Z85;* Bl – *E.coli BL21* Marker – 1kb DNA ladder (Fermentas)

RESULTS AND DISCUSSION

Since we aimed to develop a robust and convenient PCR-system for high-resolution genome typing of coliform strains, field testing of the novel system was necessary on a series of natural coliform isolates. So, the indigenous enterobacteria isolated from the feces of three horses served as the subject of the inquiry. Eighty various clones were chosen from the colonies grown on a LTA medium selective for enterobacteria.

IS1-fingerprinting system. We have developed a new system for genomic PCR-fingerprinting [6]. The PCR template is a crude DNA extract from heated cells rather than the purified DNA. The reaction uses single oligonucleotide primer annealing with inverted terminal repeats of the insertion element IS1 that is widely distributed in enterobacterial genomes [3], so that the primer 3'-end is directed. outwards of the element. Thus, the sequences amplified are those that are localized between either IS1 copies or other hybridization sites which are not associated with IS1 copies but may represent the remaiders of lost insertion elements. The length of specific reaction products depends on the relative position of IS elements or other hybridization sites in the bacterial chromosome, but it does not exceed the limit defined by the PCR conditions. The reaction products may be separated and analyzed by routine DNA electrophoresis in agarose gel [6].

The data of PCR with the IS1 primer show a distinct pattern of the reaction products for each coliform strain. All bands are well-separated in agarose gel. In most cases, their number varies from two to ten, thus simplifying evaluation of identical or closely related IS1-patterns. For instance, two identical patterns (fig.1, lanes 12 and 19) were found among the indigenous strains isolated from the first fecal sample, and two pairs of identical patterns (fig. 1, lanes 28, 31 and 37, 39) – among those isolated from the second one. Besides, two identical patterns were found between the strains from the first and second samples (fig.1, lanes 20 and 30).

Reproducibility and sensitivity of IS1-fingerprinting. The test for resistance of genomic DNA template amplification to various physical and chemical factors has shown that heating the template for 10 min at 100°C has no effect on IS1-fingerprinting, as compared to control; thus, possible deviations from heating parameters during template preparation would not influence the results. It is notable that in course of this work (about three months), the templates were stored in a freezer and repeatedly underwent thawing and freezing without any effect on the both quality and quantity of the IS1-PCR products (and coliform IS1-fingerprinting patterns as well). It is worth noting, however, that the excess of heat-lyzed biomass in the reaction mixture can inhibit PCR, so positive control is necessary in each template series, with the use of the strain certainly providing a specific pattern.

To check for the stability of IS1-fingerprinting through generations, we chose a strain with an easy-to-read IS1 pattern. Then, the strain was passed through five sequential passages in a liquid LB medium. The culture dilutions from the first and last passages were plated on LB agar for single colonies isolation. The IS1-PCR of randomized 20 colonies randomly chosen from each passage showed no deviation of subclone patterns from the initial one (Fig. 2). Some differ-

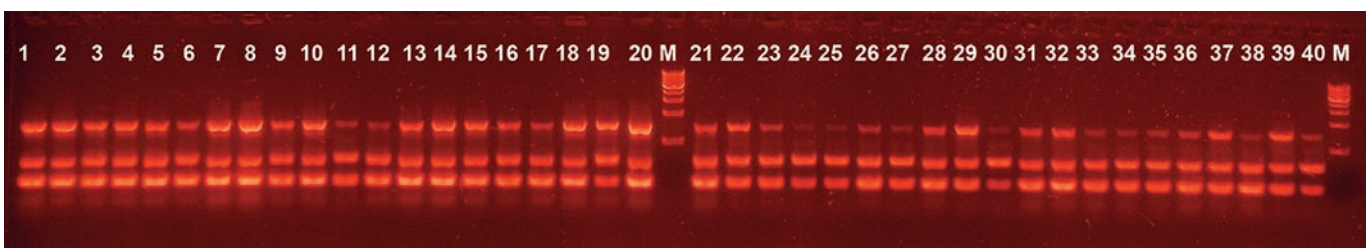

Fig. 2. Test for stability of IS1-pattern through passages of the strain in the liquid medium. No difference between subclones of initial strain (lanes 1-20) and its subclones obtained after 5 passages in liquid medium (lanes 21-40) was observed. Marker – 1kb DNA ladder (Fermentas)

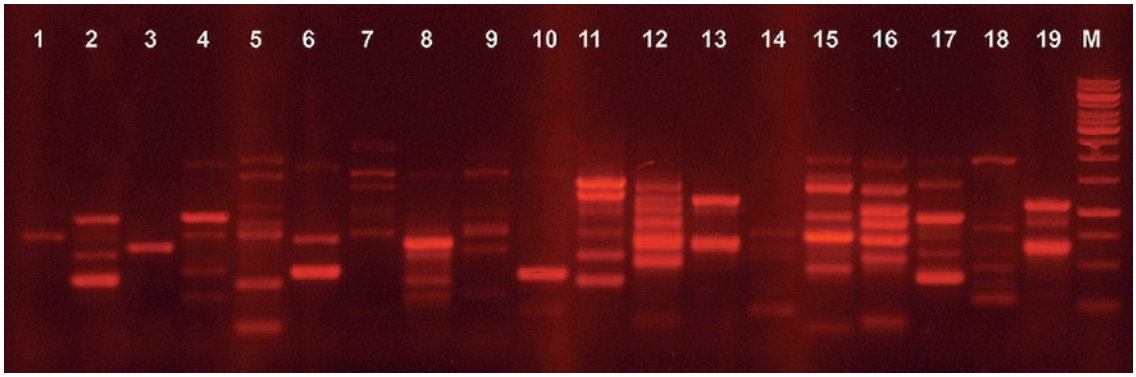

Fig. 3. BOX-PCR fingerprinting patterns. Indigenous coliform strains isolated from sample of horse feces No 3 Marker – 1kb DNA ladder (Fermentas)

ence in the intensities of individual DNA bands after electrophoresis in agarose gel might result from the nonstandardized amount of the DNA template in the PCR mixture. The indistinguishable genomic patterns of the initial strain and its offspring at limited number (about 50) of generations makes this system appropriate for long-term monitoring of populations of distinct bacterial strains in gut ecotopes and other natural biocenoses.

The IS1-fingerprinting system performs equally well both in a BIO-RAD MJ mini Personal Thermal Cycler and in the DNA-Technology Thercyc thermal cycler, which is widely available in Russia. PCR in the Thercyc thermal cycler manufactured in Russia requires the application of mineral oil over the PCR mixture to avoid evaporation. Both the yield of the PCR product and the band patterns obtained are perfectly comparable. The use of different polymerases, *Taq* or *Pfu* or their mixture, also did not influence the result (data not shown). Thus, the kinetic features of the equipment used have no definite bearing on the results, which provides an advantage over existing alternative systems for strain typing, such as BOX-PCR, which are more dependent on the quality of equipment and chemicals. The first commercial PCR thermal cycler, Perkin-Elmer Cetus (which became available in 1989), has provided a similar yield and an identical pattern of PCR products.

Comparison of IS1-fingerprinting, BOX-fingerprinting, and ribotyping of enterobacteria. We compared the novel method of genomic IS1-fingerprinting with existing methods of molecular BOX-fingerprinting and ribotyping using the same DNA templates as those used for IS1-PCR and complex optimized amplification protocols recommended by the authors of [15]. Electrophoresis of BOX-PCR products in agarose gel demonstrated faint separation of the amplified DNA fragments, whose number averages about 20-30, thus hampering the search for identical patterns without specialized software. The yield of PCR products is lower than that in IS1-PCR. The profiling revealed four identical groups (each combining 2-7 patterns) among the autostrains isolated from the third fecal sample (Fig. 3). Thus, both the discriminative capability and sensitivity of this system are lower than those of the system we offer.

Ribotyping is more labor- and materials-intensive than BOX- and IS1-PCR fingerprinting. This method includes both PCR-amplification of the required DNA sequence and the following enzymatic hydrolysis of the desalted PCR-product. This method did not allow grouping within the given series of field isolates of *E. coli*, thus demonstrating low resolution. This is determined by the highly conserved 16S rRNA gene sequence within the bacterial species and incomplete count of possible mutations in the locus (the endonuclease restriction analysis can only reveal mutations in the restriction site rather than in the entire sequence). The method of choice in this case is DNA sequencing – an expensive and slow process.

The use of IS1-PCR for differentiation of closely related strains differing in bacteriophage sensitivity. Susceptibility to infection by distinct phage races is one of the most labile properties of bacteria which rapidly evolve both in natural and laboratory microbial biocenoses. We conducted the fol-

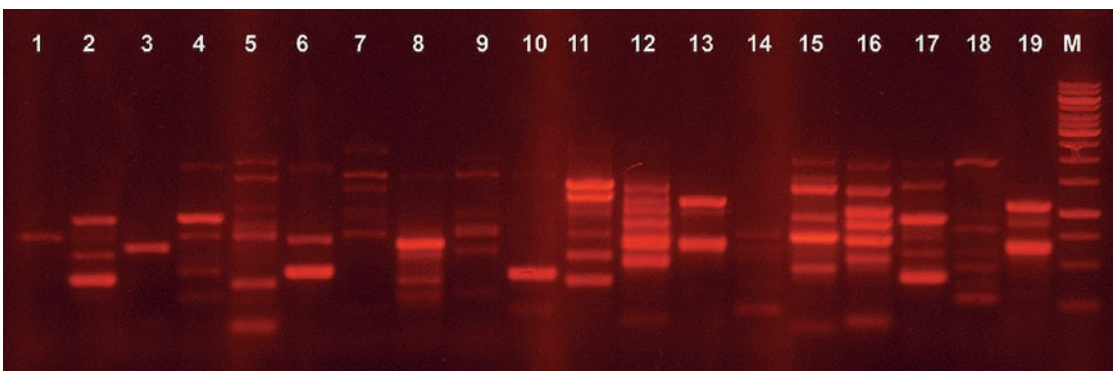

Fig. 4. IS1-fingerprinting. The set of the strains and the order are as in Fig. 3 Marker – 1kb DNA ladder (Fermentas)

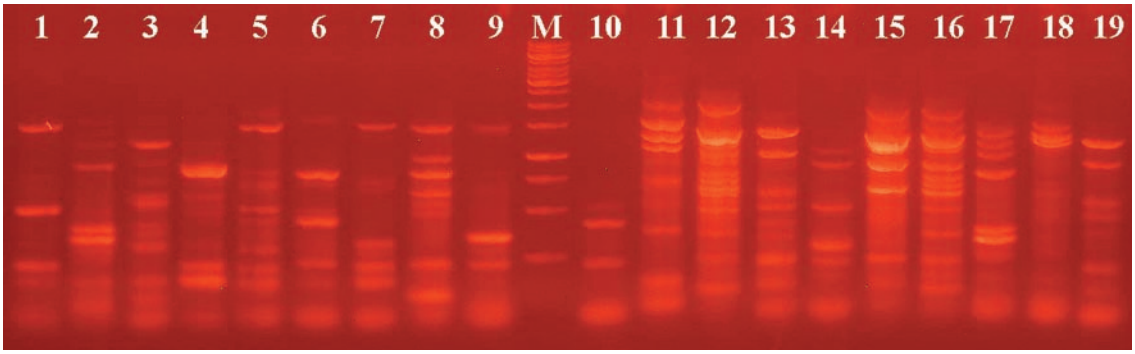

Fig. 5. Fingerprinting using the IS1 and TR8834 primer pair. The strains are the same as in Figs. 3 and 4 Marker – 1kb DNA ladder (Fermentas)

lowing experiment to find out whether the resolution of IS1-fingrprinting is high enough for the differentiation of closely related natural isolates differing in sensitivity to the phages present in the same biocenoses.

Four pairs of autostrains from horse feces were chosen: two with identical IS1 patterns (and identical BOX patterns as well) and two with identical BOX-PCR profiles. These autostrains were tested for sensitivity to a panel of 20 phages differing in specificity, which were preliminarily isolated from horse feces in our laboratory [6]. Phage lysis plaques were only found in isolates with identical IS1 patterns, when coliphages Nos 12 and 17 were applied. At the next step, our own phages were isolated from the same fecal samples from which the enterobacterial strains tested were derived. Fecal extracts were seeded onto the lawns of the tested strains on LB agar according to the bilayer method, and 200 plaques were collected. Each of the autostrains was tested for sensitivity to this phage sampling. The experiment showed that the strains with identical BOX-PCR patterns are differently sensitive to this phage sampling and, interestingly, show different IS1 patterns. In contrast, the strains with identical IS1 patterns are equally sensitive to bacteriophages.

The coliform strains unable to yield the PCR product in our system are relatively rare (5-10% of the tested strains) in samples of highly heterogeneous bacterial associations found in horse feces. In addition, sometimes the strains whosepatterns contain few bands cannot be differentiated with adequate certainty.

To increase the number of bands in patterns and thereby increase the resolution of the system, we constructed a series of primers specific to the inverted terminal repeats of other, less distributed in coliform genomes, insertion elements (IS2, IS3, IS4, IS5, and IS30), as well as the primers TR8D, TR8R, and TR8834 complementary to transposase gene sequences presented in many copies in the genomes of many *E. coli* strains.

PCR with these primers was carried out as described above. Various oligonucleotide combinations were tested, and the best result was achieved with an IS1 and TR8834 primer pair. The autostrains showing 2-3 electrophoretic bands in IS1-fingerprinting patterns (Fig. 4) might be far easier differentiated after PCR with the IS1+TR8834 primer pair yielding 5-7 bands (Fig. 5). The PCR protocol with this primer combination was the same as that used for IS1-fingerprinting. We compared this improved system with BOX-PCR and ribotyping on the same templates and were convinced of its superiority over these methods.

Therefore, the high resolution of this system can often mask the genetic kindred of distantly related strains. This makes it virtually impossible to classify IS1-fingerprinting profiles into operational taxonomic units (OTUs) or use them as a backbone for phylogenetic tree construction, as is common for other PCR fingerprinting systems, such as ERIC-PCR [9] and BOX-PCR. This reduces the applicability of our system for a series of tasks, such as the search for sanitary-representative enterobacterial strains pointing to a source of fecal pollution [5]. Besides, IS1-profiling focused on coliforms is less universal as relates to the spectrum of analyzed microflora than the above-mentioned systems. Despite our improvements, a small portion of the strains remains nontyped in our method, because of the lack of PCR-amplification.

At the same time, the high resolution of the system we have developed, which is comparable with that of phage typing, makes it the "method of choice" in studies of the microecology of enterobacterial phages in natural microbial ecotopes, such as mammalian gut and wastewater. The environmental conditions of these ecotopes often condones a quick co-evolution of phages and their hosts, leading to unusual heterogeneity of bacterial populations at the strain level [14, 20]. As a result, even closely related strains may essentially differ in sensitivity to bacteriophages inhabiting the ecosystem [6, 8]. It seems obvious that the high resolution of the system, together with its excellent reproducibility, is valuable in many other tasks, in particular, in tracing epidemiological chains in the analysis of the distribution of pathogenic enterobacteria among animals, particularly humans.

CONCLUSION

We have developed a quick system of genomic PCR-fingerprinting that essentially supplements the existing set of tools for molecular differentiation of enterobacteria and enables to resolve the tasks associated with the detailed analysis of highly heterogeneous and rapidly evolving natural populations of these bacteria.

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Tumoricidal Activity of RNase A and DNase I

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ABSTRACT In our work the antitumor and antimetastatic activities of RNase A and DNase I were studied using two murine models of pulmonary (Lewis lung carcinoma) and liver (hepatoma A-1) metastases. We found that intramuscular administration of RNase A at the dose range of 0.1–50 μg/kg retarded the primary tumor growth by 20–40%, and this effect disappeared with the increase in RNase A dose over 0.5 mg/kg. DNase I showed no effect on the primary tumor growth. The intramuscular administration of RNase A (0.35–7 μg/kg) or DNase I (0.02–2.3 mg/kg) resulted in a considerable decrease in the metastasis number into the lungs of animals with Lewis lung carcinoma and a decrease of the hepatic index of animals with hepatoma 1A. A histological analysis of the organs occupied by metastases revealed that the administration of RNase A and DNase I induced metastasis pathomorphism as manifested by the destruction of oncocytes, an increase in necrosis and apoptosis foci in metastases, and mononuclear infiltration. Our data indicated that RNase A and DNase I are highly promising as supplementary therapeutics for the treatment of metastasizing tumors.

KEYWORDS antimetastatic activity, DNase I, RNase A, Lewis lung carcinoma, hepatoma 1A.

ABBREVIATIONS Lewis lung carcinoma (LLC), hepatoma 1A (HA-1).

INTRODUCTION

Recent data on the implication of small noncoding RNAs in tumorigenesis [1–3] and tumor-derived DNAs in metastasis progression (genometastasis hypothesis) [4] gave a new initiative to the study of enzymes cleaving nucleic acids as potential antitumor and antimetastatic agents.

Extensive studies on the antitumor potential of exogenous ribonucleases are being conducted worldwide. The high antitumor activity of the RNase A family members BS-RNase [5–8] and onconase [9–11] has been shown. Of this family, RNase A was first studied for antitumor activity [12–14]. The data of these experiments were contradictory. Some authors reported high antitumor activity in RNase A [12, 13], whereas others reported its complete absence [14, 15]. The absence of any antitumor effect of RNase A was attributed to its inactivation by ribonuclease inhibitor [16, 17]; both onconase and BS-RNase can avoid interaction with the inhibitor, thus keeping their cytotoxic activity against tumor cells [18–20]. The antimetastatic potential of DNase I was demonstrated in vivo using a L5178Y-ML liver metastasis model [21, 22]. However, the use of DNase I as an adjuvant in cancer therapy was not further extended.

In this work we studied the antitumor and antimetastatic effects of RNase A and DNase I on two murine tumor models: Lewis lung carcinoma (LLC) metastasizing to the lungs and hepatoma A-1 (HA-1) metastasizing to the liver. The intramuscular administration of RNase A at a dose ranging within 0.1–50 μg/kg resulted in the retardation of tumor growth by 20–40%. The administration of either RNase A or DNase I led to a two- to threefold decrease in the number of metastases in the lungs (LLC) or a decrease of the hepatic index (HA-1). A histological analysis revealed the destruction of tumor cells, an increase in the number of necrotic and apoptotic sections in metastatic foci, and mononuclear infiltration following treatment with the enzymes.

MATERIALS AND METHODS

RNase A (mol. wt 13,700) and DNase I (2.155 kU/mg) from bovine pancreas were purchased from Sigma (United States); [γ-^{32}P]adenosine-5'-triphosphate ([γ-^{32}P]ATP) (3,000 Ci/mmole) was purchased from Biosan (Russia), and T4 polynucleotide kinase was purchased from Fermentas (Lithuania). The pHIV-2 plasmid was kindly provided by Prof. Hans J. Gross (University of Wuerzburg, Wuerzburg, Germany).

LLC and HA-1 tumor strains were obtained from the vivarium at the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences (SB RAS), Novosibirsk, Russia.

The HIV-1 RNA fragment prepared by in vitro transcription was labeled at the 5'-end using γ-^{32}P ATP and T4-polynucleotide kinase [23].

Determination of RNase A activity. A reaction mixture (10 μl total volume) containing 50 000 cpm of 5'-[^{32}P]-labeled RNA, 10^{-10}–10^{-7}M RNase A, 50 mM Tris-HCl, pH 7.0, 200 mM KCl,

1 mM EDTA, and 100 μg/ml of RNA carrier was incubated at 37°C for 1–15 min. Following incubation, the reaction mixtures were extracted with phenol and RNA was precipitated from an aqueous phase with 96% ethanol. The products of RNA cleavage were analyzed by electrophoresis in 12% denaturing polyacrylamide gel.

Determination of DNase I activity. A reaction mixture (10 μl total volume) containing 0.2 μg of pHIV-2 plasmid DNA, 0.01–1 U of DNase I, 10 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ was incubated at 37°C for 1–15 min. The reaction was quenched by heating at 60°C for 10 min. The products of DNA cleavage were analyzed by electrophoresis in 1% agarose gel.

Tumor models. Female C57Bl/6 mice (10–11 week-old) and female A/Sn mice (12–14 week-old) were housed in plastic cages (8–10 animals per cage) under normal daylight conditions. Water and food were provided ad libitum. All procedures with the animals were carried out according to approved methods and recommendations for laboratory-animal care [European Communities Council Directive 86/609/CEE].

Solid LLC or HA-1 tumor development was generated by injecting corresponding tumor cells (10^6 cells per animal) into the femoral muscle of C57Bl/6J or A/Sn mice, respectively.

Intramuscular administration of RNase A and DNase I and an examination of their effect on the primary tumor and metastases. On day 4 or 8 after the implantation of LLC tumor cells, C57Bl/6J mice were divided into groups and intramuscular injections were performed daily as follows: group 1 (control) received saline and groups 2–9 received 0.1 ml of RNase A saline solution (0.1, 0.5, 1, 10, and 50 μg/kg and 0.5, 1 and 10 mg/kg, respectively); groups 10–13 received 0.1 ml of DNase I saline solution (0.02, 0.23, 1.15, and 2.3 mg/kg, respectively).

On day 8 after the implantation of HA-1 tumor cells, A/Sn mice were divided into groups and intramuscular injections were performed daily as follows: group 1 (the control) received saline and groups 2–4 received 0.1 ml of RNase A saline solution (0.35, 0.7 and 7 μg/kg, respectively); groups 5–9 received 0.1 ml of DNase I saline solution (0.02, 0.23, 1.15, and 2.3 mg/kg, respectively).

During the experiment, animals were injected 8–10 times with either enzyme solution or saline. The tumor size was measured every three days with calipers, and the tumor volume was calculated from the equation V = (π/6 × length × width × height) [24].

On day 20 after tumor implantation, the mice were killed by cervical dislocation. Livers of A/Sn mice with HA-1 were weighted, and the hepatic index (HI) was calculated from the equation HI = (liver weight/body weight) × 100%. The average liver increment (ALI) during tumor development was calculated by the subtraction of healthy animals' HI (4.5% for A/Sn mice) from the mean HI of experimental group. Therapeutic efficacy (TE) was calculated from the equation $TE\ (\%) = 100 - ALI_{exp} / ALI_{control} \times 100\%$.

The lungs of animals with LLC and liver of animals with HA-1 were fixed in 4% formaldehyde for further histological analysis. The number of metastases in the lungs of LLC-bearing mice was enumerated using a binocular microscope.

Histological analysis. Fixed lungs and liver were treated according to routine protocol and embedded in paraffin. Histological sections (5-μm-thick) were prepared on a microtome and stained with hematoxylin and eosin (HE staining). Pathomorphological features were evaluated visually using an Axioimager Z microscope (Zeiss).

Statistical analysis. Whenever the data showed normal distribution, their statistical processing was performed using Student's *t*-test. Otherwise, the Mann-Whitney nonparametric statistics was used. Differences were regarded as significant at $p < 0.05$.

RESULTS AND DISCUSSION

Choice of Dose Ranges for RNase A and DNase I Used in Experiments In Vivo.

Since the enzymatic activities of RNase A and DNase I were assumed to be essential for the antitumor effect of these enzymes, concentrations which provide a 50% cleavage of substrates in a relatively short time were determined in experiments in vitro.

To do this, [5'-^{32}P]RNA (10^{-5}M) was incubated in the presence of 10^{-10}–10^{-7}M RNase A at 37°C for 1–15 min. Kinetics of RNA cleavage has shown a 50% cleavage of the substrate in 10 min at a RNase A concentration of 10^{-9} M. Similarly, 50% cleavage of DNA substrate was achieved in 1 min by 10 U of DNase I per ml. These concentrations of RNase A and DNase I were taken as the starting points to select the appropriate doses of the enzymes for the in vivo assay.

EFFECTS OF RNASE A AND DNASE I ON PRIMARY TUMOR GROWTH

Intramuscular administration of RNase A to LLC-bearing C57Bl/6J mice. The effect of RNase A on the primary tumor growth was examined in experiments with LLC-bearing C57Bl/6J mice. On day 4 after tumor transplantation, the animals began receiving daily intramuscular injections of saline (control) or RNase solution ranging in concentration from 0.1 μg to 10 mg per kg body weight (experiment).

Fig. 1A demonstrates changes in the size of tumors during the experiment depending on the RNase A dose. One can see a retardation of tumor growth in the LLC-bearing animals treated with RNase A at a dose ranging within 0.5–50 μg/kg. On day 8 after LLC transplantation, the tumor volume in these experimental groups was retarded by 20–40% when compared with the control. This difference was 23–33% on day 11 and 16% on day 13. No effect on tumor growth was observed in animal groups treated with RNase A at a dose above 0.5 mg/kg (Fig. 1A).

Intramuscular administration of RNase A to HA-1-bearing A/Sn mice. To ensure that the antitumor activity of RNase A is not tumor-specific, we examined it on another model, hepatoma A1 in A/Sn mice. Since RNase A showed a marked activity on the LLC model at a dose ranging within 0.5–50 μg/kg, we also used this dose range in experiments with HA-1. The LLC-bearing C57Bl/6J mice were used as positive controls in these experiments. Beginning from day 8 after tumor implantation, when the tumors became palpable, the mice with HA-1 or LLC received intramuscular injections of either saline or RNase A solution at doses of 0.35, 0.7, and 7 μg/kg.

A comparison of tumor sizes in the control group and groups of animals with either LLC or HA-1 treated with

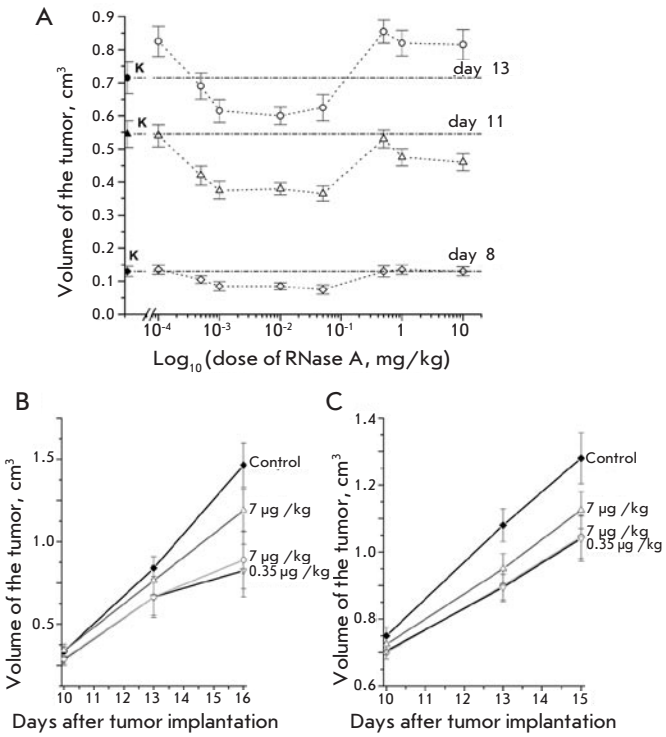

Fig. 1. Antitumor effect of RNAse A. **A.** The effect of RNase A on the growth of a primary LLC tumor in C57Bl/6J mice (concentration dependence). B. The effect of RNase A in 0.35, 0.7 and 7 μg/kg dosages on the growth rate of a primary LLC tumor in C57Bl/6J mice. **C.** The effect of RNase A in 0.35, 0.7 and 7 μg /kg dosages on the growth rate of a primary HA-1 tumor in A/Sn mice.

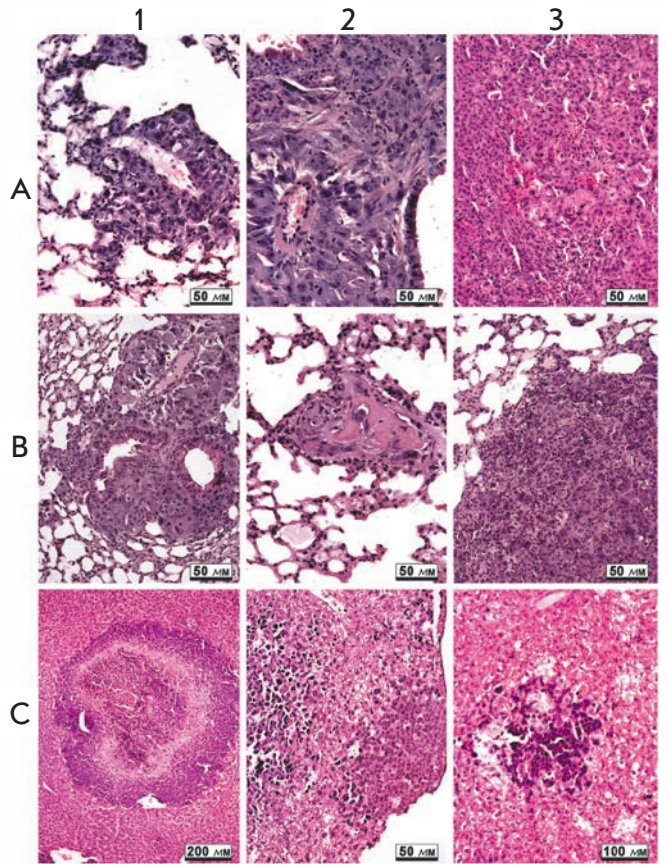

Fig. 2. A. Metastases in the lungs of animals with LLC (A1 and A2) tumors and in the liver of animals with HA-1 (A3). B. Metastases in the lungs of animals with LLC tumors after treatment with DNase I (0.12 mg/kg) (B1) and RNase A (0.7 μg/kg) (B2 and B3). **C.** Metastasis in the liver of animals with HA-1 tumors after treatment with DNase I (0.02 mg/kg) (C1), DNase I (1.2 mg/kg) (C2) and RNase A (0.35 μg/kg) (C3)

RNase A showed an insignificant difference between the groups at the initial stage of treatment (day 10 after tumor transplantation) (Figs. 1B, 1C). On day 15 the tumor size in the groups of animals with HA-1 treated with RNase A at doses of 0.35 and 0.7 μg/kg was 23% less than that in control (Fig. 1C); in the groups of animals with LLC, it was 43% less (Fig. 1B). It is worth noting that the antitumor effect of RNase A on the LLC model did not depend on which day (4 or 8) the treatment began after implantation.

Intramuscular administration of DNase I to LLC-bearing C57Bl/6J mice and HA-1-bearing A/Sn mice. The antitumor potential of DNase I was evaluated on two tumor models, LLC and HA-1. Starting at day 8 after the implantation of LLC to C57Bl/6J mice and HA-1 to A/Sn mice, the animals were injected with DNase I at a dose ranging within 0.02–2.3 mg/kg. Measuring the tumor size showed that the injection of DNase I does not lead to the retardation of primary tumor growth.

EFFECTS OF RNASE A AND DNASE I ON METASTASIS DEVELOPMENT

The antimetastatic activities of RNase A and DNase I (their capability to decrease the number of metastases in target organs) were estimated from (1) a histological analysis of target organs (the lungs for LLC and liver for HA-1), (2) a microscopic examination of metastasis number in the lungs of LLC-bearing animals, and (3) the liver weight alteration (hepatic index) in animals with HA-1.

A HISTOLOGICAL ANALYSIS OF METASTASES IN THE LUNGS OF ANIMALS WITH LLC AND IN THE LIVER OF ANIMALS WITH HA-1

Metastasis formation in the pulmonary tissue is a characteristic feature of LLC. Distinct metastases and multiple groups of tumor cells were observed in the lungs of control mice (Figs. 2A1, 2A2). Metastases of different sizes and irregular shapes were predominantly localized in the subpleural area. Some signs of mononuclear infiltration were observed in large metastases extending over several bronchi and large vessels (Fig. 2A1). Surface metastases were composed of two or three layers of tumor cells expanding along the pleura.

The development of heavy metastases in the liver is a characteristic feature of HA-1 progression. A multitude of

metastases of different sizes are found on histological sections of liver tissue (Fig. 2A3). We have revealed several morphologic types of metastases, such as (1) distinctly bordered metastases with pseudoglandular structures at the periphery of basophilic cells with pale densely packed cells at the center; (2) loose accumulations of basophilic oncocytes under hepatic capsule, and (3) small loose aggregations composed of dark basophilic oncocytes. Numerous mitoses in metastases, individual disseminated tumor cells, the lymphocyte infiltration of liver parenchyma, and dystrophic changes and necroses of hepatocytes were observed in liver tissue of mice with HA-1 (Fig. 2A3).

A HISTOLOGICAL ANALYSIS OF METASTASES IN THE LUNGS OF ANIMALS WITH LLC AND IN THE LIVER OF ANIMALS WITH HA-1 TREATED WITH ENZYMES

The administration of RNase A or DNase I to animals with LLC induced dystrophic changes in metastases in the lungs (Fig. 2B). The morphologic parameters of these changes were identical in all groups irrespective of the dose: an increase in the number of necroses and apoptoses, a dystrophic transformation of oncocytes, and a considerable mononuclear infiltration of tumor extravasates and metastases (Fig. 2B, *1–3*).

A histological analysis of the metastases in the liver tissue of mice with HA-1 treated with RNase A or DNase I at different doses has shown clear morphologic changes with similar features. Both central and perifocal necroses, tissue edema, numerous hemorrhages, and clear mononuclear infiltration were observed in metastatic foci (Fig. 2C, *1–3*). It should be noted that, unlike control animals, in which tumor infiltrates were found in the myocardium and kidney, metastases were not found in these organs of mice with HA-1 treated with the enzymes.

The state of immunity organs of animals with HA-1 also came under our notice. In particular, we observed some signs of the accidental involution of thymus, such as an increase in the amount of lymphocytes in the medulla or even an inversion of the thymus layers. Similar alterations suggesting for expressed antigenic stimulation were found in the spleen. The degree of manifestation of these signs of antigenic stimulation correlated with the enzyme dose.

Thus, a comparison between control animals with LLC or HA-1 and experimental ones treated with RNase A or DNase I has shown signs of induced pathomorphism of metastases manifested as the expressed dystrophic involution of tumor cells and an intensification of mononuclear infiltration.

COUNTING METASTASES IN THE LUNGS OF MICE WITH LLC FOLLOWING TREATMENT WITH ENZYMES

A microscopic examination of metastases on the surface of the LLC-bearing mouse lungs has shown that treating these animals with enzymes leads to a significant decrease in the metastasis number. The average number of metastases in groups of LLC-bearing mice treated with RNase A at doses of 0.5 μg/kg, 0.7 μg/kg, and 10 mg/kg were 14 ± 3, 15 ± 4, and 18 ± 4, respectively. The average number of metastases in groups of LLC-bearing mice treated with DNase I at doses of 0.02, 0.12, and 2.3 mg/kg were 10 ± 4, 16 ± 7, and 18 ± 4, respectively, whereas in the untreated animal group this amount was 30 ± 5. Thus, the observed amount of metastases

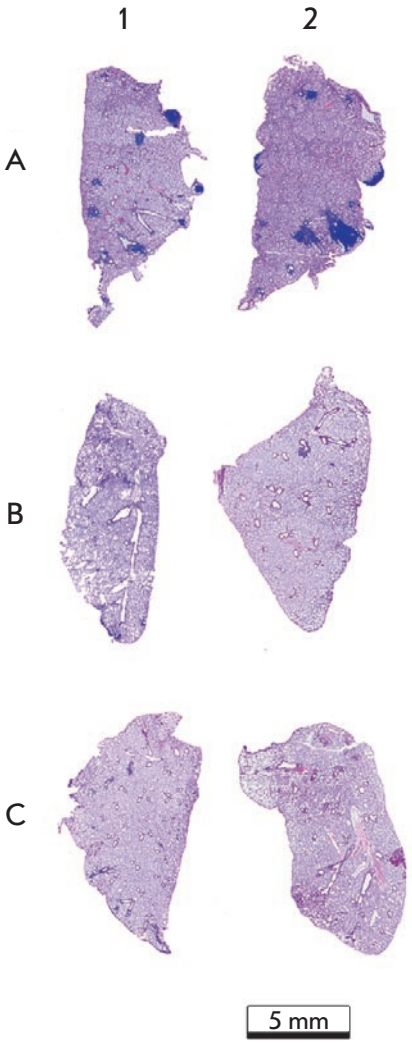

Fig. 3. Histotopogramme of the lung lobes of C57Bl/6 mice with LLC. A. Animals which received injections of normal saline solution B. Animals which received injections of DNase I at a dosage of 0.02 mg/kg. C. Animals which received injections of RNase A at a dosage of 0.7 μg/kg. Stained by hematoxylin and eosine.

in groups of LLC-bearing mice treated with the enzymes was two- to threefold less than in the control.

An analysis of metastases in the lungs of LLC-bearing animals has shown not only morphologic changes and a decrease in their amount following treatment with the enzymes, but also an existential reduction of the metastasis area and the altered localization in the organ. Fig. 3 shows the lungs of LLC-bearing animals without treatment (Fig. 3A) and after treatment with the enzymes (Figs. 3B, 3C). The decrease in both the amounts of metastatic foci and the area of metastases is plain to see.

ESTIMATION OF THE THERAPEUTIC EFFICACY OF ENZYMES IN THE TREATMENT OF ANIMALS WITH HA-1

The diffuse boundaries of metastatic foci in hepatic parenchyma made it impossible to use microscopy for counting metastases in the liver of animals bearing HA-1. Since the liver increases in weight during the metastasis development, we used the hepatic index (HI) reflecting disease severity and calculated as HI = (liver weight/body weight) × 100% to estimate the antimetastatic effects of the enzymes: the relative

Table 1. Hepatic index (HI), average liver increment (ALI), and treatment efficiency (TE) of the A/Sn mice bearing HA-1

	Control	Healthy mice	RNase A, μg/kg			DNase I, mg/kg			
			0.35	0.7	7	0.02	0.23	0.12	2.3
[1]HI, %	6.7±0.3	4.5	5.9±0.2	6.0±0.2	5.9±0.2	5.5±0.3	5.8±0.2	5.6±0.3	5.7±0.2
[2]ALI, %	2.2	–	1.3	1.5	1.4	1.0	1.3	1.1	1.2
[3]TE, %	0	–	42	30	38	53	40	52	46

[1] HI = (liver weight/mouse weight) × 100%;
[2] ALI (%) = $HI_{experiment} - HI_{healthy} = 4.5\%$; and
[3] TE (%) = $100 - ALI_{treatment} / ALI_{control} \times 100$.

HI reduction in a group of treated animals compared to the control group served as the criterion of the therapeutic efficacy (TE). The data on the average liver increment (ALI) of animals with HA-1 compared to that of the healthy ones were used to estimate TE (Table 1). A noticeable decrease in HI in HA-1-bearing animals treated with the enzymes was observed relatively to the control. The TE value varied from 30% to 42% in HA-1-bearing animals treated with RNase A and from 40% to 53% in those treated with DNase I.

DISCUSSION

As was mentioned in the Introduction, the largest representative of the RNase A family, pancreatic RNase A, demonstrated weak antitumor activity at high doses (above 10 mg/kg) [14, 15] and DNase I was capable of metastasis growth suppression [21, 22].

In this work, we studied both antitumor and antimetastatic activities of RNase A in vivo using doses ranging from 0.1 μg/kg to 10 mg/kg. We have shown that the intramuscular administration of RNase A at doses ranging within 0.5–50 μg/kg leads to the retardation of primary tumor growth by 20–40% with a more pronounced effect at early stages of tumor development (on the 8th day). Doses above 0.5 mg/kg, RNase A did not affect the tumor growth, which conforms to the previously reported data of other authors [17, 25]. The administration of DNase I at a dose in the range of 0.02–2.3 mg/kg did not result in any retardation of the primary tumor growth. We found that the intramuscular administration of any of these enzymes led to a considerable (two- to threefold) decrease in both the amount and size of metastases in the lungs of animals with LLC. In the case of hepatoma HA-1, the intramuscular administration of either RNase A or DNase I led to a decrease in the liver weight relatively to the control, with a therapeutic efficacy of 30–42% for RNase A and 40–53% for DNase I. A histological analysis of the lungs and liver has shown that both enzymes similarly destroy tumor cells and increase the number of necroses and apoptoses in metastatic foci. Our data make it possible for us conclude that both enzymes have high antimetastatic activity.

Yet there is no commonly accepted mechanism of antitumor activity for ribonucleases. The antitumor effect of RNase A that we observed can occur due to (1) the degradation of encoding intracellular RNAs and, as consequence, (a) the arrest of protein synthesis [26, 27] and (b) the alteration of gene expression profile via RNA cleavage products [28]; (2) the degradation of noncoding RNAs (pre-miRNAs, miRNAs, and siRNAs) [2, 29]; (3) the destabilization of the RNA structure [30]; (4) the blockage of RNA functions [31]; (5) the influence on signaling pathways [32–34], and (6) the cutoff of uncontrolled potassium influx via calcium-dependent potassium channels of tumor cells [35]. Also, one cannot exclude other as of yet unknown mechanisms.

We hypothesize that the antimetastatic effects of RNase A and DNase I, as well as the antitumor effect of RNase A, are associated with main function of these enzymes (the nucleic acid cleavage). Nevertheless, we cannot claim definitively that the antitumor effect of RNase A happens via the degradation of tumor intracellular RNAs, because a great pool of data univocally evidences for the binding of the enzyme penetrating into the cell with the ribonuclease inhibitor [17].

Putative targets for RNase A are RNAs circulating in blood plasma, including pre-miRNAs and miRNAs implicated in the control of oncogenesis and invasion [3, 36, 37]. The expression of most miRNAs implicated in the control of tumor-specific genes is known to be disordered [38, 39]. In particular, the elevation of miR-9 expression in breast cancer leads to a decrease in the E-cadherin level and invasion enhancement [40]. It was shown that the level of miR-184 possessing a stimulatory effect on the antiapoptotic and proliferative potential of tumor cells is increased in the plasma of patients with squamous cell carcinoma of th etongue [41]. Putative targets for DNase I are extracellular tumor-derived DNAs that, according to the genometastatic theory, are capable of the transfection of distant cells, thus providing metastatic progression in primarily unaffected organs [4].

Some small peptides show antitumor [42] and immunostimulating [43, 44] activities at extremely low doses; however, it is not really understood how they act. We cannot exclude that the antimetastatic effects of low doses of RNase A and DNase I that we found in this study might be associated with the formation of biogenic peptides due to the proteolysis of these enzymes in blood.

The disappearance of the antitumor activity of RNase A at doses above 0.5 mg/kg or upon prolonged administration (the observed decrease of antitumor effect on day 13 of tumor development) might be associated with the specific anti-RNase A antibody production. This suggestion is supported by signs of antigenic stimulation following the administration of RNase A: there is an increase in the number of lymphocytes in the medullar layer of thymus and in the spleen,

an inversion of thymus layers, and a mononuclear infiltration of metastatic foci.

CONCLUSIONS

We have shown that the intramuscular administration of RNase A or DNase I has a systemic effect on malignant tumors, which is manifested as a retardation of tumor growth (RNase A), a decrease in the amount and area of metastases, and destructive changes in metastatic foci (both enzymes). The most effective antimetastatic doses of the enzymes had no toxic effect on animals. Our data make it possible to recommend using RNase A and DNase I in the supplementary therapy of metastasizing tumors. ●

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Penicillin Acylase-Catalyzed Effective and Stereoselective Acylation of 1-phenylethylamine in Aqueous Medium using Non-Activated Acyl Donor

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ABSTRACT **Until recently the biocatalytic preparation of enantiomerically pure amines was based on stereoselective acyl transfer in an organic medium using activated acyl donors. The possibility of performing an effective and enantioselective enzymatic acylation of amines in an aqueous medium without using activated acyl donors was demonstrated for the first time as the example of direct condensation of phenylacetic acid and racemic 1-phenylethylamine. Direct condensation of the acid and the amine took place at mild reaction conditions with a high initial rate (3.3 μmole/(l·h)), degree of conversion (80% acylation of active amine enantiomer), and enantioselectivity (enantiomeric excess of the product was more than 95%). The suggested approach has remarkable advantages compared to enzymatic reactions in organic media and is of practical value for the biocatalytic preparation of enantiomerically pure compounds at mild conditions using readily available reagents.**

KEYWORDS **stereoselective enzymatic acylation in aqueous medium, direct condensation, enantiomerically pure compounds, penicillin acylase**

INTRODUCTION

Since enzymes demonstrate such unique characteristics as chemo-, regio- and stereospecificity, biocatalytic processes have significant advantages as compared to traditional organic synthesis. The advantages of enzymatic technologies are especially noticeable during the synthesis of multifunctional or enantiomerically pure compounds. Experts relate further possibilities of growth in the enantiomerically pure compound market (which has shown an annual growth rate of more than 13% in the last decade [2]) mainly to the introduction of biocatalytic technologies [1]. The synthesis of enantiomerically pure amines is of especial interest, since these compounds are important chiral synthones in the pharmaceutical and agrochemical industries [3].

Currently, most of the industrial processes involving biocatalysis are based on the hydrolysis of amides and esters, although synthetic reactions are often of more practical use [4]. This is based on the fact that in the most favorable aqueous medium for biocatalytic processes the equilibrium of the reaction is shifted towards hydrolysis. In order to shift the equilibrium towards the formation of amide and ester bonds, most sources in the literature suggest to perform the reactions in various organic solvents [5]. For a long time, enzymatic reactions in organic media have been considered to be the most promising approach for these synthetic reactions [6–9]. Therefore, researchers focused their attention on the use of enzymes that were stable in organic solvents. Until recently, the possibility of enzymatic acylation of amines had been demonstrated only in non-aqueous water-free organic media with the use of lipases as catalysts of the acyl transfer reaction from the activated acyl donors to the amines [10–12]. Even though the use of lipases has been fairly successful, biocatalysis in non-aqueous media has a number of complications, the most obvious being low enzymatic activity [13], as well as the necessity to control the use of ecologically toxic organic solvents, which constitute the bulk of pharmaceutical industrial waste [14].

A critical analysis of studies in the field of stereoselective biocatalytic transformations has indicated that at sufficient optimization the enzymatic synthesis in aqueous media could be much more effective than was previously thought. Among other examples, this was demonstrated by the highly effective and stereoselective acylation of amines due to penicillin acylase-catalyzed acyl transfer in 100% aqueous solution [15]. This demonstration was made possible thanks to the unique catalytic traits of the penicillin acylase from *Alcaligenes faecalis* [16]; namely, its high catalytic activity and stability under alkaline conditions (pH ~ 10), which were optimal for the acylation of basic amines.

This report presents the first results of an approach for the effective and stereoselective enzymatic acylation of amines in an aqueous medium by direct condensation of an amine and a carboxylic acid, which is used as an acyl donor. An effective approach for the isolation of α-phenylethylamine enantiomers is of practical importance, since they are used as resolving agents and chiral auxiliaries during the production of enantiomers of various classes of chemical compounds [17, 18].

METHODS

Reagents. This study used phenylacetyl chloride (Sigma, USA); phenylacetic acid (Aldrich Chemie, Germany); (*R*)- and (*S*)-α-phenylethylamine (Fluka, Switzerland); phenylmethylsulphonylfluoride, sodium dodecyl sulphate (Merck, Germany); acetonitrile («CryoChrome», Russia); *N*-phenylacetyl derivatives of α-phenylethylamine were synthesized according to [15]; the penicillin acylase from *Alcaligenes faecalis* was supplied by the LLC Innovations and Higher Technologies of MSU (Russia). The concentration of penicillin acylase active sites was determined according to [16].

HPLC analysis. Concentrations of the reaction mixture components and the enantiomeric purity of the synthetic reaction product (*N*-phenylacetyl-(*R*)-α-phenylethylamine) were measured by HPLC using a Perkin Elmer Series 200 (Perkin Elmer, USA) equipment as described earlier [15].

The direct enzymatic condensation of phenylacetic acid and (±)-α-phenylethylamine was performed with equimolar amounts of reagents (0.2 M), which were constantly mixed in a thermostated cell of a pH-stat (Titrino 718, Metrohm, Switzerland) at pH 7.5 and 15 °C; the penicillin acylase concentration was 12 μM. The reaction product, *N*-phenylacetyl-(*R*)-phenyletylamine, precipitated in the course of enzymatic reaction. Aliquots (50 μl) of the heterogeneous reaction mixture were added to 1.95 ml of the mobile phase in order to fully solubilize the reaction components and to stop the reaction. These samples were then diluted with eluent and analyzed using standard and chiral HPLC. The enzymatic reaction continued until it reached an equilibrium state; that is until the concentrations of the reaction components reached fairly constant values. The reaction product was then filtered onto a glass filter, washed with water, recrystallized from aqueous ethanol, and then dried in a desiccator over a layer of calcium chloride. Yield 0.145 g, (38%); e.e. 95%; mp 117-118°C; 1H NMR (250 MHz, CDCl3): 1.29 (d, 3H, CH3), 3.47 (s, 2H, CH2), 5.01 (m, 1H, CH), 5.49 (d, 1H, NH), 7.04-7.29 (m, 10H, Ph). MS m/z: 239 (62, M), 120 (49, PhCH2CH(NH)CH3), 105 (100, PhCCH3), 91 (75, PhCH2), 77 (68, Ph), 65.

RESULTS AND DISCUSSION

Stereoselective acylation is the key step in the resolution of racemic amines, and the use of enzymes as catalysts of this process seems to be a very promising approach. However, it is worth noting that primary amines are strongly basic compounds, and that they can be effectively acylated only in alkaline aqueous solutions (pH approximately 10) or a water-free organic medium, where the majority of enzymes exhibit diminished catalytic activity and low stability. Also, conducting enzymatic reactions in organic solvents requires the use of activated acyl donors, which can spontaneously acylate reactive amino groups in a non-stereoselective manner, thus lowering the enantiomeric purity of the reaction product [3, 9]. We suppose that these drawbacks can be circumvented by conducting the enzymatic acylation of amines in an aqueous medium using the direct condensation of the carboxylic acid and the amine. In this case, thermodynamically favorable conditions for the condensation reaction are achieved in a practically neutral medium (pH approximately 6–8), where most enzymes are highly active and stable. Both reacting substrates are highly soluble and stable at these conditions, which allow the use of highly concentrated solutions of the reagents and creates favorable thermodynamics for enzymatic amine acylation. The driving force of the reaction, which makes the whole process so efficient, is the shift of equilibrium towards synthesis, caused by the precipitation of the poorly soluble reaction product. Notably, direct condensation does not require activation of the acyl donor, which simplifies the enzymatic amine acylation process and reduces costs. The wide substrate specificity and stereoselectivity of penicillin acylases towards N-acylated derivatives of amino compoun-

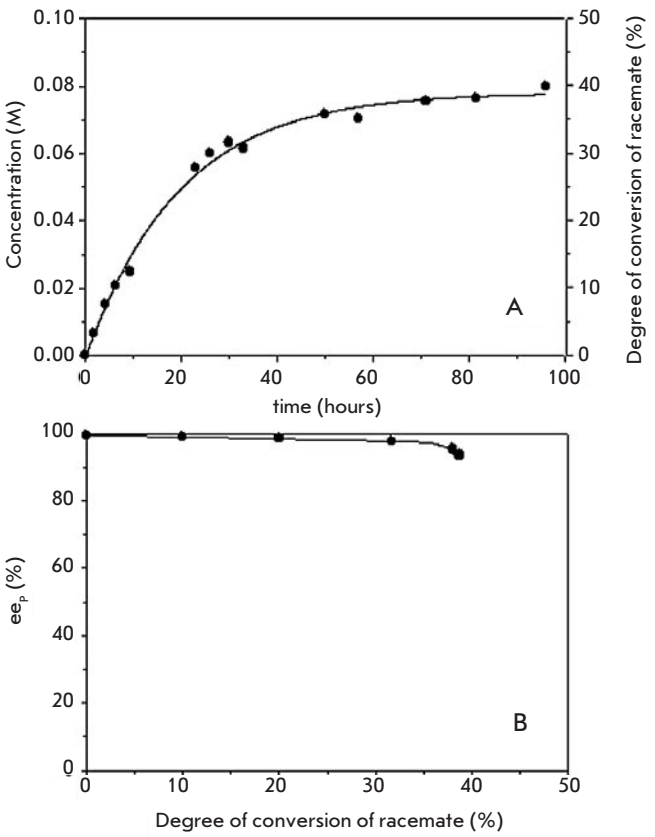

Fig. 1. Synthesis of enantiomerically pure *N*-phenylacetyl-(*R*)-phenylethylamine using direct stereoselective condensation of phenylacetic acid and a (±)-α-phenylethylamine racemic mixture in an aqueous medium catalyzed by penicillin acylase from *Alcaligenes faecalis*: (A) – The integral kinetics of product formation, (B) – Enantiomeric excess of the target product at several degrees of conversion. The conditions are described in the Methods section

ds [16, 19, 20] provides reason to hope that enzymatic amine acylation via direct condensation and using this family of enzymes will be effective.

Indeed, the first experiments show that the condensation of phenylacetic acid with a racemic α-phenylethylamine catalyzed by penicillin acylase in an aqueous medium is highly effective, with an initial rate of 3.3 mmol/(l·h). Only a few minutes after the beginning of the reaction, the target product (*N*-phenylacetyl-(*R*)-phenylethylamine) starts precipitating. The synthesis progresses quite rapidly until it reaches a 30–35 % degree of conversion of the initial reagents' concentrations (Fig. 1, A). The condensation reaction then slows down, which is probably caused by the thermodynamic equilibrium being achieved. This is confirmed by the control experiment, which shows that the enzyme is not inactivated at this point, and that the penicillin acylase retains virtually all of its initial catalytic activity.

The analysis of the enantiomeric purity of the target product at different stages of conversion validates (Fig. 1, B) a high stereoselectivity of enzymatic acylation. The enantiomeric excess of the target product is 98% and 96% at conversion degrees of 30% and 40%, respectively. After the reaction stops, the precipitated *N*-phenylacetyl-(*R*)-phenylethylamine can be easily isolated from the reaction mixture. The yield of active enantiomer of *N*-phenylacetyl-(*R*)-phenylethylamine is 80 %, and the enantiomeric excess is 95 %.

The amine acylation method described here has none of the drawbacks of enzymatic reactions conducted in organic solvents, and it is of practical use for the biocatalytic production of enantiomerically pure compounds under mild conditions out of readily available reagents. In order to assess the perspectives of this method, it is necessary to compare it with enzymatic acylation of amines in an aqueous medium using activated acyl donors, as proposed previously [15]. Both approaches have their advantages and drawbacks. The use of activated acyl donors provides a high reaction rate and depth of acylation, as well as a high enantiomeric purity of the target product. However, it involves the use of more expensive acyl donors and takes place in an alkaline medium, which makes the enzyme less stable. The most important advantage of the direct condensation method is the possibility of using inexpensive acyl donors and milder reaction conditions, in which most enzymes, including the whole penicillin acylase family, are more active and stable. These advantages may prove decisive for the use of direct condensation during preparative resolution of a wide range of racemic amines.

The results presented in this report are just the first observations, and the process of enzymatic amine acylation by direct condensation should be further studied and optimized. It is likely that stereoselective enzymatic acylation of amino compounds by direct condensation will become an important integral part of the general biocatalytic method for preparation of enantiomerically pure amino compounds [21]. In further research, we plan a detailed study of the various factors affecting the equilibrium of the reaction and a careful analysis of the kinetics of penicillin acylase-catalyzed stereoselective acylation reactions by direct condensation in an aqueous medium.

CONCLUSION

This is the first report on the possibility of effective and enantioselective enzymatic acylation of primary amines in an aqueous medium without the use of activated acyl donors. Direct condensation catalyzed by penicillin acylase is highly efficient, and the target product of high enantiomeric purity can be easily isolated from the reaction mixture with high yield. This method can be used for preparative biocatalytic synthesis of enantiomerically pure amines under mild reaction conditions using readily available substrates and a biocatalyst. ●

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Assessment of Formate Dehydrogenase Stress Stability *in vivo* using Inactivation by Hydrogen Peroxide

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ABSTRACT **Kinetic studies on hydrogen peroxide-induced inactivation of mutant formate dehydrogenase from *Pseudomonas sp.* 101 (PseFDH Cys255Ala) suggest a simple bimolecular mechanism for enzyme reaction with the inactivation agent. In the excess of hydrogen peroxide, the decrease in enzyme activity follows first-order kinetics. Therefore, the first-order effective inactivation kinetic constants determined for various FDH forms at a constant H_2O_2 concentration can be used as a quantitative measure of the enzyme stability. It was shown that two cysteine residues located in the active site formate- and coenzyme-binding domains (Cys145 and Cys255, respectively) make similar contributions to the enzyme stability, while the contribution of Cys354 is insignificant. The inactivation kinetics of wild-type PseFDH, mutant PseFDH Cys145Ser/Cys255Ala, and FDH produced under stress conditions by bacterium *Staphylococcus aureus*,** higher plants ***Arabidopsis thaliana*,** and soya ***Glycine max*, was studied. It was found that the stress-induced FDHs are at least 20 times more stable than the nonstress-induced PseFDH from *Pseudomonas* sp. 101 grown on methanol.**
KEYWORDS **formate dehydrogenase, hydrogen peroxide, inactivation, stress, mutant enzyme.**

INTRODUCTION

Formate dehydrogenase (EC 1.2.1.2, FDH), a NAD^+-dependent enzyme, catalyses oxidation of formate to carbon dioxide coupled to NAD^+ reduction into NADH.

$$NAD^+ + HCOO^- \rightarrow NADH + CO_2\uparrow$$

This is one of the key reactions providing the cell with NADH that is subsequently used for ATP synthesis. Formate dehydrogenases are very common in nature. They are present in various bacteria, such as methylotrophic and symbiotic nitrogen-fixing bacteria [1]. In addition, FDH genes are found in many pathogenic microorganisms, including bacteria (*Staphylococcus aureus*, *Mycobacterium avium subsp. paratuberculosis str.* k10, various strains of *Bordetella* and *Legionella pneumophila*, *Francisella tularensis subsp. tularensis* SCHU S4) and microfungi (*Histoplasma capsulatum*, *Cryptococcus neoformans var. neoformans* JEC21, etc.) [1]. Formate dehydrogenases are also present in various yeasts, nonpathogenic microfungi, mosses, and plants. These enzymes act as stress proteins in pathogenic microorganisms and plants, where the FDH content increases more than ten-fold under stress [2].

The FDH reaction mechanism is of great interest as well as its physiological role. The enzyme belongs to the D-2-hydroxy-acid dehydrogenase superfamily [3]. The substrate structure, the formate ion, is the simplest among those of D-2-hydroxy-acid dehydrogenase substrates. This fact, along with the absence of an acid-base catalysis stage in the FDH enzyme cycle, makes FDH a model enzyme for the entire D-2-hydroxy-acid dehydrogenase superfamily.

FDH is extensively used in fine organic synthesis, being an ideal catalyst for cofactor regeneration. A number of processes to synthesise optically active compounds have been developed using NAD(P)H regeneration catalysed by FDH [4–7]; for example, Evonik (formerly Degussa) annually produces hundreds of tons of L-tert-leucine by this method [8]. Therefore, it is important to develop new FDH-based catalysts that can be stable not only in water solutions, but also in more aggressive media.

There are three main factors contributing to the overall FDH stability. The first one is the enzyme thermal stability, i.e. its ability to remain active at elevated temperatures.

Thermal inactivation of enzymes usually occurs via protein denaturation. The thermal stability of formate dehydrogenases of different origins varies broadly. Thus, FDH from soybeans cloned in our laboratory [9], as well as FDH from bakery yeast, rapidly becomes inactive even at 45–46 ˚C. At the same time, FDH cloned from bacteria *Pseudomonas* sp.101 and *Staphylococcus aureus*, as well as from plants *Arabidopsis thaliana*, demonstrates good stability at 60–65 ˚C.

The second factor that determines the overall enzyme stability is its chemical stability, i.e. its ability to remain active in the presence of chemicals that modify protein amino acid residues, such as those located in the enzyme active site or responsible for the stabilization of the tertiary and quaternary structures. Cysteine residues are the most critical residues for formate dehydrogenases, since in almost all FDHs they play a significant role in the enzyme activity. In most cases, enzyme inactivation below 40 ˚C is caused either by chemical modification or oxidation of cysteine thiol groups.

The third factor particularly important for protein storage, is the enzyme stability in the presence of proteases. Even the lowest levels of protease impurities (down to 0.001%) may cause a complete loss of enzyme activity during storage. No systematic studies on this issue have been conducted, although it has been shown that FDH from *Pseudomonas* sp.101 does not degrade under the action of *E. coli* proteases upon cultivation of the producer strain for 72 hours and longer.

Thermal stability of FDH has been well studied and described in the literature, even though information on the chemical stability of FDH remains rather scarce. The chemical-stability experiments have been performed on only a few enzymes, such as FDH from bacteria *Pseudomonas* sp.101 [10, 11] and *Mycobacterium vaccae* N10 [12] and yeast *Candida boidinii* [13]. The choice of a method to assess the enzyme chemical stability is very important. Several agents have been used to inactivate FDH, including Hg^{2+} and Cu^{2+} ions and specific reagents for cysteine thiol groups, such as 5,5'-Dithio-Bis(2-Nitrobenzoate) (DTNB) and p-chloro mercury benzoate. However, no clear correlation between the chemical stability and the structure of the modifying agents has been established.

In this work, we chose hydrogen peroxide as an inactivation agent. This choice was dictated by the following reasons:

1. Hydrogen peroxide is a small molecule and can oxidise both surface thiols and those inside the protein globule. Bulky agents conventionally used for thiol modification, such as p-chloro mercury benzoate and DTNB, for steric reasons, first rapidly react with the highly active Cys255, and then with other unidentified Cys residues, with the more than ten times slower rate [10].

2. Hydrogen peroxide is a *naturally occurring* inactivating and signalling agent. The natural origin and small size of hydrogen peroxide make it a good chemical agent for the assessment of FDH stability *in vivo*.

3. The concentration of hydrogen peroxide in the cell increases under stress conditions. Sometimes, the FDH concentration in the cell increases dramatically under stress, too. For instance, in plants, FDH is located in mitochondria, and under stress the enzyme content in the organelle can reach 9% of the total protein [14]. FDH from pathogenic *Staphylococcus aureus* is likewise a stress protein. In *S. aureus* biofilms, the concentration of FDH mRNA ranks third among all m-RNAs in the cell. Under these conditions, the level of FDH biosynthesis is 20 times higher than that in the bacteria growing as plankton [15]. These facts suggest that FDH induced under stress may be more stable in the presence of hydrogen peroxide than FDH synthesized under normal conditions; like FDH from *Pseudomonas* sp.101 grown on methanol.

The aim of this study was to compare the stability of wild-type FDH from various sources in the presence of hydrogen peroxide as an inactivating agent and to investigate the role of certain Cys residues in the chemical stability of FDH from methylotrophic bacterium *Pseudomonas* sp.101. The experimental plan consisted of the following tasks:

1) Study the kinetics of inactivation of a model enzyme in the presence of different concentrations of hydrogen peroxide and determine its kinetic mechanism;

2) Investigate the role played by certain Cys residues in the chemical stability of FDH from *Pseudomonas* sp.101; namely, Cys 145 in the formate-binding domain of the active site, Cys 255 in the coenzyme-binding domain located on the protein surface, and Cys 354 located outside the active site, on the protein surface;

3) Compare the chemical stability of FDH from bacteriun *Pseudomonas* sp.101 (enzyme synthesised in stress-free conditions) and *S. aureus* bacteria, plants *A. thaliana* and soya *Glicyne max* (enzymes overproduced under stress).

EXPERIMENTAL

The preparations of recombinant formate dehydrogenase from bacterium *Pseudomonas* sp.101 (PseFDH) and its mutant forms with a single substitution Cys255Ala and double substitutions Cys145Ser/Cys255Ala and Cys255Ala/Cys354Ser, as well as recombinant wild-type FDH from bacterium *S. aureus* (SauFDH), plants *A. thaliana* (AraFDH) and soybeans *G. max* (SoyFDH), were kindly provided by Innovations and High Technologies MSU Ltd. (http://www.innotech-msu.com). All preparations were of 97–98% purity or higher as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Determination of FDH activity. FDH activity was determined spectrophotometrically by NADH absorption at 340 nm (ε_{340} = 6220 $M^{-1}cm^{-1}$, Schimadzu UV 1601PC instrument, 30 ˚C, 0.1 M sodium phosphate buffer, pH 7.0). NAD^+ and sodium formate were added at saturating concentrations of 1.5 mM and 0.3 M, respectively.

Inactivation of recombinant FDH by hydrogen peroxide. Inactivation of FDH by hydrogen peroxide was performed in a 0.1 M sodium phosphate buffer, 0.01 M EDTA, at pH 7.0 and 25 ˚C. The hydrogen peroxide concentration was determined by absorption measurements at 240 nm (ε_{240} = 43.6 $M^{-1}cm^{-1}$). For the experiments, 0.3 ml of H_2O_2 solution at varied fixed concentrations was added to 0.7 ml of FDH solution with activity of 2.5–4 units/ml. The temperature of the solutions was maintained at 25 °C prior to mixing. The mixture was stirred vigorously and placed into a thermostat maintained at 25 ±± 0.1 ˚C. At fixed time intervals, 20 μl samples were taken for residual activity measurements. Hydrogen peroxide working

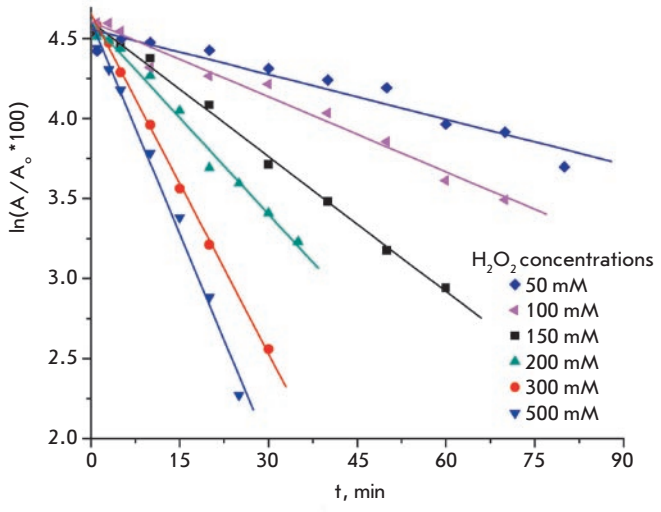

Fig. 1. Inactivation of mutant PseFDH Cys255Ala at different hydrogen peroxide concentrations (0.1 M sodium phosphate buffer, pH 7.0, 25 °C)

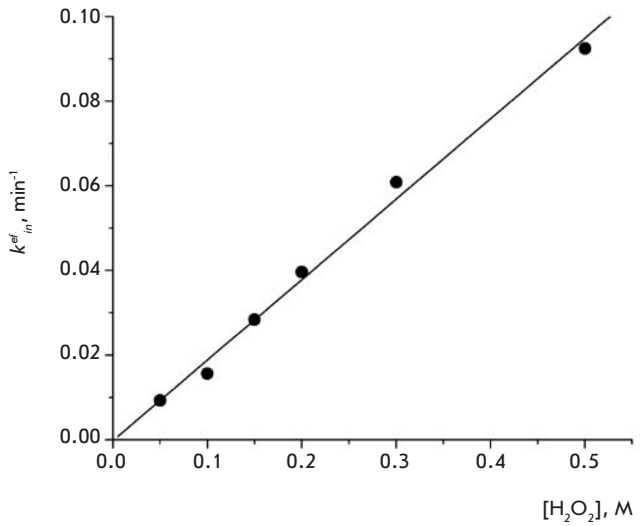

Fig. 2. Dependence of the effective kinetic constant (k^{ef}_{in}) for mutant PseFDH Cys255Ala on the hydrogen peroxide concentration (0.1 M sodium phosphate buffer, pH 7.0, 25 °C)

solutions were prepared by diluting a 33% H_2O_2 stock solution (9.1 mol) with bidistilled water.

RESULTS AND DISCUSSION

INACTIVATION OF MUTANT PSEFDH CYS255ALA BY HYDROGEN PEROXIDE AT VARIOUS CONCENTRATIONS

The mutant enzyme from *Pseudomonas* sp.101 PseFDH GAV was used to study the dependence of the FDH inactivation rate on hydrogen peroxide concentration. Two Cys residues at positions 145 and 255 are present in the PseFDH active site. Modification of either of these residues may lead to enzyme inactivation. It is possible that, by analogy with DTNB modification, hydrogen peroxide could demonstrate a different reactivity to the above residues, since Cys145 is located inside the active site, whereas Cys255 is on the surface of the protein globule and exposed to the solution. We were mostly interested in studying the contribution Cys145 into PseFDH inactivation by H_2O_2, therefore, a mutant PseFDH, with Cys255Ala substitution was used in these experiments.

We found an exponential decay dependence of the enzyme residual activity on time at all hydrogen peroxide concentrations used, as represented in linear semi-log graphs in *Fig. 1*. The effective kinetic constants of inactivation, k^{ef}_{in}, calculated from the slopes did not depend on the enzyme concentration.

Assuming a bimolecular mechanism for the reaction between the enzyme and H_2O_2,

$$FDH_{act} + H_2O_2 \rightarrow FDH_{in} + H_2O,$$

the reaction rate equation will look as follows:

$$v_{in} = k_{in} * [FDH_{act}] * [H_2O_2],$$

where $[FDH_{act}]$ and $[H_2O_2]$ are the active enzyme and hydrogen peroxide instant concentrations. Taking into account that $[H_2O_2] >> [FDH_{act}]_0$, the decrease in the H_2O_2 concentration in the course of the reaction is negligibly small; hence, $[H_2O_2] \approx [H_2O_2]_0$. In this case, the enzyme inactivation kinetics will be of the first order, with the effective first order constant

$$k^{ef}_{in} = k_{in} * [H_2O_2]_0.$$

The true second-order constant of the inactivation kinetics can be derived from the dependence of the effective constant (k^{ef}_{in}) on the hydrogen peroxide concentration. Indeed, k^{ef}_{in} exhibits a linear dependence on the H_2O_2 concentration (*Fig. 2*). We obtained $(3.17 \pm 0.14)*10^{-3}$ $M^{-1}s^{-1}$ as the value of a bimolecular inactivation kinetics constant for PseFDH Cys255Ala.

Thus, our results show that the enzyme's inactivation is a bimolecular reaction, and that the enzyme directly reacts with hydrogen peroxide with no intermediates formed. In the excess of hydrogen peroxide, the inactivation shows first-order kinetics; therefore, the effective first-order constant (k^{ef}_{in}) should not depend on the enzyme's concentration (this has been confirmed experimentally). This allows us to use k^{ef}_{in} as a quantitative measure of FDH stability in experiments with FDH from different sources (or different mutant forms) at a constant H_2O_2 concentration. In all subsequent experiments, 0.15 M H_2O_2 was used.

INACTIVATION OF MUTANT PseFDHs BY HYDROGEN PEROXIDE

The results of hydrogen peroxide inactivation of the wild-type and three mutant FDH enzymes from *Pseudomonas* sp.101 with Cys substituted at various positions are presented in *Fig. 3*. We had previously prepared a number of mutant PseFDH enzymes with various substitutions for Cys 145, 255, and 354. For this study, we selected only those mutants that had demonstrated the best kinetic behaviour.

The hydrogen peroxide inactivation of all FDH forms studied showed first-order kinetics. As we expected, the wild-

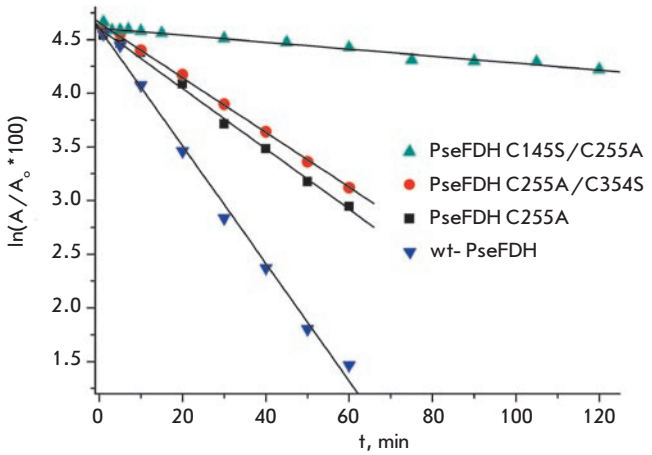

Fig. 3. Inactivation by hydrogen peroxide of the wild-type PseFDH (wt-PseFDH) and its various mutant forms (0.15 M H_2O_2, 0.1 M sodium phosphate buffer, pH 7.0, 25 °C)

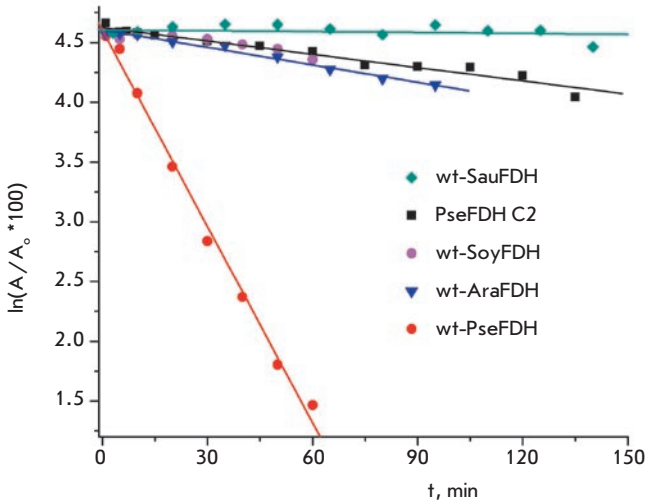

Fig. 4. Inactivation by hydrogen peroxide of formate dehydrogenases from different sources. wt-SauFDH, wt-PseFDH, wt-SoyFDH, and wt-AraFDH are wild-type recombinant FDHs from bacteria *S.aureus* and *Pseudomonas* sp.101, soya *Glycine max* and plant *A.thaliana*, respectively. PseFDH C2 is mutant PseFDH Cys145Ser/Cys255Ala (0.15 M H_2O_2, 0.1 M phosphate buffer, pH 7.0, 25 °C).

type enzyme was the least stable (*Fig. 3*). The substitution of Cys255 with Ala reduces the inactivation constant by a factor of two (k^{ef}_{in} = 9.13*10^{-4} and 4.69*10^{-4} s^{-1}, respectively). In the other mutant PseFDH, the Cys354Ser substitution was added to Cys255Ala. The substitution of Cys354 with Ser had only a negligible effect on the enzyme stability towards H_2O_2, a confirmation of our conclusion that the Cys354 residue played no significant role in the enzyme activity [1]. The highest stability was demonstrated by the mutant PseFDH with substitutions in the enzyme active site, Cys145Ser and Cys255Ala.

The comparison of the effective inactivation kinetic constants (k^{ef}_{in}) for the wild-type PseFDH and mutant Cys255Ala and Cys145Ser/Cys255Ala PseFDHs (*Table 1*) provides an estimate for the contribution of each of these residues into the enzyme stability. The contribution of the Cys255 residue to the value of k^{ef}_{in} is 4.40*10^{-5} s^{-1} calculated as the difference between the k^{ef}_{in} values for the wild-type and Cys255Ala PseFDH. The contribution of the Cys145 residue to the value of k^{ef}_{in} is 4.17*10^{-5} s^{-1} calculated as the difference between the k^{ef}_{in} values for Cys255Ala and Cys145Ser/Cys255Ala PseFDHs. These calculations indicate the similar reactivity of these two residues towards hydrogen peroxide, despite their different locations in the protein. As mentioned above, the difference between the reactivities of the Cys145 and Cys255 residues was at least 2–3 orders of magnitude when DTNB was used as a modifying agent [10].

Table 1. Effective first-order kinetic constants for hydrogen peroxide inactivation of the wild-type and mutant PseFDHs (0.15 M hydrogen peroxide, 0.1 M sodium phosphate buffer, pH 7.0)

Enzyme	Wild-type PseFDH	PseFDH C255A	PseFDH C255A/C354S	PseFDH C145S/C255A
k^{ef}_{in} * 10^{-5} (s^{-1})	91.3 ± 3.2	46.9 ± 1.2	42.5 ± 0.8	5.20 ± 0.37

INACTIVATION OF FDH FROM BACTERIA AND PLANTS BY HYDROGEN PEROXIDE

We studied the stability of FDH from various sources to inactivation by hydrogen peroxide. We selected three FDH enzymes, the biosynthesis of which increases sharply under stress conditions: SauFDH of bacterial origin and AraFDH and SoyFDH of higher plants origin (*Fig. 4*). The wild-type FDH from *Pseudomonas* sp.101 and its most stable to hydrogen peroxide inactivation Cys145Ser/Cys255Ala PseFDH mutant were used for reference. PseFDH is not a stress protein; its biosynthesis is induced in bacterium *Pseudomonas* sp.101 under grown on methanol. The stress-induced FDH of plant, as well as bacterial, origin show very high stability to inactivation induced by hydrogen peroxide (*Fig. 4*). They are much more stable than the wild-type PseFDH; only the best mutant, PseFDH Cys145Ser/Cys255Ala, has the stability comparable to those of FDH of plant origin. Although plant FDHs show almost identical stability to inactivation by hydrogen peroxide, they differ in their thermal stability by more than 5000 times [9]. FDH from pathogenic bacterium *S. aureus* was the most stable to inactivation by hydrogen peroxide. As shown in *Fig. 4*, the residual activity of this enzyme after 4 hours of incubation in the presence of 0.15M H_2O_2 was more than 90%. SauFDH also possesses high thermal stability, being second only to PseFDH among all known formate dehydrogenases.

The results obtained in inactivation experiments provide evidence for the activity loss upon treatment with hydrogen peroxide for all the enzymes studied, even those without Cys residues in the active sites, which inactivate more slowly (*Fig. 4*). The values of the effective inactivation kinetic constants for single mutant PseFDH Cys255Ala and double mu-

tant Cys255Ala/Cys354Ser (*Table 1*) suggest that, most likely, their activity decreases not due to the oxidation of the Cys residues located outside the enzyme active site, but rather because of the modification of other amino acid residues in the active site. This means that hydrogen peroxide is not a reagent specific for Cys residues in FDH. It is this nonspecificity of H_2O_2 that reveals the contribution of other amino acid residues into FDH stability; these residues upon oxidation result in a decreased enzymatic activity. Using the k^{ef}_{in} value, one can quantify the contribution of those residues into the enzyme chemical stability. The contribution of non-cysteine residues is 6 times lower in the case of FDH of plant origin than in the case of SauFDH (*Fig. 4*).

The higher stability of SauFDH to hydrogen peroxide-induced inactivation compared to that of FDH of plant origin correlates well with the stability requirements for these enzymes under stress *in vivo*. Plant cells can withstand much milder stress conditions for a much shorter period of time before dying than the conditions in which the *S. aureus* biofilms can survive. Apparently, the better stress resistance of *S. aureus* must be supported by the high stability of all cell components responsible for survival under stress, including FDH. Therefore, our results confirm the hypothesis put forward in the introduction, that FDHs upregulated under stress conditions should be more stable with respect to inactivation by hydrogen peroxide: the stronger the stress, the higher the stability. In conclusion, the experiments on the inactivation of purified FDH preparations by hydrogen peroxide can be used for comparative analysis of stability of formate dehydrogenases *in vivo*. ●

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Changes in the Proteasome Pool during Malignant Transformation of Mouse Liver Cells

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ABSTRACT **Multiple forms of proteasomes regulate cellular processes by destroying proteins or forming the peptides involved in those processes. Various pathologies, including carcinogenesis, are related to changes in functioning the proteasome forms. In this study, we looked at the changes in the pool of liver proteasomes during nodular regenerative hyperplasia and formation of adenoma and hepatocellular carcinoma in mice treated with Dipin, followed by partial liver resection. The relative content of various proteasome forms was determined using Western blot analysis. The chymotrypsin-like activity of proteasomes was assessed from the hydrolysis of the commercial Suc-LLVY-AMC substrate. It was found that changes in the proteasome pool appeared already during the formation of diffuse nodules, the changes being the increased expression of the X(β5) constitutive subunit and the LMP7(β5i) and LMP2(β1i) immune subunits, accompanied by the increase of the total proteasome pool and the decrease in the chymotrypsin-like activity. These changes were more pronounced in hepatocellular carcinoma. The content of the total proteasome pool and the LMP2(β1i) immune subunit and the chymotrypsin-like activity in adenoma were intermediate compared to those in the samples of liver with diffuse nodules and carcinoma. In addition, the level of the Rpt6 subunit present in the 19S proteasome activator was increased in carcinoma. Our results indicate that nodular regenerative hyperplasia and adenomatosis may be stages preceding carcinogenesis. We also conclude that there is a need to find signalling pathways that change the expression of various proteasome subunits during carcinogenesis. The 19S proteasome activator, which is overexpressed in malignant tumours, can be a promising target for the development of new anticancer drugs.**

KEYWORDS **immunoproteasomes, 19S proteasome activator, chymotrypsin-like activity of proteasomes, Western blot analysis, nodular regenerative hyperplasia of the liver, adenoma, hepatocellular carcinoma, mouse liver.**

ABBREVIATIONS **nNOS - neuronal nitric oxide synthase, dipin - 1,4-Bis-[N,N'-di(Ethylene)-phosphamide]piperazine, Suc-LLVY-AMC - N-succinyl-leu-leu-val-tyr7-amido-4-methyl coumarin, MG132 - Z-leucyl-leucyl-leucinal, mAb - monoclonal antibody, pAb - polyclonal antibody**

INTRODUCTION

Understanding the molecular mechanisms underlying the malignant transformation of cells is of ever vital importance. The new protein hydrolysis system discovered in the 1980s involving proteasomes and affecting all cellular processes provided a new impulse to the studies of the mechanisms of mammalian cell malignant transformation. Proteasomes, multisubunit multiproteinase protein complexes, are present in mammalian organs and tissues in a multitude of forms of different structures and physiological functions [1–4]. Proteasomes can be divided into two groups—constitutive proteasomes and immunoproteasomes—depending on the nature their active protease subunits. The constitutive proteasomes contain two of each of the X(β5), Y(β1) and Z(β2) subunits, possessing chymotrypsin-like, caspase-like, and trypsin-like activity, respectively. The immunoproteasomes contain the LMP7(β5i), LMP2(β1i), and LMP10(β2i) immune subunits instead of the above-mentioned protease active subunits of the constitutive proteasomes. When foreign proteins are hydrolysed by immunoproteasomes, the amount of antigen epitopes formed is several times higher. The antigen epitopes are capable of incorporating into the Bjorkman gap of the major histocompatibility complex class I molecules for further their presentation to T lymphocytes. In addition, immunoproteasomes participate in the regulation of the differentiation and proliferation of some cell populations, perhaps, by producing biologically active peptides [5, 6]. They are also an essential part of the signalling pathway responsible for the quenching of oxidative stress [7].

Both constitutive proteasomes and immunoproteasomes form 26S and 20S proteasome pools [3]. The 26S proteasomes consist of the 20S proteolytic core particle and one or two of the 19S regulatory particles responsible for binding to ubiquitinated target proteins, the unfolding of those proteins, and directing them into the proteolytic chamber. Thus, the 26S proteasomes regulate cellular processes by degrading proteins or forming the peptides involved in those processes.They also trigger the reactions associated with the T-cell immune

response. The 26S proteasomes are usually dependent on ATP and ubiquitin. The 20S proteasomes, on the contrary, degrade proteins and short peptides independently of ATP and ubiquitin. The number of proteins identified as substrates of 20S proteasomes increases every year; these include, for example, proteins with a damaged tertiary structure [8] and some virus proteins [9, 10].

The functions of proteasomes are very diverse, and determining the changes occurring in the proteasome pool during malignant cell transformation is important for understanding the transformation mechanism, as well as for finding new targets for anticancer therapy among the multiple forms of proteasomes. The scarce published data on this matter concern the comparison of separate proteasome form content in malignant and control cells [11–16]. Information on how the proteasome pool functions during the growth of nonmalignant and malignant tumours could shed light on some of the mechanisms of cell transformation into the malignant state. The aim of this study was to determine the changes in the proteasome pool during the growth of nonmalignant and malignant tumours using the same model animals. We used a previously developed model to induce malignant transformation of liver cells in mice CBA/Lac x BL/6 F1 by alkylating drug, Dipin, followed by partial liver resection [17, 18]. Dipin causes irreparable damage of the genetic material in hepatocytes leading, after mitoses stimulated by partial liver resection, to chromosome breakage and rearrangement. The cells damaged in such a way are not viable, and they eventually die. The parenchyma is regenerated by means of activation of stem cells and clonogenic growth of neoplastic hepatic nodules, which coalesce and displace the original degenerating hepatocytes and form new tissue. This nodular regenerative hyperplasia is diffuse in nature, but, eventually, the separate nodules can progress and give rise to large adenomas and hepatocarcinomes. In this paper, we report on the comparative study of the chymotrypsin-like activity and total proteasome pool content, as well as the 26S proteasome and immunoproteasome content, in the intact liver and induced nonmalignant and malignant liver tumours.

EXPERIMENTAL

Reagents. The following reagents were used: Suc-LLVY-AMC and MG132 (Sigma, USA), anti-β-actin mouse mAb (Santa Cruz, Germany), anti-Rpt6 and anti-α1,2,3,5,6,7 mouse mAb, anti-X(β5), anti-LMP7(β5i) and anti-LMP2(β1i) rabbit pAb (Biomol, UK), anti-nNOS rabbit pAb (Abcam, UK), and ECL kit, Hybond-ECL nitrocellulose membranes and peroxidase conjugated antibodies to mouse and rabbit IgG (Amersham Biosciences, UK).

Animals. Male mice CBA/Lac x BL/6 F1, three months old, 20–22 g weight, were used in the study. Dipin at 60 microgram per 1 g of weight was injected to a group of male mice. A standard partial liver resection operation (up to 70 %) was performed according to a previously developed procedure [17]. Mice with intact liver and mice subjected to partial liver resection were used as controls. After 12 months, the livers of the control and test animals were studied.

Histological study of the liver. The fragments of the liver and large tumour nodules were fixed in 10% formalin. The fixed material was processed following the standard procedure: 5-micron-thick sections were prepared after paraffin embedding. After removing the paraffin, the specimens were H&E stained, embedded in balsam, and analysed with Olympus AHBT3 optical microscope.

Preparation of clarified homogenates of liver and tumour fragments. All procedures were performed at 0–4 °C. Liver and tumour fragments were washed with a standard sodium phosphate buffer, dried, weighed, and homogenised (glass-glass, homogeniser Braun Melsungen, Germany) in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerine, 5 mM $MgCl_2$, 1 mM ATP, 10 mM $Na_2S_2O_5$, leupeptin (0.5 μg/ml), pepstatin (1 μg/ml) and aprotinin (1 μg/ml) at 1 : 3 ratio. The homogenates were centrifuged at 10,000 g for 30 min. The supernatants (clarified homogenates) were used in the studies. The protein concentration in the clarified homogenates was determined by the Lowry method [19].

Determination of proteasome activity. The proteasome activity was determined by measuring the hydrolysis of the Suc-LLVY-AMC fluorogenic oligopeptide, which is a substrate for proteasome chymotrypsin-like sites [20]. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 30 μM Suc-LLVY-AMC, 5 mM $MgCl_2$, and 1 mM ATP. In order to eliminate the contribution of the proteolytic activity of impurities, 10 μM MG132 (inhibitor of the proteasome chymotrypsin-like sites) was added to some samples. The reaction was carried out at 37 °C for 20 min after adding 0.5–2 μl of clarified homogenate (to a total volume of 100 μl), and the reaction was stopped with 1% SDS. The product mixture was measured in a fluorimeter with excitation and emission at 380 and 440 nm, respectively. The difference between the total and residual activity in the presence of MG132 was calculated. The activity was expressed as nanomol of Suc-LLVY-AMC hydrolysed in 20 min by proteasomes contained in 100 μl of clarified homogenates.

Western blot analysis. Western blot analysis was used to determine the relative content of proteasome subunits, nNOS, and β-actin in the clarified homogenates. Gel electrophoresis of the proteins from clarified homogenates was performed in 10–13% PAA gel in the presence of SDS (5 μl per lane, 120–148 μg of protein). The polypeptides were transferred from the gel to a nitrocellulose membrane using the semi-dry method. The membrane was incubated for 2 hours at 20 °C in TNT buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20), and then for 1 hour in TNT buffer containing 2–5% of non-fat milk and mouse mAb to β-actin (1 : 200) or to Rpt6 (1 : 2500), or to α1,2,3,5,6,7 (1 : 2500) (or rabbit pAb to nNOS (1 : 500) or to X(β5), or to LMP7(β5i), or to LMP2(β1i) (1 : 1250)). The membrane was washed several times with TNT buffer and incubated for 1 hour in TNT buffer containing 2–5% of nonfat milk and peroxidase conjugated antibodies to mouse (or rabbit) IgG (1 : 2500). Then, the membrane was washed with TNT buffer and analysed using the ECL kit following the standard procedure.

ImageJ software was used for image processing. The relative content of proteins in the clarified homogenates was determined by measuring the density of corresponding bands on the X-ray film, using previously prepared calibration plots of density vs. analysed protein content. Further experiments

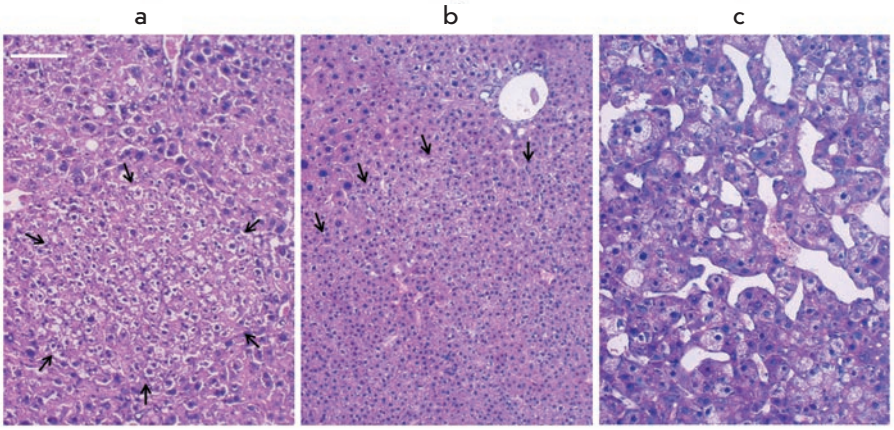

Fig. 1. Hepatic tumors developed under chronic regenerative liver condition in the 12 months after treatment with Dipin followed by partial liver resection. *a* — microadenoma-nodule consisting of small hepatocytes with diploid nuclei. *b* — large hepatocellular adenoma lacking typical liver lobule and vasculature structure. *c* — trabecular hepatocellular carcinoma with cytomegaly, anomalous trabecular and sinusoid structure. H&E staining. Arrows indicate tumour boundaries. Scale bar 100 microns

were carried out within the range of protein concentrations for which the calibration plot showed linear behaviour.

Statistical analysis. The data are presented as average ± confidence interval (δ):

$$\delta = \pm t\sigma n^{-0.5},$$

where t is the Student's criterion value at significance level $p < 0.05$, σ is the standard error, and n is the number of experiments.

RESULTS AND DISCUSSION

Histological study of mouse liver. The results of mouse liver histological study performed 12 months after Dipin injection and partial liver resection are presented in Fig. 1. Multiple nodules (benign tumours, microadenomas) were revealed in the liver tissue (Fig. 1 a*)*; they formed during the diffuse nodular regenerative hyperplasia of hepatocytes. In addition, we detected large benign tumours, adenomas, (Fig. 1 b*)* and malignant tumours whose biological properties corresponded to hepatocellular carcinoma of the trabecular type (Fig. 1 c) [21]. We performed a comparative study of the chymotrypsin-like activity of the total proteasome pool and the content of various proteasome subunits in the tumour samples and liver fragments with diffuse nodular hyperplasia versus liver samples of the control animals.

Technical notes on the study of changes in the proteasome pool during tumourogenesis. The relative content of the total proteasome pool in the samples was studied by Western blot analysis using antibodies to α1,2,3,5,6,7 subunits present in all proteasome forms. In a similar way, we determined the relative content of the 26S proteasomes using antibodies to the Rpt6 subunit contained in the 19S particles of the 26S proteasomes, as well as the relative content of the X(β5), LMP7(β5i), and LMP2(β1i) proteolythic subunits, using corresponding antibodies.

In addition to measuring the concentration of proteasome subunits in the clarified homogenates of liver and induced tumours, we studied the concentration of total proteins and β-actin, which are normally used for standardisation of the activity and concentration of proteins in tissues. The total protein content in the clarified homogenate of hepatocellular carcinoma was slightly—but reliably—lower than that in the clarified homogenate of the intact liver (Table 1). At the same time, the β-actin content was significantly higher in the hepatocellular carcinoma sample (Fig. 2, Table 1). This meant

Table 1. Chymotrypsin-like activity of proteasomes and the content of the proteasome subunits, nNOS, β-actin, and total protein in the clarified homogenates of mouse liver and induced liver tumors.

Parameter		Value in the clarified homogenate of			
		intact liver	liver with hepatic nodular hyperplasia	adenoma	hepatocellular carcinoma
Chymotrypsin-like activity of proteasomes in a 100 µl sample (nanomol Suc-LLVY-AMC)		18.6 ± 1.3	13.7 ± 0.5	11.2 ± 0.5	5.3 ± 0.3
Content of proteasome subunits (%)	α1,2,3,5,6,7	100 ± 4	135 ± 3	147 ± 2	220 ± 6
	Rpt6	100 ± 3	98 ± 3	101 ± 5	150 ± 6
	X(β5)	100 ± 3	125 ± 5	–	210 ± 8
	LMP7(β5i)	100 ± 5	150 ± 4	170 ± 5	169 ± 7
	LMP2(β1i)	100 ± 3	200 ± 7	290 ± 17	400 ± 15
nNOS content (%)		low	100 ± 3	–	450 ± 13
β-actin content (%)		100 ± 2	102 ± 3	97 ± 8	270 ± 7
Protein concentration (mg/ml)		29.5 ± 0.9	26.5 ± 1.1	26.0 ± 0.7	24.0 ± 0.8

A 100% level of proteasome subunits and β-actin correspond to their content (optical density of bands) in the clarified homogenate of the intact liver; A 100% level of nNOS correspond to its content in the clarified homogenate of the liver with hepatic nodular hyperplasia. The data are represented as the average value ± δ. For each data point, $p < 0.05$, $n \geq 5$.

that in this study both these parameters could not be used as an internal reference for the standardisation of the protein properties. The more appropriate method would be to compare the activity and concentration of proteasome subunits in the control mice liver and hepatic tumours normalised to the raw tissue weight.

As an additional reference, we used liver regenerated after the partial resection, which was not treated with Dipin. For the clarified homogenates of that liver and the intact liver, no difference was found in the proteasome chymotrypsin-like activity, nor in the content of all studied proteasome subunits, β-actin, and total protein (data not shown).

Differences and similarities in the change in the proteasome pool during benign and malignant tumour formation. The changes in the proteasome pool appear as early as when diffuse nodules are being formed, manifested by the increase of the total proteasome pool and the expression of the X(β5) constitutive subunit and the LMP7(β5i) and LMP2(β1i) immune subunits (Fig. 2, Table 1). The extent to which the expression of these subunits is increased is demonstrated by the following pattern: LMP2(β1i) > LMP7(β5i) > X(β5). The increase in the LMP2(β1i) subunit content is equal to that of the third immune subunit LMP10(β2i), since they are always incorporated into proteasomes together, whereas the LMP7(β5i) immune subunit can be incorporated into proteasomes independently from the other two [22, 23]. The increase in the content of the subunits studied in the total proteasome pool was accompanied by a decrease in the total pool activity with respect to the Suc-LLVY-AMC oligopeptide hydrolysed by the chymotrypsin-like sites of the X(β5) constitutive subunit and the LMP7(β5i) immune subunit (Table 1).

The formation of a malignant tumour caused an even stronger decrease in the proteasome chymotrypsin-like activity, as well as a stronger increase in the total proteasome pool and the content of the immune subunits and the X(β5) constitutive subunit (Fig. 2, Table 1). The pattern of the increase of these subunits was different: LMP2(β1i) > X(β5) > LMP7(β5i). It should be noted that although the content of the LMP7(β5i) immune subunit in the hepatocellular carcinoma was higher than that in the control liver tissue and the liver fragments with diffuse nodules, it was the same as that in the adenoma. The content of the total proteasome pool and the LMP2(β1i) immune subunit, and the chymotrypsin-like activity in the adenoma were at an intermediate level compared to those in the samples of liver with diffuse nodules and hepatocellular carcinoma (Fig. 2, Table 1). These results indicate that, during the formation of benign and malignant tumours, the increase in the total proteasome pool occurs due to multiple immunoproteasome forms expressed at different ratios. These proteasomes include those containing all three immune subunits LMP7(β5i), LMP2(β1i), and LMP10(β2i); proteasomes containing the LMP7(β5i) immune subunit and the Y(β1) and Z(β2) constitutive subunits; and proteasomes containing the X(β5) constitutive subunit and the LMP2(β1i) and LMP10(β2i) immune subunits.

The decrease in chymotrypsin-like activity during tumour formation cannot be explained only by the change in the ratio of the X(β5) and LMP7(β5i) subunits responsible for that type of activity, since there is no correlation between those values (Table 1). It is likely that incorporation of the LMP2(β1i) subunit into proteasomes and/or intracellular regulation have more effect on the chymotrypsin-like activity.

In this study, we uncovered fundamental differences between proteasome pools in malignant and benign tumours. Of all the tumours studied, only hepatocellular carcinoma contained an increased amount of the 19S activator, which is present in the 26S proteasomes and controls their level (Fig. 2, Table 1). The increased level of the 26S proteasomes in hepatocellular carcinoma is easy to understand. High protein metabolism is typical for malignant tumours, including liver cancer [24, 25], which, in turn, requires more proteolytic enzymes, such as the 26S proteasomes.

The reason for the increased content of immunoproteasomes in hepatocellular carcinoma is not so clear, however. One can speculate that immunoproteasomes are expressed in the transforming cells so that the immune system can recognise and destroy those cells. It is possible that in our model some other links necessary for the immune reaction fail to function, and, regardless of the amount of immunoproteasomes generated in tumour cells, the immune system cannot destroy the cells. This is one issue we will study further. On the other hand, tumour cells may generate immunoproteasomes, which are known to possess an antioxidant function, in order to protect themselves from metabolites and other factors that would cause oxidative stress and apoptosis.

Possible mechanism of immunoproteasome regulation in tumours. The NO-dependent signalling pathway intended for quenching the oxidative stress in endotheliocytes causes

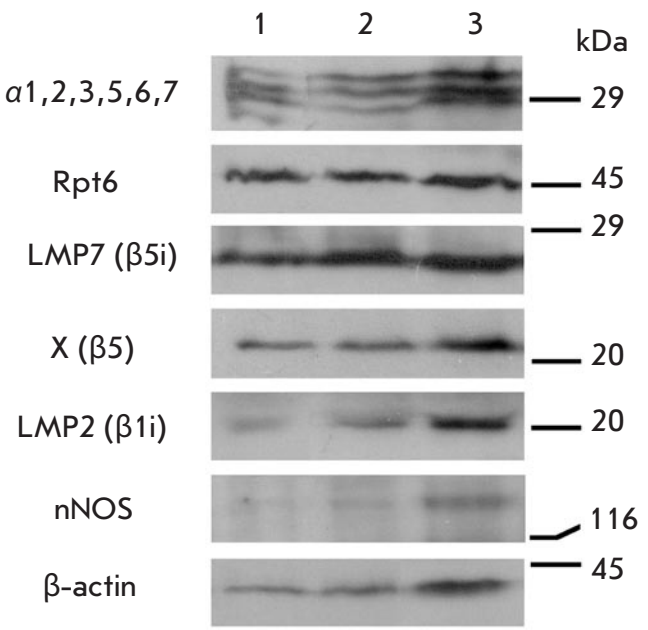

Fig. 2. Western blot analysis of proteins in the clarified homogenates of the intact liver (*1*), liver with diffuse nodules (*2*), and hepatocellular carcinoma (*3*) with use of antibodies to proteasome subunits α1,2,3,5,6,7, Rpt6, LMP7(β5i), X(β5), and LMP2(β1i), nNOS and β-actin. Markers: carboanhydrase (29 kDa), ovalbumin (45 kDa), trypsin inhibitor (20 kDa), and β- galactosidase (116 kDa)

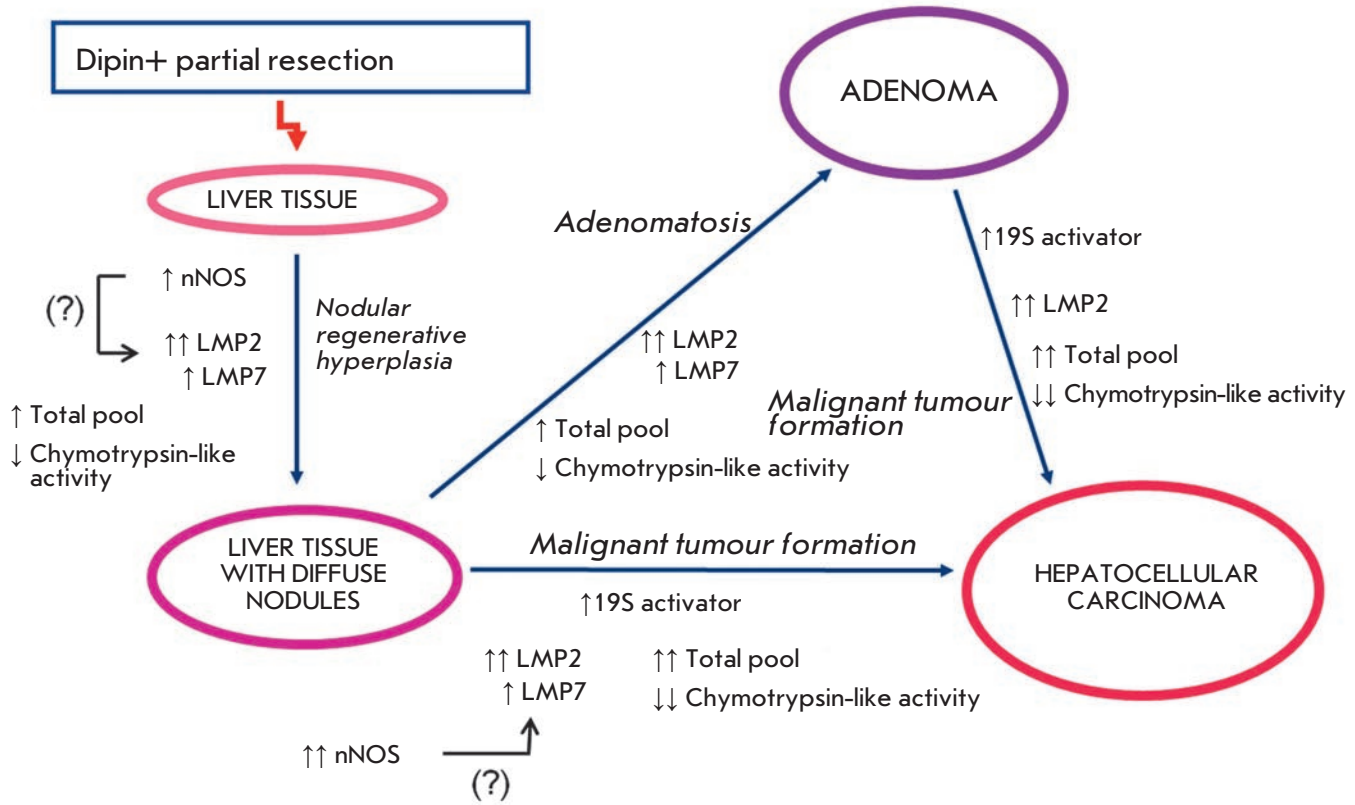

Fig. 3. Schematic representation of liver cell malignant transformation based on the changes in the proteasome pool

additional expression of the LMP2(β1i) immune subunit to a larger extent than that of the LMP7(β5i) immune subunit [7], which is coincides with our results on the expression dynamics of immune subunits during hepatic tumourogenesis (Table 1). The antioxidative function of immunoproteasomes in endotheliocytes is to eliminate the transferrin receptor and block free radical oxidation chain reactions involving Fe(II) [7]. It has been proven that NO acts as an antioxidant in malignant cells, too [26–28]. It is possible that immunoproteasomes in hepatocellular carcinoma participate in the NO-dependent signalling pathway that protects the tumour from oxidative stress. This hypothesis is confirmed by our data on the increased expression of nNOS in hepatocellular carcinoma (Fig. 2, Table 1), while there was little nNOS found in the control mouse liver. This result is in accord with data in the literature pointing to the fact that the amount of nNOS in adult mouse liver drops dramatically compared to that in foetal liver, where this enzyme regulates haematopoiesis [29].

In this study, we have shown that the formation of tumours in mouse liver is accompanied by significant changes in the proteasome pool. These changes are less pronounced in nodular hepatic hyperplasia and adenomatosis than in hepatocellular carcinoma. This suggests that nodular hepatic hyperplasia and adenomatosis may be stages preceding carcinogenesis. A schematic representation of liver cell malignant transformation based on our results is given in Fig. 3.

Our results indicate that there is a need to identify the signalling pathways that change the expression of various proteasome subunits during tumourogenesis. In addition, we can conclude that the 19S proteasome activator overexpressed in malignant tumours can be a potential target for the development of new anticancer drugs. At the moment, the first proteasome inhibitor anticancer drug, Bortezomib (Velcade), is being used clinically [30]. Bortezomib is injected into a patient's bloodstream, and it is administered along with other anticancer medications. Bortezomib, a boronic acid derivative, selectively inhibits the chymotrypsin-like activity of all proteasome forms and temporarily induces apoptosis, primarily of neoplastic cells. The prolonged inhibition of proteasome activity, however, induces feedback mechanisms and the generation of new proteasomes [31]; hence the drug's temporary therapeutic effect. At the same time, Bortezomib affects the total proteasome pool in all organs, thus causing side effects such as fatigue, atony, gastrointestinal disorders, peripheral neuropathy, and significant deterioration of the general wellbeing of patients [30]. In this regard, suppressing the functions of the 19S activator while maintaining the proteasome's proteolythic activity appears to be a more efficient and safer approach to anticancer therapy.

CONCLUSION

The formation of hepatic nodular regenerative hyperplasia, adenomatosis, and carcinoma is accompanied by changes

in the proteasome pool, the changes having similarities, as well as differences. The similarities are the increase in the content of immunoproteasomes and in the total proteasome pool, and the decrease in the proteasome chymotrypsin-like activity in all tumour types compared to the control samples. The difference is in the behavior of the 19S proteasome activator content, which is increased only in hepatocellular carcimona.

The dynamics of changes in the proteasome pools in liver with diffuse nodules, adenoma, and carcinoma indicates that nodular regenerative hyperplasia and adenomatosis may be stages preceding carcinogenesis.

The 19S proteasome activator, which is overexpressed only in malignant tumours, can be a promising target for the development of new anticancer drugs. ●

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New 5-Modified Pyrimidine Nucleoside Inhibitors of Mycobacterial Growth

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ABSTRACT **The WHO has declared tuberculosis (TB) a global health emergency. Therefore, there is an urgent need to discover and develop new anti-TB drugs. Here we report a new category of 5-substituted pyrimidine nucleosides as potent inhibitors of *Mycobacterium tuberculosis* growth in vitro. A series of 2′-deoxy-, 3′-azido-2′,3′-dideoxy-, and 3′-amino-2′,3′-dideoxypyrimidine nucleoside analogues bearing lengthy flexible alkyloxymethyl substituents exhibited marked inhibitory activity against *M tuberculosis* in vitro. 5-Dodecyloxymethyl-2′-deoxyuridine was found to be a potent inhibitor of *M. tuberculosis* propagation in vitro. In contrast, monophosphates of the tested nucleosides were devoid of antimycobacterial activity. This new class of inhibitors seems to be a promising chemotherapeutic agent against TB and merits further studies.**

KEYWORDS **tuberculosis; *Mycobacterium tuberculosis*; anti-TB drug; nucleoside; 5-substituted pyrimidine; inhibitor**

ABBREVIATIONS **TB - tuberculosis, HIV - human immunodeficiency virus, MDR - multi-drug resistance, MIC - minimal inhibitory concentration, CFU - colony-forming unit**

INTRODUCTION

Tuberculosis (TB) is one of the largest problems of modern health services. At the beginning of the 21st century, TB is one of the most widespread infectious diseases: about one-third of the world's population (more than two billion people) is currently infected with *Mycobacterium tuberculosis*. According to WHO reports, about nine million people are infected every year, and more than two million people, of whom 10% are also infected with HIV, die from tuberculosis [1, 2]. Infection with HIV increases the chance of latent TB reactivation [3] or leads to rapid TB development soon after (re) infection with TB bacillus [4]. Latent-to-active TB transition risk reaches 50% among people with AIDS and is about 10% among the rest of the population.

Effective and accessible TB treatment schemes based on combinations of different drugs were developed at the beginning of the 1950s. More than ten anti-TB drugs may be included in the chemotherapy scheme [5]. Since this time, the broad use of anti-TB drugs, as well as vaccination, has led to a significant decrease in TB-related mortality. On the other hand, the drug usage induced the selection of strains resistant to several pharmaceuticals of various types. HIV infected, drug-dependent, and transplant patients have extremely weakened immunities and, hence, become TB victims. These circumstances led to the fact that the frequency of TB cases started to grow at the end of the 1980s. The WHO announced a global TB health emergency in 1993. New *Mycobacterium tuberculosis* strains should be particularly noted: multidrug and extensively drug-resistant tuberculosis (M/XDR-TB) strains [1] that are barely affected by standard chemotherapy schemes. M/XDR-TB generally results from incorrect treatment, when patients receive insufficient amounts of anti-TB drugs [6]. Usually, it takes more than half a year from the beginning of anti-TB treatment until the patient recovers, and during this time any contact with an infected patient can result in contamination [5]. The main factor determining the resistance development under the action of anti-TB pharmaceuticals is the selection of drug-resistant mycobacteria with genome mutations.

TB is one of the largest problems in Russia, because its current incidence rate is 190.5, prevalence is 85.1, and mortality rate is 17.9 cases per 100 000 people. At the same time, the occurrence of MDR-TB is 13.6% in the patients with new TB cases and 28.8% in patients with relapses, according to the statistics of the Ministry of Health and Social Development of the Russian Federation [7]. In connection with the above, the search for new anti-TB drugs is necessary.

Therapy for viral infections is frequently based on using natural nucleoside derivatives [8]. The anti-TB activity of nucleosides has not been revealed until recently. Recent reports have appeared on several groups of modified nucleosides displaying a remarkable anti-mycobacterial effect in experimental models [9–14].

Recently, 5-modified pyrimidine nucleosides with lengthy 1-alkinyl radicals have demonstrated an inhibitory effect on *Mycobacterium tuberculosis* and *M. bovis* in vitro [11–14]. The best antibacterial activity has been demonstrated for

№	X	R1	R2	R3
1	OH	$C_{10}H_{21}$	OH	OH
2	OH	$C_{12}H_{25}$	OH	OH
3	OH	$C_{10}H_{21}$	H_2PO_4	OH
4	NH_2	$C_{10}H_{21}$	H_2PO_4	OH
5	OH	C_9H_{19}	H_2PO_4	OH
6	NH_2	$C_{12}H_{25}$	OH	OH
7	NH_2	$C_{14}H_{29}$	OH	OH
8	OH	$C_{12}H_{25}$	OH	OH
9	OH	$C_{12}H_{25}$	OH	N_3
10	OH	$C_{12}H_{25}$	OH	NH_2

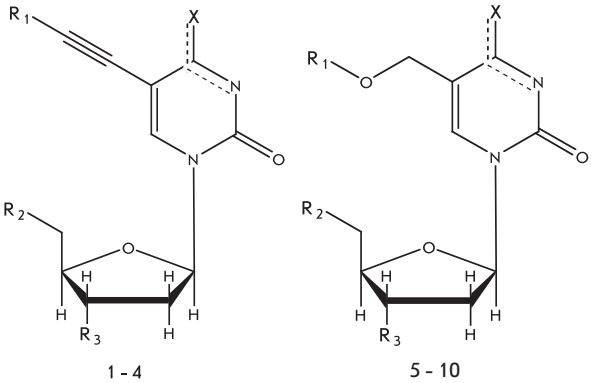

Fig. 1. Structures of tested substances

nucleoside 5-(1-dodecynyl) and 5-(1-tetradecynyl) derivatives. The study on the influence of the carbohydrate fragment modification on antibacterial properties of 5-modificed nucleosides has demonstrated that virtually all 2′-deoxy-, 2′,3′-dideoxy-, 3′-fluoro-2′,3′-dideoxy-, and 2′-fluoro-2′,3′-dideoxynucleosides, as well as acyclic and arabinonucleosides with long 1-alkynyl radicals, have displayed anti-TB activity [11–14]. This work is devoted to an investigation of the *Mycobacterium tuberculosis* growth inhibitory capability of newly synthesized 2′-deoxy-, 3′-azido-2′,3′-dideoxy-, and 3′-amino-2′,3′-dideoxy-pyrimidine nucleosides containing lengthy alkyloxymethyl radicals at their position 5.

MATERIALS AND METHODS

Tested substances (Fig. 1, *1–10*). 5-(1-Dodecynyl)- and 5-(1-tetradecynyl)-2′-deoxyuridine (*1* and *2*, respectively) used as a control were prepared by the method [11]. 5-Alkyloxymethyl-pyrimidine nucleoside derivatives (*6–10*) were synthesized using our method [15], and nucleoside 5′-monophosphates (*3–5*) were synthesized by the method [15–16].

Mycobacterial strain. These substances were tested using the *Mycobacterium tuberculosis* H37Rv laboratory strain sensitive to anti-TB drugs. A suspension of individual mycobacterial cells was equalized by the growth phase and standardized by CFU [17]. Cells were grown in a liquid medium Dubos with 5% BSA (Difco).

Estimation of antibacterial effect of tested substances. The effect that these substances have on the growth of the mycobacterial strain was examined using a BACTEC™ MGIT™ 960 Mycobacterial Detection System (BD, United States) for 24 days. Mycobacterium cell suspension (500 μL) was inoculated into 7.9 mL liquid medium 7H9 Middlebrook supplemented with OADC. The final concentration of *M. tuberculosis* in the sample was 10^5–10^6 CFU/mL. Each of the experiments with different tested substance concentrations, including control samples without any drug, was triplicated.

The antimycobacterial effect of tested substances was estimated from the growth kinetics of *M. tuberculosis* H37Rv in presence of a varied concentration of the tested substances compared with that in the absence of any drug [17]. Growth was monitored automatically every hour and recorded using Epicenter software (BD, United States). The mycobacterial growth was expressed in reference fluorescence units (RFU).

RESULTS AND DISCUSSION

We have studied the inhibitory effect of synthesized 2′-deoxynucleoside derivatives (Fig. 1, *6–10*) carrying a long linear alkyl moiety introduced at position 5 of pyrimidine base via a oxymethyl group providing a higher flexibility of the hydrocarbon chain than 1-alkynyl derivatives (*1* and *2*) described in literature [11–14] on the growth of *M. tuberculosis*. The method of pyrimidine nucleoside methoxyalkyl derivative synthesis we have developed [15] is essentially easier and cheaper than the proposed method of 5-(1-alkynyl)-nucleoside synthesis [11–14]. To reveal the role of 3′-modification of the carbohydrate moiety in the anti-TB activity of 5-modified nucleosides, we have synthesized the following 2′-deoxyuridine derivatives with the same substituent at position 5 of the base: 2′-deoxy-, 3′-azido-2′,3′-dideoxy-, and 3′-amino-2′,3′-dideoxy-5-dodecyloxymethyluridine (Fig. 1, *8–10*).

The data on the bacteriostatic activity of the studied substances from the growth of mycobacteria in the automated Bactec MGIT960 system has demonstrated that the *M. tuberculosis* H37Rv culture began to grow after 3.59 days in a medium without any drugs. The growth curve had a classic sigmoid shape with three phases: latent growth (before 3.59 days), exponential (log-phase or phase of active mycobacterial cell division) from 3.59 to 10.25 days, and a stationary one from 10.25 days until the end of the experiment (Fig. 2). The duration of active cell replication phase was 6.66 days.

To confirm the antimycobacterial activity of nucleosides and adjust the experimental conditions, we primarily tested 5-(1-dodecynyl) and 5-(1-tetradecynyl)-2′-deoxyuridine (*1* and *2*) at concentrations of 2, 20, and 200 μg/mL. According to published data [11] compounds *1* and *2* inhibited the growth of both *M. bovis* (MIC_{90} = 50 and 10 μg/mL, respectively) and *M. avium* by 50–70% at high concentrations. We have shown that both substances taken at a concentration of 200 μg/mL completely inhibited the growth of *M. tuberculosis* culture.

5-Alkyloxymethyl derivatives of pyrimidine nucleosides (*6*, *7*, *9*, and *10*) and nucleoside monophosphates (*3–5*) were tested at concentrations of 0.2, 2, 20, 100, and 200 μg/mL, and 5-dodecyloxymethyl-2′-deoxyuridine (*8*) was tested at concentrations of 0.2, 2, 20, 50, and 100 μg/mL. The data of an experiment on determining the antimycobacterial activity is shown in Fig. 2.

A determination of the inhibitory effect of the substances *6–10* on the culture of *M. tuberculosis* H37Rv has shown that

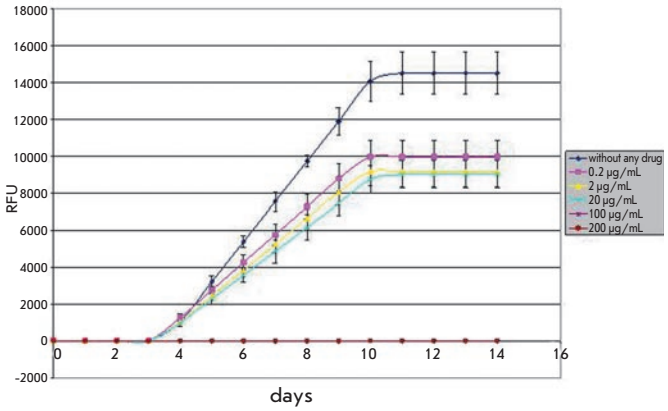

Fig. 2. Experiment on testing the antimycobacterial activity of substances. The growth kinetic plot is drawn for the *M. tuberculosis* H37Rv culture exposed to a varied concentrations of substance *9*. RFU is the reference fluorescence unit monitored using the Epicenter software (BD, United States)

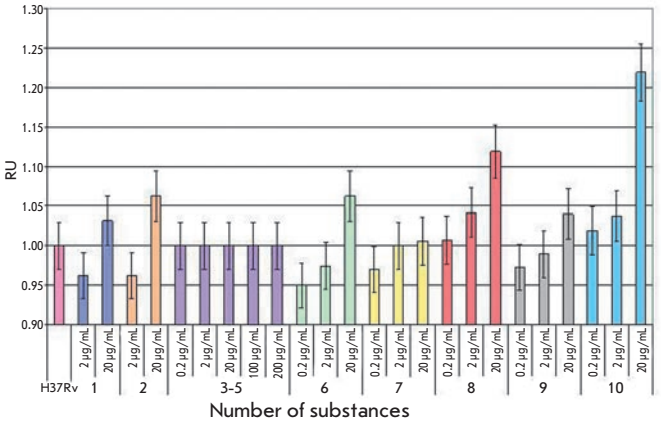

Fig. 3. The efficacy of the substances inhibiting the *M. tuberculosis* culture growth at different concentrations. RE is the ratios between the active replication phase durations of the *M. tuberculosis* H37Rv culture exposed to the tested substances and that of the control culture, which was taken to be unity

substance *6* led to 100% growth inhibition at concentrations of 200 and 100 μg/mL, *7* at concentrations of 200 and 100 μg/mL, *8* at concentrations of 100 and 50 μg/mL, *9* at concentrations of 200 and 100 μg/mL, and *10* at concentrations of 200 and 100 μg/mL (Fig. 2). Thus, the minimal inhibitory concentration (MIC) is 100 μg/mL for the substances *6*, *7*, *9*, and *10* and 50 μg/mL for the substance *8*.

Nucleosides with large hydrophobic groups at position 5 of the nucleic base are hardly soluble in water. To increase the solubility, we have synthesized nucleoside 5′-monophosphates (*3*–*5*) by the method [16]; however, these substances do not inhibit the growth of mycobacteria even at high (200 μg/mL) concentrations, and the growth kinetics in the presence of these substances is the same as in the control (in the absence of any drug).

We have evaluated the active growth duration in the culture exposed to tested substances taken at concentrations not inducing 100% inhibition of the culture growth in comparison with the control (Fig. 3). The data is given in relative units (RU) calculated as the ratio of the active replication time of the culture growing in the presence of a substance to that of the control culture (*M. tuberculosis* H37Rv without any substance added). Fig. 3 shows that a more prolonged active replication phase is observed under exposition with 5-dodecyloxymethyl-2′-deoxyuridine (*8*) and 5-dodecyloxymethyl-3′-amino-2′,3′-dideoxyuridine (*10*) taken at a concentration of 20 μg/mL, which is significantly different from the control ($p < 0.01$) and suggests a decrease in the intensity of mycobacterial-cell replication in the culture. Note that 24 and 18 h delays (for the substances *8* and *10*, respectively) of mycobacterial growth when compared with the control were shown for these substances.

CONCLUSIONS

The inhibitory effect that 2′-deoxyuridine and 2′-deoxycytidine derivatives with lengthy alkyloxymethyl moieties have on the growth of the *M. tuberculosis* H37Rv culture was first demonstrated in this work. 5-Dodecyloxymethyl-2′-deoxyuridine (*8*) can be regarded as the most active against *M. tuberculosis* because of the lowest MIC of all tested compounds (50 μg/mL) and the most prominent capability of prolonging active cell replication and delaying the growth initiation at concentrations that do not cause 100% inhibition. Nonetheless, we have not revealed any fundamental difference in the inhibitory effect of pyrimidines differing both in the structure of the substituent (1-alkynyl or alkyloxymethyl moiety) at position 5 of the base and the carbohydrate fragment (2-deoxy-, 3-azido-2,3-dideoxy-, or 3-amino- 2,3-dideoxyribofuranose).

Thus, we have shown the capability of pyrimidine 2′-deoxynucleoside 5-methyloxyalkyl derivatives to inhibit the growth of *M. tuberculosis* in vitro; the most effective derivatives could serve as prototypes for the development of new anti-TB drugs. ●

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Induction of a Protective Heterosubtypic Immune Response Against the Influenza Virus by Using Recombinant Adenoviral Vectors Expressing Hemagglutinin of the Influenza H5 Virus

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ABSTRACT **Influenza viruses are characterized by a high degree of antigenic variability, which causes the annual emergence of flu epidemics and irregularly timed pandemics caused by viruses with new antigenic and biological traits. Novel approaches to vaccination can help circumvent this problem. One of these new methods incorporates genetic vaccines based on adenoviral vectors. Recombinant adenoviral vectors which contain hemagglutinin-encoding genes from avian H5N1 and H5N2 (Ad-HA5-1 and Ad-HA5-2) influenza viruses were obtained using the AdEasy Adenoviral Vector System (Stratagene). Laboratory mice received a double intranasal vaccination with Ad-HA5-1 and Ad-HA5-2. This study demonstrates that immunization with recombinant adenoviruses bearing the H5 influenza virus hemagglutinin gene induces a immune response which protect immunized mice from a lethal dose of the H5 influenza virus. Moreover, it also protects the host from a lethal dose of H1 virus, which belongs to the same clade as H5, but does not confer protection from the subtype H3 influenza virus, which belongs to a different clade.**

Our data allow us to conclude that adenoviral vectors may become a universal platform for obtaining vaccines against seasonal and pandemic strains of the influenza virus.

KEY WORDS **adenoviral vector, influenza virus, hemagglutinin, immunization, heterosubtypic protection.**

INTRODUCTION

Influenza A viruses can cause severe epidemics. These viruses are widespread in nature and can infect humans, many mammalian species (horses, pigs, seals, etc.), and all species of birds [6, 17]. Different strains of the influenza virus are traditionally named after the numerical indexes of their surface antigens (hemagglutinin (HA) and neuraminidase (NA)). Overall, there are 15 subtypes of hemagglutinin and 9 subtypes of neuraminidase with minimal cross-activity in serological reactions between subtypes [18]. Two subtypes of hemagglutinin (H1, H3) and two types of neuraminidase (N1–N2) are currently circulating in the human population [18, 20]. Another potential threat to humans comes from avian influenza viruses, which have H5 hemagglutinin, since numerous cases of humans being infected by the H5N1 have been reported as far back as 1997. This virus was first detected in a human organism in China more than 10 years ago [7]; according to the WHO, the overall number of humans infected with H5N1 is 478, and 286 of such cases resulted in death. Influenza A viruses are characterized by a very high degree of antigen variability. The most variable entities are the surface glycoproteins of the viral particle (hemagglutinin and neuraminidase). Two mechanisms of variation are known. The first is antigenic drift. When a viral population is under pressure from the immune system, mutations that allow the virus to escape this controlling influence give the virus a serious advantage, and thus these mutations are conserved. This means that new antigenic variants of hemagglutinin and neuraminidase are constantly replacing each other. This creates the basis for epidemics, since immunity against the previous virus strain, even if it still belongs to the same subtype, is not sufficient to neutralize the new strain. Unfortunately the use of modern subunit and inactivated vaccines does not solve the problem, since they confer protection only from the strain which was used to obtain the vaccine. For this reason new vaccines for the protection of the population need to be created constantly.

The second mechanism of influenza virus variation is the antigenic shift, which is the alteration of the antigenic formula of a virus via the exchange of a gene (genes) and the corresponding protein (proteins). Antigenic shift is based on the reassortment or recombination of genes, which can take place if an organism is infected by two or more virus strains [19]. Shifts most often affect the antigenic structure of hemagglutinin, shifts in neuraminidase being less common. Thus, pandemic variants of the influenza virus with new antigenic and biological traits can emerge at irregular time periods, causing severe diseases and the deaths of a large number of people [6, 17]. For instance, the pandemic of the so-called "Spanish flu" – an H1N1 influenza virus in 1918–1920 – caused the death of around 50 million people worldwide. In June 2009, the World Health Organization (WHO) called the new H1N1 virus, cases of which started cropping up in April 2009, a 6th degree pandemic threat. This virus was the result of a reassortment, which combined the genes of avian, human, and porcine viruses [8]. If a new pandemic virus emerges, the creation of a vaccine takes too long a time, thus preventing a swift end to the spread of the dangerous virus. Circumventing this problem requires the creation of novel approaches to vaccinations against influenza viruses. One such approach is the creation of genetic vaccines based on viral vectors [5, 11]. The most promising type of genetic vaccine is a vaccine based on adenoviral vectors [1, 2]. Until recently it was thought that influenza-targeted vaccines, including genetic varieties which used variable surface antigens of influenza viruses (hemagglutinin and/or neuraminidase), were strictly specific to the strain whose antigen was used during the creation of the vaccine. However, studies performed in recent years (2008–2009) demonstrate that genetic vaccines based on adenoviruses which contain the hemagglutinin gene from an A-type influenza virus can induce cross-immunity both inside a single subtype of influenza virus [3] and between different subtypes of a virus that share the same subtype of hemagglutinin [15]. Such an effect may be caused by the fact that an organism vaccinated by an adenovirus receives an influenza virus hemagglutinin gene, which is then effectively expressed in the cell and is exposed on the plasma membrane, retaining its native ternary structure. This induces both cellular and humoral responses to the conservative epitopes of the influenza virus hemagglutinin. It was demonstrated that antibodies obtained from human plasma cells infected by an H5N1 influenza virus could also neutralize H1-subtype viruses as well [4]. Data published in 2008 also show that influenza viruses can be divided into two groups according to the presence of highly conservative conformational epitopes in the hemagglutinin molecule which can be identified by broad-range antibodies. The first group includes subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16; the second includes subtypes H3, H4, H7, H10, H14 and H15. Antibodies against the antigenic determinants of the first group are not equally effective for the neutralization of viruses of this group, but they do not neutralize viruses from the second group [14]. These groups were divided into four subgroups or clades according to the presence of conservative epitopes: H1 clade (H1, H2, H5, H6, H11, H13 and H16 hemagglutinins), H9 clade (H9, H8 and H12), H7 clade (H15, H7 and H10) and H3 clade (H3, H14 and H4).

Thus we have proposed that vaccination by an adenoviral vector bearing the the hemagglutinin gene of an Influenza A virus can lead to the production of antibodies against conservative epitopes (some of which may be conformational) of the major Influenza A surface antigen. This provides a cross-subtype immune response not only against influenza A viruses from a single subtype, but also against viruses of various subtypes which belong to the same clade.

This study used recombinant human adenoviruses of the fifth serotype which bore the hemagglutinin genes from the avian influenza viruses H5N2 and H5N1, since the H5 subtype avian viruses are likely candidates for the next pandemic strain. It was shown that immunization with recombinant adenoviruses induced the induction of a protective immune response, which allowed mice to survive lethal doses of its "native" H5 strain, and protected against a lethal dose of H1, which belongs to the same clade as H5. However this immune response did not protect the mice from an H3-subtype virus belonging to a different clade.

EXPERIMENTAL PROCEDURES

Viruses. This study used an avian influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2) and human influenza viruses A/USSR/90/77 (H1N1) and A/Aichi/2/68 (H3N2) adapted for use on mice [9]. The viruses were accumulated in the alantoic fluid of chicken embryos at 37˚C for 48 hours. The virus-containing alantoic fluid was stored at −70˚C. The titer of the virus was calculated by titration in chicken embryos. The 50% lethal dosage (LD_{50}) was calculated by titration on mice.

Obtaining recombinant adenoviruses. Plasmids and recombinant adenoviruses were obtained by using hemagglutinin genes from an avian influenza A/Mallard/Pennsylvania/10218/84 (H5N2) virus and a A/Duck/Novosibirsk/56/2005 (H5N1) (H5-2 and H5-1, respectively). The cDNA of the viral genome of A/Mallard/Pennsylvania/10218/84 (H5N2) was obtained by reverse transcription using a Reverse Tpanscription System (Invitrogene, United States). The hemagglutinin HA5-2 was obtained by amplifying the cDNA with primers which flanked the hemagglutinin gene. The H5-1 hemagglutinin gene was supplied by the Viral Disease diagnostic laboratory of the All-Russian Research Institute of Animal Health (Vladimir).

Recombinant adenoviruses Ad-HA5-1 and Ad-HA5-2 were obtained by homologous recombination in *E.coli* cells using the AdEasy Adenoviral Vector System (Stratagene). The obtained adenoviruses were purified and concentrated by double ultracentrifugation in a cesium chloride gradient. A recombinant serotype 5 human adenovirus with no expression cassette in the region of deletion in the E1 area of the genome was used as a control (Ad-null). The titers of the Ad-HA5-1, Ad-HA5-2 and Ad-null preparations were calculated using plaque formation in a HEK-293 cell culture.

Detection of hemagglutinin gene expression by IFA. Hemagglutinin gene expression by the recombinant viruses was performed on HEK-293 cells inoculated with the Ad-HA5-1 and Ad-HA5-2 vectors at a dosage of 100 particles per cell. After 24 h of incubation, the cells were lysed and ELISA was performed using a kit for the detection of H5 avain influenza strain hemagglutinin (BioAssay).

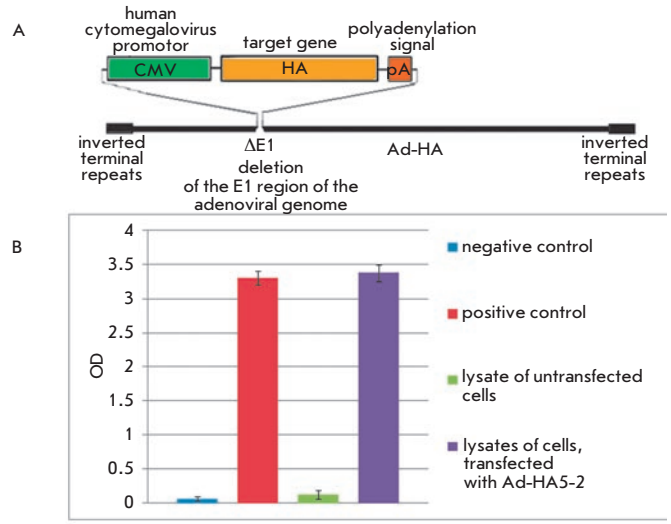

Fig. 1. The recombinant human adenovirus of the 5th serotype expresses the hemagglutinin gene of the avian H5N2 influenza virus (A/Mallard/Pensylvania/10218/84) in infected cells. (A) A schematic of the genome of the recombinant adenovirus, which bears the influenza virus hemagglutinin. (B) Expression of the H5N2 virus hemagglutinin by line 293 human embryo kidney cells infected with Ad-HA5-2 (ELISA): (-) negative control, (-) positive control, (-) lysates prepared from cells untransfected by Ad-HA5-2, and (-) lysates prepared from cells transfected by Ad-HA5-2

Measuring levels of hemagglutinin-binding antibodies in murine serums. Antibody levels in murine serums were assayed 21 days after the second immunization. The hemagglutinin-binding antibody level in murine serums was assayed by a hemagglutination-inhibition reaction performed according to the WHO/CDS/CR S/NC S/2002.5 protocol using chicken erythrocytes.

Mice. The mice used in this study were females of the BALB/c line and weighed about 7–9 g.

Animal immunization. The mice were divided into groups (ten animals per group) and immunized twice by an intranasal dose of recombinant Ad-HA5-1 and Ad-HA5-2 viruses, 10^8 PFU/mouse. The interval between immunizations was 21 days. The control groups were immunized with the Ad-null virus or treated with PBS solution instead.

Animal infection. 21 days after the second immunization, the mice were lightly anaesthetized with ether and then received a lethal dose (50 LD_{50}) of the A/Mallard/Pennsylvania/ 10218/84 (H5N2) strain or lethal doses (10 LD_{50}) of A/USSR/90/77 (H1N1) and A/Aichi/2/68 (H3N2) strains. The survival and changes in mouse weight were measured for 16 days after infection.

RESULTS AND DISCUSSION

Detection of hemaglutinnin gene expression in recombinant Ad-HA5-1 and Ad-HA5-2 adenoviruses. Schematics of the genomes of the recombinant Ad-HA5-1 and Ad-HA5-2 adenoviruses, which were obtained by homologous recombination in *E.coli* cells, are presented in Fig. 1A. The obtained preparations of adenoviral vectors were assayed for the presence of avian H5N1 and H5N2 influenza hemagglutinin gene insertions in the genomic DNA by PCR (data not shown).

Lysates obtained from HEK-293 cells infected with the recombinant Ad-HA5-2 adenovirus were assayed for the expression of recombinant hemagglutinin by ELISA using a kit for the detection of avian H5 influenza hemagglutinin (Fig. 1B). The expression of avian H5N1 influenza hemagglutinin by the Ad-HA5-1 adenovirus was assayed similarly (data not shown).

Assay for the immunogenicity of Ad-HA5-2-expressed hemagglutinin and the cross-immunogenicity of Ad-HA5-1-expressed hemagglutinin. The level of antibodies secreted against the recombinant hemagglutinin produced by the Ad-HA5-2 adenovirus was assayed using a hemagglutination-inhibition reaction. Mice of the BALB/c line were immunized with the Ad-HA5-2 adenovirus. Mice injected with PBS buffer were used as controls. Mice sera obtained 21 days after repeated immunization were analyzed for the presence of specific antibodies against avian influenza strain A/Mallard/Pennsylvania/10218/84 (H5N2). The hemagglutination-inhibition reaction results are presented in Fig. 2. The sera from mice immunized with the recombinant Ad-HA5-2 adenovirus appeared to have high levels of antibodies, which inhibited the agglutination of erythrocytes by the H5N2 virus. These data indicate the induction of a humoral immune response specific to the H5N2 avian influenza virus following the intranasal injection of a recombinant adenovirus expressing the H5 hemagglutinin gene.

The cross-immunogenicity of Ad-HA5-1-expressed hemagglutinin was assayed in a similar manner. The sera ob-

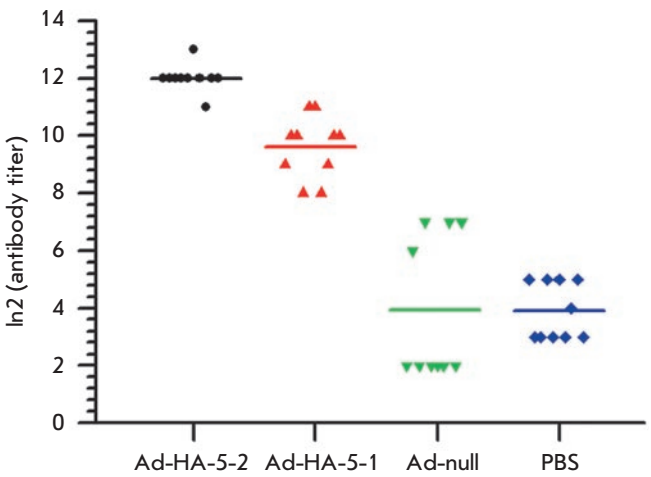

Fig. 2. Level of specific hemagglutinating antibodies which neutralize the avian H5N2 influenza virus in sera obtained from mice immunized by the recombinant Ad-HA5-1 and Ad-HA5-2 adenoviruses. Mice were immunized with Ad-HA5-1 and Ad-HA5-2 adenoviruses twice. Mice immunized by either Ad-null or PBS solution were used as controls. Antibody titers in mouse sera were assayed by HRR against the influenza A/Mallard/Pensylvania/10218/84 (H5N2) virus. A significant difference was detected between the control groups immunized by Ad-null and PBS and the groups vaccinated by Ad-HA5-1 and Ad-HA5-2 ($p < 0.05$)

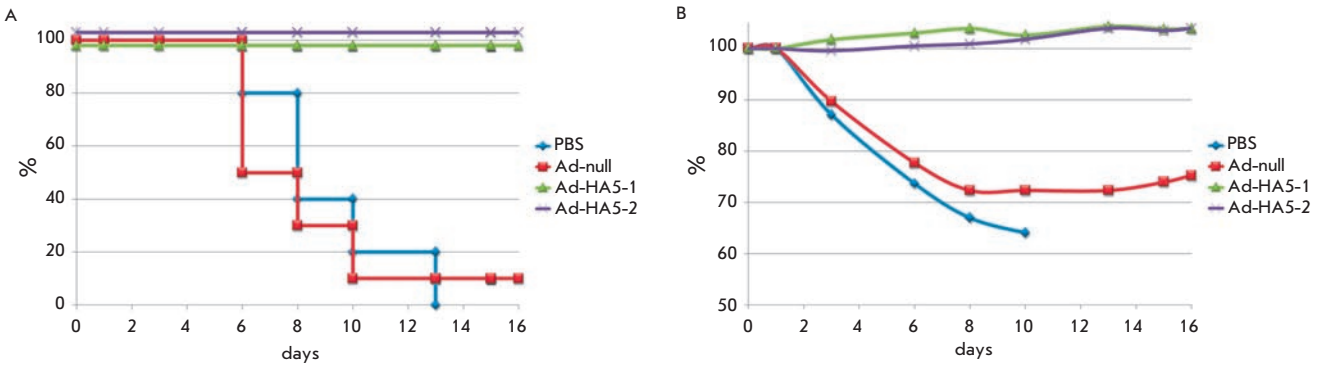

Fig. 3. Analysis of the protective hemagglutinin-specific immune response in mice immunized by recombinant Ad-HA5-1 and Ad-HA5-2 adenoviruses and later infected by a lethal dose of avian influenza virus H5N2. (A) Survival of mice infected by a lethal dose (50LD50) of the A/Mallard/Pensylvania/10218/84H5N2 (H5N2)-strain virus. Mice were immunized twice with the Ad-HA5-1 and Ad-HA5-2 adenoviruses. Control mice were immunized twice either with Ad-null or with PBC solution. The differences in survival between the Ad-HA5-2 (Ad-HA5-1) groups and the PBS control group ($p < 0.00001$) and the Ad-null control group ($p < 0.0001$) were statistically significant. (B) Changes in mouse weight as measured for the mice treated with 50LD50 of the A/Mallard/Pensylvania/10218/84H5N2 (H5N2) virus.

tained after immunization were assayed for the presence of specific antibodies against avian influenza A/Mallard/Pennsylvania/10218/84 (H5N2) hemagglutinin using hemagglutination-inhibition reaction (Fig. 2.). The tested mice sera had high levels of antibodies that prevented the agglutination of erythrocytes by the H5N2 influenza virus.

These results indicated the induction of a heterosubtypic humoral immune response specific to the H5N2 avian influenza virus following the immunization of mice by adenoviruses expressing the hemagglutinin gene from H5N1 avian influenza virus. The amount of antibodies specific to the H5N1 viral hemagglutinin was lower than in the case of immunization by an adenoviral vector bearing the H5N2 influenza hemagglutinin, but was still relatively high compared to the control group.

In control (PBS) sera, some nonspecific hemagglutination inhibition was observed due to the components of the murine serum.

Protective immunity of mice immunized by Ad-HA5-2 and cross-immunity of Ad-HA5-1-infected mice against H5N2 avian influenza virus infection. In order to study the protective effect of a double immunization with Ad-HA5-2, the immunized animals were infected with a lethal dose (50 LD_{50}) of A/Mallard/Pennsylvania/10218/84 (H5N2) influenza virus. The control mice were injected with the Ad-null recombinant adenovirus and with PBS solution. Mice which were immunized with the AdHA5-2 adenovirus did not lose any weight after infection and had 100% survival. The control PBS group, on the other hand, showed a 100% mortality rate over the course of 10 days and a 20% reduction of weight. The Ad-null group showed 20% survival and 30% weight reduction (Fig. 3). Thus, immunization with the recombinant Ad-HA5-2 adenovirus protected mice from a lethal H5N2 influenza virus infection. In order to study the induction of protective cross-immunity, mice were immunized as was described above by a recombinant Ad-HA5-1 adenovirus and infected with a lethal dose (50 LD_{50}) of A/Mallard/Pennsylvania/10218/84 (H5N2) virus. The immunization of mice with the Ad-HA5-1 adenovirus induced a heterosubtypic cross-immune response against the H5N2 influenza virus. The immunized mice showed no reduction in weight and had a 100% survival score (Fig. 3).

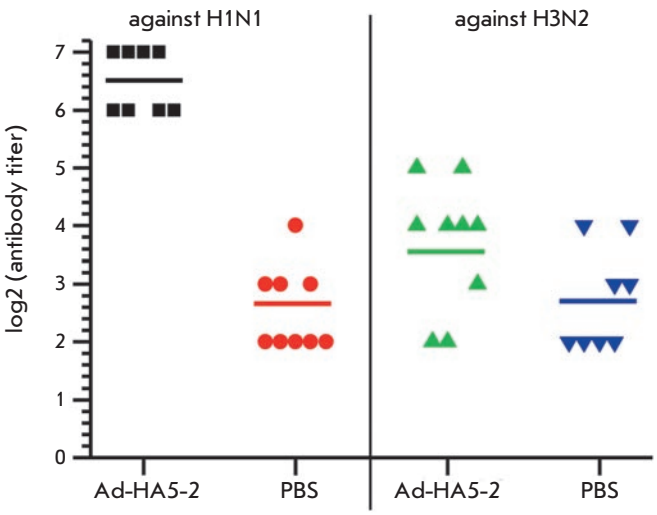

Fig. 4. The level of specific hemagglutinating antibodies against H1N1 and H3N2 viruses in the sera of mice immunized with the recombinant Ad-HA5-2 adenovirus. The mice were immunized with the Ad-HA5-2 adenovirus twice. Control mice were immunized with PBS solution twice. The antibody titer in mice sera was assayed using HRR against A/USSR/90/77 (H1N1) and A/Aichi/2/68 (H3N2) viruses. The difference between the vaccinated and control groups as assayed by HRR against the H1N1 was statistically significant ($p = 0.05$), while the HRR against the H3N2 virus showed no significant difference ($p > 0.05$).

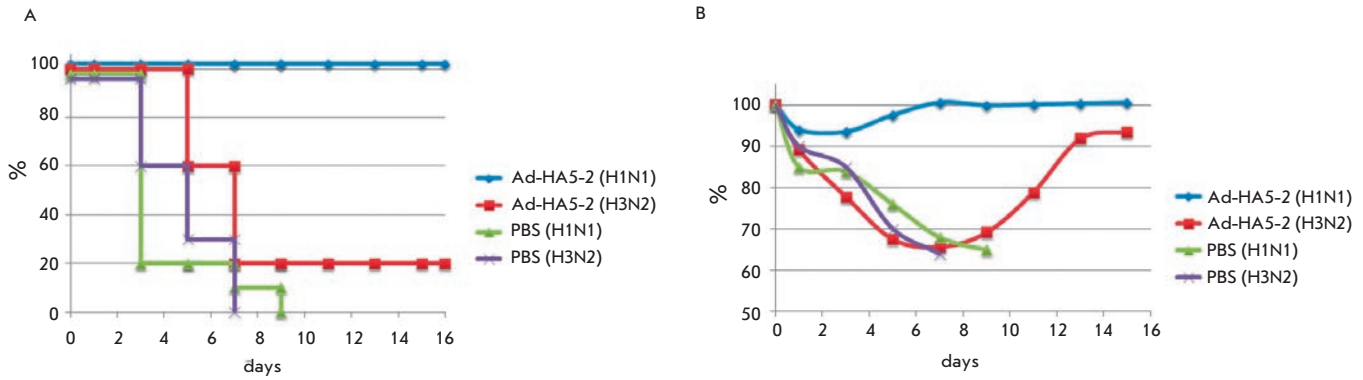

Fig. 5. Analysis of the protective hemagglutinin-specific response in mice immunized by the recombinant Ad-HA5-2 adenovirus and later infected by a lethal dose of either the H1N1 or H3N2 virus. (A) Survival of mice infected with a lethal dose (10LD50) of A/USSR/90/77 (H1N1) or A/Aichi/2/68 (H3N2) influenza virus. Mice were immunized with the Ad-HA5-2 twice in concentration 108 PFU/mouse. Control mice were injected with PBS solution twice. The differences in survival between the Ad-HA5-2 group (infected with H1N1) and the PBS control group were statistically significant, while the differences between the survival in the Ad-HA5-2 group (infected with H3N2) and the PBS control group were not ($p = 0.5$). (B) Changes in the weight of mice infected with a lethal dose (10LD50) of A/USSR/90/77 (H1N1) or A/Aichi/2/68 (H3N2) virus

Assaying the cross-immunogenicity of Ad-HA5-2-expressed hemagglutinin against H1N1 and H3N2 viruses. The cross-immunogenicity of recombinant hemagglutinin expressed by the Ad-HA5-2 adenovirus was assayed by hemagglutination-inhibition reaction. BALB/c mice were immunized by the Ad-HA5-2 adenovirus as previously described. Control mice were intranasally injected with PBS solution. Samples of mice serum were collected 21 days after the second immunization. These samples were assayed for the presence of antibodies specific to A/USSR/90/77

(H1N1) and A/Aichi/2/68 (H3N2) influenza hemagglutinin (results show on Fig. 4). Sera from mice immunized with the recombinant Ad-HA5-2 adenovirus were found to exhibit a pronounced increase of anti-H1N1 antibody titers in a hemagglutination-inhibition reaction assay when compared to the control group. There was no statistically significant difference in the hemagglutination inhibition levels between the anti-H3N2 reaction in sera from the Ad-HA5-2-immunized mice and the nonspecific inhibition due to components of the control murine serum.

The obtained result indicated the induction of a humoral immune response specific to the H1N1 influenza virus in response to immunization by a recombinant Ad-HA5-2 adenovirus. The efficacy of the humoral immune response against the H3N2 influenza virus proved to be insignificant.

Assaying the cross-protection of mice immunized with Ad-HA5-2 against the influenza A viruses H1N1 and H3N2. After the second Ad-HA5-2 immunization, mice were injected with lethal doses (10 LD_{50}) of A/USSR/90/77 (H1N1) and A/Aichi/2/68 (H3N2) viruses. Control mice were intranasally injected with PBS solution. This experiment showed that immunizing animals with the recombinant Ad-HA5-2 adenovirus protected mice from a lethal dose of H1N1 influenza virus. Mice did not die during the whole observation period, and their weight reduction was approximately 5%. The control group injected with PBS showed a 100% mortality over 9 days and a weight reduction of 30%. Immunizing mice with the Ad-HA5-2 adenovirus did not protect mice from a lethal dose of H3N2 influenza virus. The immunized mice exhibited 20% survival and almost 40% weight reduction (Fig. 5).

Discussion. Protecting the human population from the constant threat of influenza is problematic due to the difficulty of predicting the appearance of new pandemic and epidemic strains, as well as to the low cross-reactivity of inactivated and subunit vaccines, which are effective only against the strain of virus they were manufactured from. Vaccines which would provide a broad range of protection from potentially dangerous influenza virus strains are still a goal that is hard to achieve.

It is known that influenza infection can confer heterosubtypic immunity based on both the humoral and cellular immune responses. A heterosubtypic immune response can considerably decrease the duration of the disease and reduce the symptoms of infection by a different viral strain [21]. The induction of a heterosubtypic immune response occurs, among other things, due to the presence of conformational epitopes in hemagglutinin, which are conserved in various strains of influenza virus.

The existence of conformational epitope shared between several subtypes has been demonstrated for influenza A hemagglutinin [10]. In recent studies, various subtypes of the influenza virus have been divided into groups (clades) according to the presence of conservative hemagglutinin epitopes, which are recognized by antibodies that thus are able to neutralize a wide range of viral subtypes. This can be explained by the genetic relations between hemagglutinins from different viral subtypes. Fig. 6 shows a phylogenetic tree of the amino acid sequences of influenza virus A hemagglutinins for various subtypes. The H1, H2, and H5 subtypes are all related and thus have common conformational epitopes, while H9, H7, and H3 virus subtypes have a low degree of homology and thus do not exhibit cross-immunity among themselves.

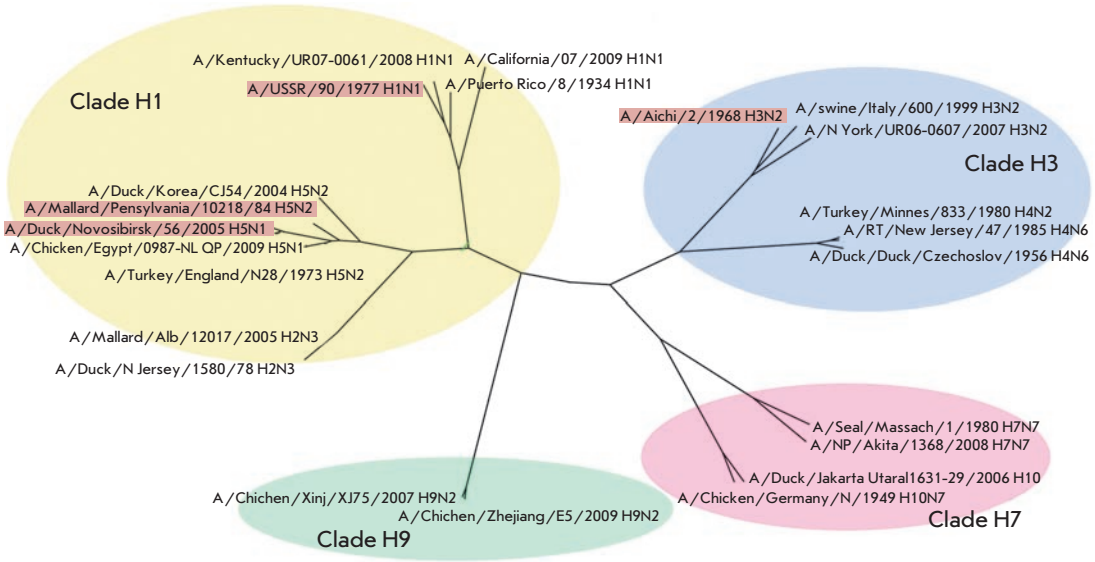

Fig. 6. A phylogenetic tree of the amino acid sequences of hemagglutinins of the influenza A viruses of various subtypes. The various subtypes are divided into four clades based on their degree of phylogenetic relatedness. The strains used in this study (highlighted by color) belong to clades H1 and H3

Since the first clade includes potentially pandemic viral subtypes (H5, H1, and H2), this makes broad-specificity vaccines against this clade especially valuable [12]. Thus, vaccines that preserve the stable ternary structure of surface antigens should provide effective protection from various influenza virus strains. The preparation of subunit and inactivated vaccines can often disrupt the structure of viral antigens. The use of live influenza vaccines does yield antibodies with a broad specificity, but this method has its own significant drawbacks.

Immunization by recombinant adenoviral vectors which bear the surface protein genes of the influenza virus leads to the expression of influenza virus antigens on the surface of the cell without disrupting their ternary structure, which in turn makes the induction of a heterosubtypic immune response, including the production of broad-specificity antibodies recognizing conservative hemagglutinin epitopes, possible. This study used adenoviral vectors with deleted E1 and E3 genomic regions, which allowed us to obtain replication-defective adenoviral particles and gave the potential possibility to use them as vaccine vectors. The advantages of using recombinant adenoviruses are the high level of transgene expression in a wide range of eukaryotic host cells, the induction of both humoral and cellular responses to the transgene, and their safety for humans (as tested on volunteers) [16]. The first phase of clinical trials for a nasal vaccine, based on a recombinant replication-defective human adenovirus of the fifth serotype bearing a hemaglutinin gene from the H5 influenza virus, was successfully performed in 2008 in the United States [13].

Our study used hemagglutinin genes from avian influenza viruses H5N1 and H5N2, since avian influenza viruses, H5N1 especially, are a cause of increasing anxiety. This virus is characterized by a mortality rate in excess of 50%, and, if it were to acquire the ability to spread from human to human, it would lead to a pandemic and cause huge casualties throughout the world. We have constructed adenoviral vectors Ad-HA5-1 and Ad-HA5-2 based on a serotype 5 human adenovirus, which contain hemagglutinin genes from viral strains A/Duck/Novosibirsk/56/2005 (H5N1) and A/Mallard/Pennsylvania/10218/84 (H5N2). An analysis of the amino acid sequence of these hemaglutinins shows a homology of 94.6% (http://align.genome.jp/). An analysis of the immunogenicity of the obtained Ad-HA5-2 adenovirus showed that a double instranasal injection of a recombinant virus expressing the H5N2 viral hemagglutinin gene induced the production of high titers of antibodies specific towards the avian influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2).

We have also demonstrated that a double injection of the Ad-HA5-1 virus expressing the H5N1 viral hemagglutinin gene induced the production of high titers or cross-reactive antibodies against the avian influenza virus A/Mallard/Pennsylvania/10218/84 (H5N2).

Immunizing mice with Ad-HA5-2 and Ad-HA5-1 adenoviruses protected the animals from a highly lethal dose (50 LD_{50}) of the A/Mallard/Pennsylvania/10218/84 (H5N2) influenza virus. A control immunization of mice by the Ad-null vector, which did not carry any gene expression cassette, allowed us to establish the role of the vector itself in the protection against influenza A viruses. It turned out that Ad-null immunization protected 20% of the animals from the influenza virus. This seems to be caused by the induction of a nonspecific antiviral immune response initiated by the injection of an adenoviral vector in the animal organism. Thus, we have demonstrated that recombinant adenoviruses, which bear the influenza virus H5N2 hemaggliutinin gene, can induce geterosubtypic immunity between H5N1 and H5N2 influenza virus subtypes.

In order to verify the hypothesis that recombinant adenoviruses can induce broad-specificity antibodies that can neutralize viruses of different subtypes belonging to the H1 clade (H1, H2, H5, H6, H11, H13 and H16), we used the A/USSR/90/77 (H1N1) virus, which belonged to this clade, and A/Aichi/2/68 (H3N2), which did not. According to a study

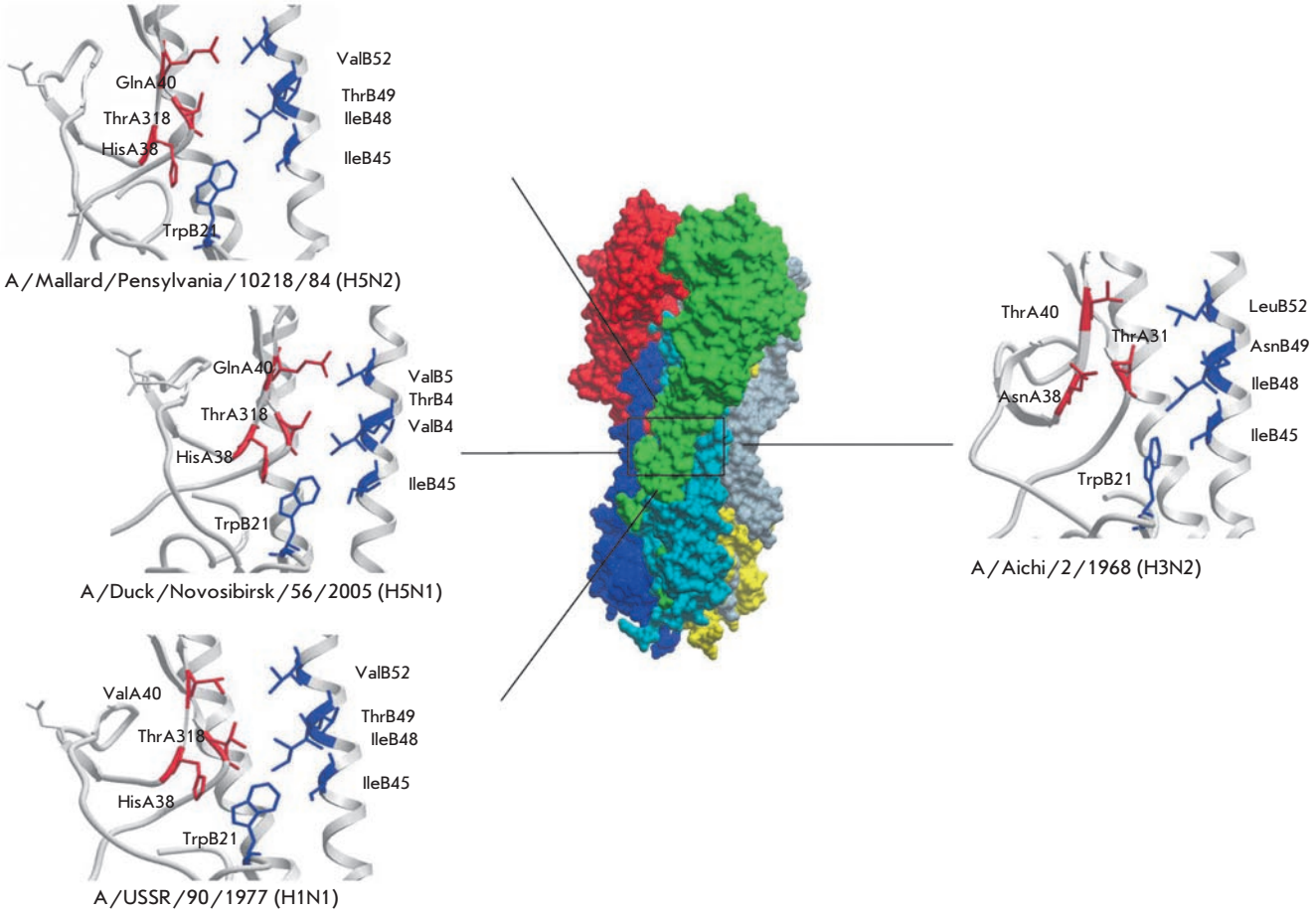

Fig. 7. Conserved conformational epitopes of hemagglutinins recognized by neutralizing antibodies specific to the influenza strains used in this work and belonging to caldes H1 and H3. The surface of the hemagglutinin trimer is depicted in the middle; the HA1 chains are depicted in red, green and grey; and the HA2 chains are shown in dark blue, light blue and yellow. The region which contains the epitopes for the neutralizing antibodies is marked by a black box. The epitopes and the key amino acids that form them are depicted in HA1 (red) and HA2 (blue) hemagglutinin chains of various influenza viruses: A/Aichi/2/68(H3N2) (1EO8), A/USSR/90/77(H1N1) (1RVX), A/Mallard/Pensylvania/10218/84H5N2(H5N2) (1JSM) and A/Duck/Novosibirsk/56/2005(H5N1) (2IBX)

by M. Throsby and his colleagues [14], H5 and H1 virus hemagglutinins have a common conservative epitope which is formed by the amino acid residues His38, Gln40, and Thr318 in the HA1 subunit of hemagglutinin and Ile45, Ile48, Thr49, and Val52 in the HA2 subunit. The hemaglutinins from H3 viruses have Asn and Thr in the 38th and 40th positions in the HA1 subunit, as opposed to His and Gln, and Thr and Val in positions 49 and 52 instead of Asn and Leu, respectively. An analysis of the amino acid sequences of influenza virus hemagglutinins in strains A/USSR/90/77 (H1N1), A/Mallard/Pennsylvania/10218/84 (H5N2), A/duck/Novosibirsk/56/2005 (H5N1), and A/Aichi/2/68 (H3N2), which were all used in this study, showed that the described hemagglutinin epitopes are highly homologues in subtypes H1 and H5. Only the strain A/duck/Novosibirsk/56/2005 (H5N1) has a single substitution (the Gln in position 40 is substituted for Val) (Fig. 7). This epitope is, however, significantly different in strain A/Aichi/2/68 (H3N2), which does not belong to the first clade of influenza viruses. It was shown that double intranasal immunization with Ad-HA5-2 induced the production of antibodies specific to the A/USSR/90/77 (H1N1) virus strain. The titer of antibodies that inhibited the agglutination of erythrocytes by the H1N1 virus in sera extracted from immunized mice was significantly higher than that in the control group sera. On the other hand, the antibody titers against the A/Aichi/2/68 (H3N2) virus as assayed by hemagglutination-inhibition reaction in immunized mice were statistically insignificant when compared with the control for nonspecific inhibition by serum components.

In order to assay the protective characteristics of the recombinant Ad-HA5-2 adenovirus against lethal doses (10 LD_{50}) of A/USSR/90/77 (H1N1) and A/Aichi/2/68(H3N2) viruses, we performed a double intranasal immunization of mice. We demonstrated that immunization by a recombinant adenovirus conferred 100% protection against a 10 LD_{50} dose of influenza virus A/USSR/90/77 (H1N1) and, on the other hand, protected only 20% of the mice from a 10 LD_{50} dose of the A/Aichi/2/68 (H3N2) virus, which probably indicates that this moderate pro-

tection was due to the nonspecific antiviral immune response induced by the injection of an adenoviral vector.

CONCLUSIONS

This study is the first to demonstrate that the introduction of an influenza virus hemagglutinin gene via an adenoviral vector into animal cells induces a protective heterosubtypic cross-immune response not only against the viruses of the same subtype as used for the immunization, but also against viruses from various subtypes which belong to the same clade.

These data allow us to conclude that adenoviral vectors can work as a universal basis for the production of vaccines against both seasonal and pandemic influenza virus strains. ●

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The article should include the following sections:

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].

There should be an abstract in English, which is to be a translation of the abstract in Russian.

RECOMMENDATIONS ON THE TYPING AND FORMATTING OF THE TEXT

We recommend the use of Miscrosoft Word 2003 for Windows text editing software.

The Times New Roman font should be used. Standard font size is 12 and the interval between the lines is 1.5. Using more than one 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 have spaces between them, for example A. A. Ivanov.

Throughout the text, except for the date of acceptance, all the dates should be presented in the "day.month.year" format, for example 02.05.1991, 26.12.1874, etc.

There should be no periods after the UDC code, 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 use a period and not a comma (0.25 and not 0,25).

Abreviated units in the Russian text should always use Russian lettering (мкМ, not μM; нм, not nm).

The hyphen ("-") is surrounded by two spaces, while the "minus." "interval," or "chemical bond" symbols do not require spaces.

The only symbol used for multiplication is "×"; the "x" 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.).

Numeric data should be presented in tables.

Formulas must use the letter of the Latin and Greek alphabet.

Latin genera and species names should be written in italic, while the taxa of higher orders should be written in regular font. Gene names (except for yeast genes) should be italicized, while names of proteins should be written 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.

GUIDELINES FOR ILLUSTRATIONS

Figures should be supplied in separate files.

Figures should have a resolution of no less than 300 dpi for color and half-tone images and no less than 500 dpi for black-and-white illustrations.

Files should not be saved with unused Path and Alpha Channels and they should not have any additional layers.

Files in Adobe Photoshop (up to 7.0) format, TIFF is accepted.

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 for print. 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 that are specialists in this field of research, and the editorial board makes its decisions based on the reviews of these specialists. 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 print. The published article has the submission and publication acceptance dates printed at the beginning.

The return of an article to the authors for improvement does not mean that the article has been accepted for print. After the revised text has been received, a new decision is made by the editorial board. The author must return the improved text together with the original text and responses to all the 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 received the comments; if it is not, the article is considered a resubmission.

E-mail is used in all the stages of communication between the author, editors, publishers, and reviewers, so it is of vital importance that the authors should monitor the address that they list in the article and inform the publisher of any changes in due time.

After the layout for the relevant issue of the journal is ready, the publisher sends out PDF files to the authors for final review.

Changes other than simple corrections in the text, figures, or tables are not allowed on the final review stage. If this is necessary, the issue is resolved by the editorial board.

FORMAT OF REFERENCES

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.

Bressanelli S., Tomei L., Roussel A., et al // Proc. Natl. Acad. Sci. USA. 1999. V. 96. P.13034–13039 (If there are more than five authors). If there are less than five authors, all the authors must be listed.

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 placed on a separate page. The tables should be placed on a separate page, and figure captions should also be placed on a separate page.

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