To the readers of the first issue of *Acta Naturae*

We present the first issue of a new journal covering a wide range of problems related to life sciences. What could be the purpose of one more journal focused on these issues? The advanced capabilities of the Internet allow us to see hundreds of publications with various "impact factors" in the sphere of biomedical science. There are quite reputable and serious journals with long-standing histories. Every year, the number of these journals increases. It is rather difficult for journals to find their own niche and demonstrate originality. Nevertheless, we decided to publish a national journal in both Russian and English and hope that it will be of interest to our readers. Our hope is based on the likely interest of both Russian and foreign readers to the recent developments of the most advanced laboratories and scientific centers of Russia. This peer-reviewed information will be presented as brief journal reports. In spite of pessimistic prognoses, Russian science survived the deep crisis of the 1990s and, in the last 3–4 years, many academic and university centers have showed progress in their development thanks, in large part, to an increase in domestic financial support and specific personnel decisions. Reviews of invited foreign and Russian authors devoted to the actual problems of biomedical science will be the second-most important constituent of our new journal. We believe that these sections will be of interest to both young students and veteran researchers. We don't plan on avoiding the social and organizational aspects of scientific development, either. Hence, we are planning to publish interviews with leading scientists, representatives of business interested in knowledge-intensive production, and officiaries. Hopefully we'll manage to create a tribune for discussing the relevant aspects of scientific development in Russia. We are aware that "living science" is closely related to the applied aspects of biopharmaceutics, and it is not improbable that the lack of information and awareness of Russian researchers in this sphere will be compensated for by our journal. We would like to see representatives of Russian and foreign biopharmaceutical businesses as authors in our new journal. If we succeed, we hope to see experimental articles by foreign authors on the pages of *Acta Natura* to make it more international.

We wish all the readers of our new journal success in their scientific studies and practical work, and we are looking forward to cooperating with them.

Editorial Board

Dear Colleagues!

On behalf of the Russian Federation`s Agency for Science and Innovations, I would like to congratulate each and every one of us on the first issue of the journal *Acta Naturae*, which is a radically new and totally unique journal in our country.

The modern post-industrial world is interested in those areas of knowledge which are related to both the fundamental problems in biological sciences, as well as to the practical applications of biology. Biotechnologies have not only become a priority for scientific development, but they also encourage significant growth in new economic sectors of the leading countries in the world. Applied problems solved with the help of biotechnologies have an effect on several industries and, on a different level, affect the country´s national security.

The field of "Living Systems" represents one of the most important priorities for the research and engineering development of our country. The state reasonably considers it one of its major tasks to encourage the development of this sphere, both from the standpoint of developing science-driven and competitive economic branches and from the standpoint of bolstering security. The development of this priority direction is actively supported by the Federal Target Program Research and Investigation of First-Priority Aspects of Russian Science and Technology in 2007-2012.

The "Living Systems" priority direction is second only to the Industry of Nanosystems and Nanomaterials in terms of the amount of financing within the framework of the Federal Target Program. We assume that this direction will become the first priority for the state, since its development can positively serve a social purpose. It is biotechnology products that have such a great influence on the quality of life of the population.

Let us briefly consider some indicators that can reflect the activity of the Federal Agency for Science and Innovations within the framework of the Federal Target Program Research and Investigation of First-Priority Aspects of Russian Science and Technology in 2007-2012 devoted to the priority direction of "Living Systems."

Currently, work is being conducted under 299 state contracts within the framework of the "Living Systems" direction (as a whole, 7 different priority directions are being developed under 1,361 state contracts within the framework of the Federal Target Program). Some 403 organizations (1,391 in all) are named in the contract for the "Living Systems" direction as the lead organization or as co-executors. Some 939 applications for theme formation (3,180 in all) and 396 competition applications (1,957 in all) were registered and logged. Organizations representing 23 different departments took part in the competitions within the framework of the "Living Systems" priority direction. Some 17 departments on.

As for the financial support for projects within this priority direction, in the period from 2005 to 2008 the budgetary funds spent for this purpose increased from 1,168.7 to 3,242.6 mln roubles (23.5% of the total sum spent on the financing of projects on behalf of the Federal Target Program).

One of the factors that favor the rapid development of the biotechnologies is the interdisciplinary nature of the projects being conducted in this sphere. Not only biologists, but also specialists from different fields of science, such as physicists and chemists, are involved in these projects.

Inna Bilenkina: "Biotechnologies are one force driving the intense growth of new types of economies in advanced countries"

Therefore, when we speak of creating a unique journal, it is because this journal will foment improved relations between representatives from different fields of science. Moreover, by virtue of the fact that research projects under the priority direction of "Living Systems" are carried out within institutions or organizations with different departmental affiliations, we hope to see this new journal as a unique vehicle for interdepartmental exchange of information, which will be of interest to scientific organizations, investors, and federal departments involved in the development of the state, innovative policy in those spheres being profiled.

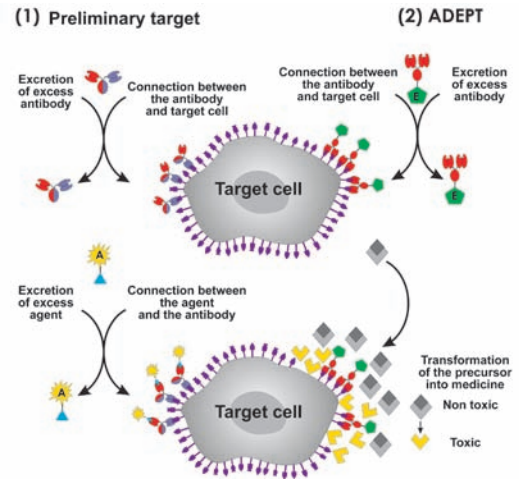
We hope that *Acta Naturae* will be useful to both representatives of basic sciences and to specialists within applied sciences. It is quite possible that applied sciences will be the object of greater attention, since the journal is devoted to technologies. Therefore, we would like to see the results of work which will find practical use in the materials published in our journal. In our opinion, the information policy of our journal shall pay significant attention to analytical industrial surveys focused on the development of different spheres within the living systems, their condition in Russia and in the world, the mechanisms of the commercialization of products produced during the course of conducting projects within the framework of the "Living Systems" priority direction. This approach will increase the interest paid by investors to this priority direction.

In conclusion, I would like for *Acta Naturae* to create its own particular niche within the range of modern scientific and engineering periodicals, while contributing to the progress of Russian biotechnologies and helping them become one of the fundamental pillars of Russian innovative economics.

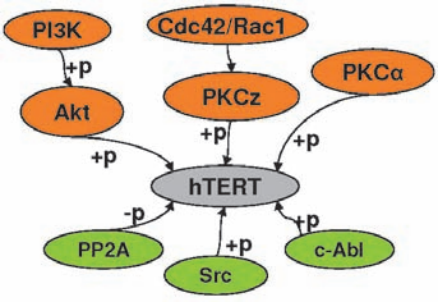
Inna Petrovna Bilenkina,
Deputy Director, Federal Agency for Science and Innovation, Russian Federation

S. M. Deyev and E. N. Lebedenko

Modern Technologies for Creating Synthetic Antibodies for Clinical Application



Scheme of two-stage target-killing technologies.
(1) Pretargeting using antibodies with the following target-killing by the involved A agent.
(2) Preliminary delivery of E ferment to the target. At the second stage, E ferment transforms the nonactive medicine precursor into an active form (ADEPT, antibody-directed enzyme pro-drug therapy).



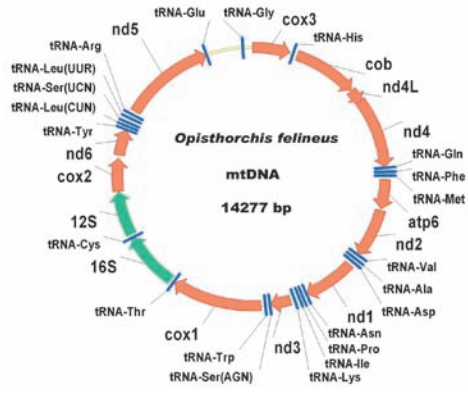
D. A. Skvortzov, M. P. Rubzova, M. E. Zvereva, F. L. Kiselev, O. A. Donzova

The Regulation of Telomerase in Oncogenesis

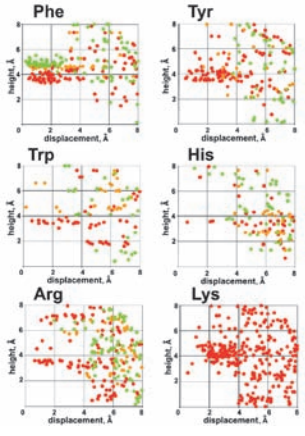
Regulating the activity of the protein hTERT by phosphorilation–dephosphorilation. Activators are shown in orange, inhibitors are shown in green. "+p" means phosphorilation, and "-p" means dephosphorilation.

V.A. Mordvinov, A.V. Mardanov, N.V. Ravin, S.V. Shekhovtsov, S.A. Demakov, A.V. Katokhin, N.A. Kolchanov, and K.G. Skryabin

Complete Sequencing of the Mitochondrial Genome of *Opisthorchis felineus*, Causative Agent of Opisthorchiasis



Genetic map of the *O. felineus* mitochondrial genome.



T. V. Pyrkov, D. V. Pyrkova, E. D. Balitskaya, and R. G. Efremov

The Role of Stacking Interactions in Complexes of Proteins with Adenine and Guanine Fragments of Ligands

The distribution of aromatic rings and positively charged side chain groups of amino acids around the guanine moiety of various ligands in complexes with protein receptors. The color red corresponds to $\cos^2\alpha = 0.6 - 1.0$ (parallel orientation), green corresponds to $\cos^2\alpha = 0.0 - 0.4$ (T-shaped orientation), and yellow corresponds to intermediate geometry. Here, α is the angle between the planes of both rings. For Lys this value is not defined.

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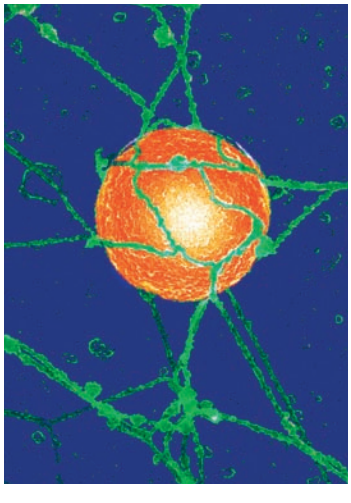
CONTENTS

RESEARCH ARTICLES

The photo on the cover shows Kahal's body in frog oocyte. The picture was taken by E.V.Kiselyova with the use of Field Emission in Lenz scanning microscope ISI DS 130F, Topcon Corporation, Tokyo, Japan in the laboratory of Prof.T.D.Allen of the Patterson Institute of Cancer Research, Manchester.
The photo was awarded the first prize in the Beautiful Science contest held by *Science and Technologies in the Russian Federation - STRF.RU* journal with the support of the Federal Innovation Agency of the Ministry of Education and Science of the Russian Federation.



VAK: Protecting High Standards in Science

On the role played by the High Attestation Commission of the Russian Federation (VAK) of Russia in managing domestic science, criteria, and professional selection, as well as the role the of scientific periodicals in this process; *Acta Naturae* talked to the president of this commission, academician Mikhail Kirpichnikov of the Russian Academy of Sciences.

Mikhail Kirpichnikov: "we do not subdivide journals into bad and good ones; we subdivide journals into those can be used for expertise and those cannot."

Science as a systematic study of natural objects and social and humanitarian phenomena does not have any well-defined national framework or boundaries. Science separated from the international community withers and dies. Due to the supranationality of science, the role of scientific periodicals, especially in our country, is constantly under discussion. In particular, the question arises of whether it is possible to use formalized publication requirements to estimate the state of scientific research in Russia. And how extensive is the need for national and international periodicals?

This is a rather complicated question. I'll take a roundabout approach to the subject. My guess is that the formalization level will always change. Many things may not be formalized in principle, not only in science, but in acts of creativity on the whole, largely (but not only) because of their creative constituent. This is why this discussion will never end. Indeed, it is simpler to formalize all things, to call for "police" control of the "scientific movement" in accordance with current legislation than to create a regular expert system. I consider it essential to estimate the quality level, especially when speaking about an outstanding specialists, by formal quotation and the impact-factor of the journal where his or her articles are published. However, a non-formalized estimation is important as well. Thus, I stand for balance between formal requirements and a normal expert system. It ought to be noted that the expertise level and points will change with time. But a definite number of points will remain beyond formalization. That is the major problem.

It is widely believed that Russian authors are often discredited against in international periodicals: i.e., they are rarely referenced to due to their Russian affiliation. Do you agree with this opinion?

I have similar views on this problem. I think that discrimination exists. I don't know if it's significant, but I am sure it is present. In my opinion, the factor of having lived in a closed society played a negative role in that situation. When our society became open (I avoid the word "economics," because "society" is a wider term), pressure was removed. However, this process is

slow, it may take years.... For the time being, the "closed-society" mentality remains.

ANALYSIS OF RUSSIAN SCIENTIFIC PERIODICALS

Where do you consider the difference to be between the quality of Russian science and the quality of science management, including the regulation of the appraisal system of higher scientific qualification for the personnel controlling the quality of scientific periodicals?

Indeed, Russian science has some problems, as does the appraisal system of higher scientific qualification personnel, by which I mean the High Attestation Commission of the Russian Federation (VAK). What role do scientific periodicals play in the appraisal system? I emphasize, not for me as a scientist, but for the appraisal system? How do we try to use it? First of all, as an external expert. Moreover, a priori we are at liberty to choose the rules of ordering external experts, and being among these external VAK experts is not a special indicator for a journal. If the results of VAK performance are useful for scientific periodicals, we'll be very glad. As often happens in science, an unintended result can be as useful as the achievement of the stated goal. There are many examples: the creation of a collider led to the invention of the Internet. What is more important now? They are at least comparable. However, when construction began at the European Organization for Nuclear Research, nobody planned to create the World Wide Web, this much is certain. My position is as follows: I don't want the problems of the appraisal system to be confused with the problems of Russian scientific periodicals.

Not long ago, you took part in a conference titled Scientific Periodicals in Russia: Topical Issues and Developmental Prospects in Present-Day Conditions, which was held at the Moscow Polytechnical Museum. What is your assessment of the conference's results?

First, I must note that we were not the initiators of that conference, but when we learned about it, we joined with enthusiasm, because it was a useful event. In any case, we planned to gather editors and publishers to discuss our prospects. In this context I am fully satisfied with the conference's results. Actually, the conference organizers set a more important and more relevant mission: to discuss the problems of Russian scientific periodicals. I'm talking not as the president of VAK, but as a representative of the commonwealth of scientists. My speech at the conference was devoted to that very question of separating the problems of scientific periodicals from external expertise related to the VAK List.

THE VAK LIST OF JOURNALS IS A TEMPORARY MEASURE

What place is given to the so-called VAK List (the list of Russian and foreign journals where the results of scientific investigations are to be published for defending a thesis) in this aspect of science?

We regard the VAK List as an element of the existing scientific attestation system. The attestation begins with an applicant. Then it involves a reviewer; leading organization; thesis council; then the VAK Expert Council; and, finally, VAK proper. All of this is the attestation system that should come to mind when you pronounce the word VAK. I am deeply convinced that the main problem in the attestation system that remains relevant to this day is the catastrophic decline in the level of theses written in the 1990s and the early 2000s. It was not just this decline, but "loss of genre," as I usually call it. By this I mean that, according to the original and current rules, thesis work is meant for educational research attestation; this is scientific qualifying work, not the draft laws often offered today or projects of certain instructions. However, I by no means diminish the importance of this activity. A draft law enacted may be more important than thousands of theses, but it is another genre. There are many different methods to increase the theses quality level. Some of these methods are within the framework of the attestation system considered. In my opinion, the thesis council is the most "complicated," yet most efficient, component of this system. Alterations at this level may lead to significant changes in the system proper. External expertise is another way to that quality. One problem with external expertise is that of scientific periodicals, and it is only one of several important problems. Currently, patents, as an element of external expertise, are legal, and I think this was a great achievement. Patents are as important as publications for thesis defense in engineering sciences. In their turn, monographs are of prime importance for humanities and social sciences. I mean serious reviewed monographs.

Hence, today the VAK system needs the same external collective expertise as scientific periodicals. This problem appeared a long time ago, and we were not the first who started discussing it. As early as 2001–2002, our predecessors offered the first draft of the VAK List. However, as often happens in Russia, the documents existed as a normative basis rarely taken into account even by their creators. Until 2005, the VAK List was discussed rather rarely, and it attracted attention only after it began to be applied. Then, finally, people felt that it was a matter of great significance.

I understood from the very beginning that it was essential to get off the VAK List, as such, and since the end of 2006 I have publicly discussed that problem. It cannot be emphasized enough that this measure is absolutely compulsory and temporary. As was mentioned above, this problem arose due to the decline in the theses' quality and the violation of the "purity" of that genre. As a consequence, we finally escaped the "cursed heritage of the past" (appeals from the doctorate theses of 1999-2000 defended without a single publication) only by the end of 2007. Several kinds of reports, conference theses, and so forth were offered as publications. Eventually, the good idea of involving external expertise was offered. However, external expertise was rarely used, but that is a secondary problem. From the mid-1990s to 2006, theses defense was characterized by exponential growth rates (almost 15% a year) and it was only in 2006 that an end was put to the chaos.

Let's get back to the List. The rules for getting onto the VAK List were assumed to be made maximally transparent so that the choice of the jour-

nals would depend neither on the VAK President, nor on VAK (minimally, at least), nor on expert councils. The primary task of VAK, the VAK President, and the experts is to offer requirements. As soon as the requirements are written, it is essential to meet these requirements in order to get on the VAK List.

Initially, those rules were observed.... Did we succeed? I don't think so. However, from the very beginning it was clear that it was a temporary measure, and later on we planned to get off the special VAK List and proceed to public databases. This goal has been declared, manifested, and presented on the VAK site since 2008.

Will the VAK List be replaced with a Public online Database?

How do you envision future VAK policy concerning the question under discussion?

It is important that we are the first, and, in contrast to our previous activity, we are going to be offering this kind of VAK policy for the List for five years. Today, the presence of journals in the public international bases of scientific periodicals, such as Web of Science and Scopus (this list may be extended with time), is sufficient enough to be included in the VAK List. If a journal falls to the above-mentioned databases, it automatically rates as a compulsory publication in the VAK List. It will be the same for Russian journals. Today, this requirement is too stringent for them (only 200–300 journals meet this requirement). And we are still carrying out attestation in Russia, which is why we offer the five year transition period.

What do you mean by "stringent"? The quality of articles or the publishing business?

I don't want to discuss the publishing business, because it can sidetrack the question under discussion. I guess that is a problem for the corresponding authorities. As for us, we adopted some amendments to our requirements at the recent VAK conference and are planning to publish them. Moreover, the requirements to be published will be valid for five years. These requirements take into account many of the proposals offered at the conference in the Polytechnical Museum, and they will be valid for five years. However, if we see any changes in Russian journals, the requirements will change as well.

As for the journals, initially they were not happy because they didn't want to change anything. After collective serious discussions, we found that they were not ready for change. For instance, the journals could not afford online full-text versions. We removed that requirement. Previously, the journal issue should have been followed by the issue of its online version. Now there has to be an online version issued by subscription or it has to be free after one year. Thus, all claims concerning the commercial problems of the publishers and all questions related to their opposition to that deal were settled. If you want, you get one additional year, if you don't, I return to my first declaration. We represent VAK and thus are entitled to choose the experts who will meet our requirements. If you don't like something, nobody forces you. I have already said at the conference and can repeat now that we do not subdivide the journals into bad and good ones; we subdivide the journals into those that can be used for expertise and those that cannot. You must admit that it would be absurd to compare Playboy with Nature.

Actually, you control only one instrument: the List. You cannot close it, can you?

No, I cannot. I know what is behind this considerable interest in the problem of journals, but it is a drop in the ocean compared to attestation. For us this drop is one of numerous expertise instruments, but it can incidentally relieve scientific periodicals of journals that are not reviewed or poorly reviewed. In spring of 2006, the government submitted in its decree a claim to have at least one compulsory publication in the journal from the VAK List for thesis defense. At the same time, compulsory publications of doctorate and Ph.D. theses abstracts on the Internet became obligatory as well. Today, these requirements are being fulfilled and it matters a good deal. That measure did not cause violent indignation: all went down well from the very beginning of its introduction. Which is why this measure is unavoidable. We are proceeding to move in this direction, though it is rather difficult due to poor financial and technical support. I think that security systems with online access will appear in the course of

time, and it will be possible to get past the security system and ask an applicant any question online.

In principle, the list is a disputable and temporary thing; it is really an administrative measure. Today it is the only real method that can have an effect on the current situation.

Today, if you want your article to be published in certain journals, including good ones, you have to pay…

It is common practice. However, it is important to know the publication rules of a particular journal beforehand. A journal should publish rules for the authors. This is one of the new requirements. If a journal states that one page costs one ruble, then it shouldn't cost one thousand rubles. The difference appears due to "under the table" calculations, "black market," etc. And I speak out against this very practice. There is a range of authoritative international paid journals; it is a common practice. Another problem, likely related to the realities of our country, is the requirement for all journals wishing to be in the VAK List to institute free publication for graduate students. This requirement included in the new criteria often gives rise to criticism. Clearly, this requirement is not perfect, but there are no perfect solutions. What should we do with an article written by a graduate student with six doctors of science as co-authors?

In 2006–2007, many complaints such as "they take money" were heard. But give me at least one address where the rules are being violated. The only thing VAK can do is to remove a journal from the VAK List. But no applications demanding this have been submitted yet. You see, it is difficult to solve this problem without a special document. On the other hand, it is clear that authors are apprehensive about their fates.

You have data on all candidates and doctors of science. How deep is this database?

I would say that it exists and is in order. In general, intensive use of IT-technologies is one of the most important directions of our work. For instance, we are ready for a storm of protests which are likely to arise if we offer to publish full-text versions of theses online.

On the other hand, if we wish to be united with the whole civilized world, we are obliged to turn to online publishing. However, taking into consideration the fact that this whole system of passwords and so forth will barely work in Russia (technically it is quite possible, but mentally it is not), I accept the position of the publishers that was declared at the conference at the Polytechnical Museum. With time I think we'll manage to put this measure into execution as well.

One more important thing. We are going to fight for the equality of online publications included on our list. And this is a rather complicated task. Sometimes it is difficult to explain that an online journal is as good as a printed one. In the past, it was difficult enough just to explain the necessity of online publication of theses abstracts. It is essential to convince people that the publications will no longer be stolen.

PURCHASE, SALE, AND THEFT OF THESES

Does VAK have any computerized system for fighting plagiarism?

We have the Antiplagiat system, which was launched and advertised in a big way. This system exists today, but due to financial and technical problems it operates in the pilot regime. Few terminals have access to this program. I guess that in a country as big as Russia the Antiplagiat system is doomed to operate in the pilot regime, because about 30,000 theses will scarcely be subject to check by this program. However, it has proven to be effective several times.

I think it is essential to come to a kind of solution that may be comparable to a stoplight. It is obvious that many people do not observe traffic rules, some of them run a red light, but most people do stop because they know that they may be caught, penalized, etc. Thus, the fact and declaration that we use the Antiplagiat program is more important than the capture of one or two unprincipled authors and public punishment.

Nevertheless, public responsibility is quite a useful measure. This measure concerns all the attestation stages (from applicant through opponents and public councils); i.e., everyone is responsible for their actions, and if somebody violates the rules, his or her reputation can suffer significantly. However, public responsibility has some disadvantages. Declarations in the presence of the mass media can lead to court proceedings, because people caught red-handed tend to be fearless and are of the conviction that those who shout louder are right. This situation causes a lot of problems. The commission consists of only 45 people who have to withstand this pressure.

But the problem appears to be political; many officiaries, deputies, and businessmen are eager to defend theses in the sphere of economics, sociology, and management, discrediting the system of scientific attestation. They wish to become academicians, doctors of science, and so forth, having written the thesis New Information about Management in the Middle of Nowhere.

In regards to the problem of bought theses, it is rather difficult to calculate their percentage in the total number of theses defended, but I do not think it is high. Nevertheless, it does happen. I don't often travel by subway, but once I had to and heard people discussing the places where defending one's thesis was cheaper. It is disgusting. The moral consequences may be really disastrous. I realize how much such facts damage scientific prestige. But in similar cases I always make reference to the entropy increase law, which acts in both nature and in the social sphere, everywhere. The first provision of this law is as follows: if you do nothing, chaos will grow; if you want to create order, you will have to deploy great effort.

BAD GRADUATE STUDENTS AND BAD THESES

Is the low quality of theses prepared for defense related to the low quality of postgraduate education?

Unfortunately, today, according to the normative basis, VAK is responsible only for the attestation of highly qualified personnel. Preparing highly qualified personnel does not fall with-

in VAK's duties. Moreover, VAK can by no means influence this situation. I often bring up this question. This is a great gap in the normative basis, and we must solve this problem somehow. The situation is as follows: organizations that have few candidates open postgraduate schools and send someone to ask VAK to approve the opening of a thesis council. Thus, postgraduate students appear without the participation of VAK and ask for VAK's expertise only to open a thesis council.

Comparing the postgraduate schools of 1996, which had 60,000 students, to those of 2006–2007, which had 150,000 students, we observe a three increase fold in the number of postgraduate students. It is clear that no attestation system, strict or not, can work properly in these conditions. Moreover, colossal external pressure exists, we try to fight against it, but it is rather hard because it is objective. Almost half of all postgraduate positions are given to social and humanitarian sciences, while only 5% of scientists carry out investigations in this sphere. This situation reflects on the attestation system. About 50% of theses defended belong to these spheres. As a result, we see a theater of the absurd. However, this picture does not reflect the interests of society and the state.

It turns out that VAK has minimum authority in this situation, correct?

We have no authority at all in preparing graduate students. As for postgraduate studies, I quite agree with Bauman Institute Rector Igor Borisovich Fedorov: postgraduate studies in the sphere of technical and natural sciences must extend over at least four years, or we'll have profanation. I think that the Ministry of Science and Education shares my opinion, but the force of inertia is too great.

ATTESTATION SYSTEM IN THE FUTURE

What is the difference between defending a PhD and a doctorate thesis?

The doctorate thesis to be defended is examined by the VAK expert council. The PhD thesis is considered to be defended as per the decision of only the thesis council, which is why PhD theses cause more problems. To confirm this decision, the PhD thesis is directed to VAK, and the expert council studies it selectively. There are exceptions when a person who does not work in the scientific or educational sphere plans to defend a thesis. These authors, by decision of the Presidium, are called by the Expert Council to check their qualification. Hence, the PhD thesis becomes an object of VAK expert council attention under exceptional circumstances. This brings about the following question: do we need VAK or not?

In the future, the personal attestations of both PhD students and doctors of science will be entrusted, not completely, but to a large extent, to universities and strong scientific organizations. Today, conditions are unfavorable for achieving this idea.

First it is necessary to create a system of our own Cambridges, Oxfords, and MITs with the same type of authority and image consciousness, and then plan the creation of a new normative basis. We'll need many years to do this. However, if we remove this barrier, soon we'll have not 30,000 but 300,000 theses a year. That's what we'll get. I guarantee it will happen immediately. That is our psychology.

Moreover, it must be kept in mind that the system of social support is related directly to scientific degrees.

Finally, one more idea is related to the attestation system and its development in the future. I don't know when we'll be ready to entrust the attestation to certain institutions of higher education, especially in spheres where the participation of the state must be significant: medicine, science, education, and national safety.

Does this mean that the state must not participate in the attestation at all, as many carelessly claim? No, I think that only personal attestation must be eliminated from the state functions. However, our state, like all civilized countries, is obliged to attest those thesis councils which carry out attestation and regulate their level. Such authorities are present in every country.

Will those who were attested under softer conditions be subject to reattestation?

No, I think that a law cannot be retroactive. As I understand it, you are asking if it will be unfair to those who will defend the theses in tougher conditions? If we have admitted the mistake of "lowering the bar" (I think everybody has admitted it), we should correct it.

You see, the main problem is the competitiveness of our cadres. There can be tragic consequences, as platitude produces platitude. But this is a question of scientific prestige!

I'll tell you my opinion about the attestation system of the academic and teaching staff: in spite of all the disadvantages of VAK, I am sure it is the best expert system in Russia, the most developed and advanced. It can be used as a model for creating public state expertise concerning all the most important questions. We pretty much have only one such example. VAK is a dual system, consisting of state and civil institutions made up of scientific and scholastic corporations combined with academies, communities, rector unions, etc. It makes sense.

Cooperation within this system is rather complicated, and we understand that. For instance, why does the expert system, which works in the West, barely work in Russia? There are two major reasons. First, free expertises have never been good, and VAK has not received any money for ten years. Now we get financial support but cannot obtain confirmation on how to spend it.

The second cornerstone, which serves as a basis for expertise, is public responsibility. Experts should know that if they make a mistake, they will be outcasts in their communities. The issue is not about corruption, which is spoken about much more often than necessary; the plain truth is that Russia is known for so-called "nepotism" in a wide sense of the word. For instance, Ivan Ivanovich does not demand money from Ivan Petrovich, because he has known him for 30 years and simply cannot refuse.... There is only one method to fight against nepotism. When I became VAK president, the first thing I offered was the replacement of 40% of the expert council. I did so not because people were stealing, but because I believe that no one can be a member of the expert

council for 30 years; you become a link in the chain, even if you don't wish to. The expert system must be subject to rotation; no one can be a member of the expert system for ever.

The next stage is the thesis councils. I am sure it is the largest component of the attestation system. In 2006, we called off all the one-time councils. What was a one-time council? A very important person in a big car arrived at VAK and, using administrative and occasionally monetary resources, forced the creation of a council. In recent years, no similar council has been created. The network of reserve councils (about a thousand) was liquidated. Actually, there were some good councils. All good councils of the reserve network were transferred into the main network. That was the first stage of work aimed at optimizing the thesis councils. In 2007–2008, we carried out complete reattestation of the councils. Today all rearrangements have been completed, and the system has achieved a dynamic balance. The number of closed councils is similar to that of opened councils. In early 2006, a little over four thousand thesis councils existed. Today only three thousand are working.

Is it necessary to introduce special levels of scientific qualification for managers (economists)?

I think something is necessary, but not scientific qualification. Let's leave aside for a minute different kinds of impostors. We are talking about (a) honest, (b) ambitious, and (c) brilliant specialists in their sphere. They wish to be attested by society and by the state. The same situation is characteristic of scientific and educational attestation. We can either try to fight affectedly against this situation, inevitably creating niches for corruption, or take the road of "horizontal conformity." Today we have a system of scientific and educational attestation represented by the existing VAK. Administrators, practicing lawyers, and businessmen must get a chance to receive a corresponding status. It is possible to do this within the framework of the current attestation system. However, the rules of the game will change, and new experts will be needed. People will get a chance to defend theses within the framework of this state and public attestation system. This system is not entirely analogous to the "magister/doctor of business administration" system, but they have some similar features. We'll be able to tell to these honest and ambitious people that a doctor of economic sciences is no better or worse that a doctor of business administration. The same rule works for a doctor of business administration: he is no better (or worse!) than a doctor of physical and mathematical sciences. Thus, it is essential to travel the road of "horizontal conformity." How will this situation work out? First of all, I'll repeat again: the social order must be satisfied. I consider it senseless to fight this situation. This is just one idea. I would like to talk about it. How will the situation work out then? These measures will surly reduce the pressure on the "boiler of scientific and educational attestation." On the other hand, our governors, politicians, and businessmen still want to be "great" scientists rather than "great" administrators (there are some objections to that).

As a matter of fact, the state can set a standard and declare that it will accept people for work in the organs of state administration who have a Masters of Business Administration degree (for instance).

"OLD" AND "NEW" SCIENCES

Is it necessary to change the classification of scientific qualifications due to the appearance of "new" sciences which study phenomena at the junction of "old" sciences? What is VAK's attitude towards this question? And what should people working at the interdisciplinary level do?

The last time scientific qualifications were reconsidered was when I was a minister. In 2006, I as VAK president wrote a letter to Andrei Alexandrovich Fursenko, where I offered to reconsider the qualifications. Science does not stand still, it constantly develops. Moreover, according to normative documents, this procedure shall be carried out every five years. I did it consciously in order to explain that was not the responsibility of VAK, but of the Ministry of Science and Education. This is a problem of scientific and technical policy. Scientific and technical programs and so forth, as well as qualifications for defense (and, accordingly, the thesis councils), should be shaped on the basis of scientific qualifications. It is essential to raise the issue of the correspondence of scientific and higher educational qualifications, which has never been done. This is more relevant than comparing non-conformity between our qualifications and Western qualifications. Now the ministry has all the required authority, and, thus, a unique chance to solve all the problems. Hence, scientific qualifications are not a problem of VAK, but of the state's scientific and technical policy. VAK is a consumer of this system as are the institutions of higher education.

Now I'll express my own attitude to the situation under discussion. Honestly, nature knows nothing about physics, chemistry, or biology. It simply exists as a unique phenomenon. Then, human beings, thanks to the peculiarities of their mentality, started constructing models that led to the appearance of biology, chemistry and then to objective consequences. Absolutely objective physical, chemical, and biological methods appeared. But nature knows nothing about it! That is why, in my opinion, the most interesting things appear on the boundaries between the sciences. Even if we carry out more delicate subdivisions into biophysics, biochemistry, bioorganics, and molecular biology, the most interesting things will still happen not "under the lamp," but at the junctions. Everything happens there. I see no contradictions therein. It is impossible to build a model "for all times." The models must change. Our ideas about the sciences must change in accordance with the development of the natural science. I think this is relevant for society as well. Though, for sure, that is a subject for aspecial discussion. ●

The Intellectual Property of Scientific Organizations and Rights to It

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One of the most acute problems inhibiting the establishment of innovative economics in our country is the imperfection of the legislation regulating the production of marketable goods on the basis of new technologies. Many specialists—both scientists and representatives of business entities—are certain that the current laws cannot effectively influence the creation of innovative chains. It is not an exaggeration to say that the current laws are not focused on building a knowledge-oriented economy. It could be said that the problem of nascent intellectual property rights is the most important problem of the day. Who should be the proprietors of these rights? The scientists, business, or the state? And to what extent? Let's try to debate this situation and consider herein the rights of scientific organizations to the results of intellectual property obtained within the framework of their activity.

INTELLECTUAL PROPERTY RIGHTS FROM THE STANDPOINT OF CURRENT LEGISLATION

Up to the end of the last century, legislation placed a high emphasis on the defense of the state's interests in the spheres of military, special and dual-purpose research, engineering, and production work. In particular, Presidential Edict N 556, dated May 14, 1988, was issued. Within the framework of that Edict, the following government decrees were adopted: Government Decree N 1132 (d/d September 29, 1998) on High Priority Measures to Protect State Interest in the Process of Economic and Civil Turnover of Military, Special and Dual-Purpose Research, Engineering, and Production Work Results; Government Decree N 982 (d/d September 2, 1999) on the Use of Scientific and Engineering Activity Results; and, finally, Regulations on the State Control of Military, Special and Dual-Purpose Research, Engineering, and the Production Results of Work which Belong by Right to the Russian Federation approved by the Russian Federation Government Decree N 131, dated February 26, 2002.

All those documents gave the state a priori the right to the results of military and special and dual-purpose work carried out and specified the requirements for recording all resulting work.

The Russian Federation Government Decree N 982 (d/d September

2, 1999) on the Use of Scientific and Engineering Activity Results (with amendments d/d November 17, 2005) stated that the rights to the results of scientific and engineering activity obtained using funds from the federal budget are to be conferred to the Russian Federation, if
(1) the rights to these results are not included in privatized property in accordance with the applicable procedure;
(2) these results are not the exclusive rights of physical or legal bodies;
(3) these results have not been applied for in accordance with the applicable procedure to gain exclusive rights.

Most results of civil scientific and engineering activity obtained in the Soviet Union were assigned to the state or were not identified or registered at all, because at the time, scientific organizations did not give due attention to copyrighting the created material due to a lack of funds and commercial interest.

All this led to the following situation: most intangible assets were not included (or were partially included) in the balance sheet of scientific organizations and were not supported by any title documentation, in spite of the fact that the intangible assets were objects of economic activity (although not always legal).

The first step of state policy towards considering and defending the interests of direct executors and the commercial sector concerning the allocation of intellectual property rights in the scientific sphere was the adoption of the Russian Federation Government Decree N 685 (d/d November 17, 2005) on the Order of Disposition of Rights to the Results of Scientific and Engineering Activity, which approved the Regulations on the Assignment and Transfer of Rights to the Results of Scientific and Engineering Activity Obtained using Funds from the Federal Budget to Commercial Entities. (It should be noted that currently amendments to this Decree are being prepared due to the adoption of the Federal Law on Technologies to be considered below.) Then, the amendments initiated were adopted at the level of federal laws.

On January 1, 2008, Part 4 of the Civil Code of the Russian Federation (CC RF) came into force. This part regulates the legal relationships concerning the creation and turnover of the results of intellectual activity. At the end of 2008, the Federal Law on the Transfer of Integrated Technology Rights (N 284-FL, d/d December 25, 2008) was adopted in accordance with the CC RF program.

What is meant by assigning rights to the results of intellectual activity, including those obtained using budget funds in the context of new legislation?

The interaction between the scientific organization and customers is based on the agreement (for the creation, transfer, and use of scientific, research and engineering products; for performing scientific research and engineering and consulting and engineering services; for joint scientific, research, and engineering activity and allocation of profit). State contracts constitute a significant part of these agreements.

The CC RF places priority in allocating the rights to the results of research, engineering, and production work (hereafter referred to as "R&D"), including those subject to legal protection and contracts. According to Article N 1298 of the CC RF, the exclusive rights to the scientific work created by state contract belong to the executor, unless stated in the contract that this right belongs to the Russian Federation or to the executor jointly with the Russian Federation.

If, in accordance with the state contract, the **exclusive right is conferred to the Russian Federation**, the executor is obliged to acquire all rights through the conclusion of corresponding contracts with his employees and third parties for the further transfer of the rights to the Russian Federation.

If the **exclusive right is conferred to the executor**, he or she is obliged to submit a request as a state customer for a free ordinary (nonexclusive) license for the use of the corresponding scientific work for state needs to a third party.

If the **exclusive rights are conferred to the executor jointly with the Russian Federation**, the state customer is entitled to submit a free ordinary (nonexclusive) license for use of the corresponding scientific work for state needs to a third party after notifying the executor.

The order of allocating rights to any invention, useful model, or production piece created within the terms of the execution of a state contract is virtually analogous to the one that existed earlier and specified in the Patent Law (the Federal Law N 22-FL d/d February 7, 2003).

In particular, the right to gain a patent for an invention, useful model, or production item in fulfilling a state contract belongs to the executor (contractor), unless otherwise specified in the state contract.

The executor (contractor) is obliged to notify in written form the state customer about obtaining a patentable result, such as an invention, useful model or production item. After the outset date of notification and within the following six months, the state customer has the right to apply for a patent in its own name. The term specified is limited, and if the state customer does not apply for a patent, the right to a patent goes to the executor (contractor).

If, in accordance with the state contract, the rights belong to the Russian Federation or to the executor jointly with the Russian Federation, an order similar to the above-mentioned as related to the creation of scientific work is applied

The state contract shall contain specified conditions for allocating the rights to the use of the results of scientific activity.

The Federal Law N 94-FL (d/d July 21, 2005) on the Placement of Orders for Goods Delivery, Work Performance, and Provision of Services for the State and Municipal Needs specifies the following procedure of preparing the final contract with the competitive tender winner: the inclusion of conditions for the execution of a contract offered by the competitive tender winner in applying for participation in the tender into the draft of the contract annexed to the tender documentation.

On the basis of the foregoing, it is essential to specify the order of allocating rights to the results of work in the contract, especially if these rights are conferred to both the Rus-

sian Federation and the executor. Unfortunately, this procedure is rarely followed out in actual practice. The section of the state contract devoted to allocating rights to objects created simply copies the norms of the law and does not specify a clear mechanism of interaction between the parties both at the stage of creating a patentable product and then at the stage of employing this product. As a result, the product created in the framework of the state order does not correspond to the product described in the accounting documents, encouraging legal disputes.

The result of research and engineering work is often a complex object which includes a combination of inventions, useful models, production pieces, software, technical data, information, etc. Within the limits of legislation, that complex object is called "**integrated technology**" and is covered by Part 4 of the CC RF. It ought to be noted that the integrated technology itself is not an object of intellectual rights. However, by virtue of the fact that the integrated technology can consist both of protectable elements and those not subject to legal protection, the considered object falls within the scope of Part 4 of the CC RF.

Currently, the scientific research results of state budget establishments and other development work financed through the budget make up a major part of integrated technology. Unfortunately, law enforcement practice concerning the transfer of rights to the technology created using the budget or by borrowing budget funds does not exist today. Thus, properly identifying the results of scientific activity as an integrated technology, specification of uniform rules of technological rights disposition, and, finally, obligations of the state and private sector to provide the following introduction to the technology require clear legal regulation at the federal level.

The Federal Law on Technologies establishes a procedure for giving up rights to technology by the Russian Federation or its constituent entity. The rights may be given up on the basis of competitive tenders or auctions. The competitive tender has priority. According to the general rule, competitive tenders and auctions are open. Closed competitive procedures may be carried out only when the technological information is a state secret.

Properly identifying the results of scientific activity requires clear legal regulation at the federal level

In accordance with Point 3 of Article N 1547 of the CC RF, the Federal Law on Technologies confers a priority right to a contract for the acquisition of technological rights between the Russian Federation or its constituent entity and the executor who organized the creation of that technology. The executor shall be invited to participate in the competitive tender or auction, irrespective of whether it is open or closed. The person who offers the best conditions for practical application (use or introduction) of the technology in the Russian Federation, including the economic indexes scheduled, is proclaimed the winner of the competitive tender and gains the right to conclude a contract either to give up the rights to the integrated technology or to conclude a license agreement.

The person who offers the highest payment under the contract to give up the technology rights is proclaimed the winner of the auction. Under otherwise equal conditions, the executor is proclaimed the winner.

In the last sentence, the formulation of the phrase "under otherwise equal conditions" sounds questionable. The current practice of holding competitive tenders and choosing a winner focuses significant attention on the price offered for the contract (this refers to providing the introductory conditions as well).

Hence, the executor, having incurred costs for obtaining a defined result of scientific and engineering work (including the use of development work provided and acquisition of rights to the results of intellectual activity), has to compete with third parties on the basis of price, and the result does not always turn out in the executor's favor.

The obligation of introducing the technology into practical activity is one indispensable condition in the contracts. To support and stimulate the practical realization of the solutions obtained, the Federal Law on Technologies introduces into practice a competitive conclusion for the contracts for the performance of additional work aimed at implementing integrated technologies in practice. The considered work is carried out with non-budgetary fund and in the interests of the person who finances these works with the purpose of acquiring the rights to the technology. Performing such a range of work is realistic when the technology rights are conferred primarily to the Russian Federation or if the primary executor of the state contract does not provide all actions required for assigning his rights to the obtained results.

In accordance with the contract for performing additional work aimed at reducing integrated technologies to practice with regard to the needs of the party in interest (investor in the project), the executor is obliged to carry out additional work, while the person interested in adapting the integrated technology with due regard to his needs is obliged to finance this

work; the person who disposes of the integrated technology right on behalf of the Russian Federation is obliged to conclude a contract giving up his or her integrated technology rights to the person interested in adopting the integrated technology with regard to his interests.

The economic constituent of the contracts for transferring the rights to integrated technologies should be noted as well. In addition to the requirements of the CC RF for the conditions of the contract to transfer the rights to integrated technologies, the Federal Law assigns the presence and execution of a plan focused on the realization of integrated technology and its practical application as an indispensable condition. Nonfulfillment of the approved plan is enough reason for cancellation of the contract and loss of the rights to technologies.

Hence, the major legal procedures of technology rights transfer to the private sector are specified in theory. It is time to put them into practice.

One more important subject was considered in the new legislation: the production secret (know-how) will be provided with the guaranteed legal protection at the highest legislation level. Scientific and engineering information (which is not a result of intellectual property) is occasionally completely or partially confidential. The scientific and engineering information (which is of actual or potential commercial value due to the fact that it is unknown to third parties, and therefore is hardly accessible on a legal basis and is subject to being considered a commercial secret by its owner) is considered a commercial secret as well. Any information, including data on the results of intellectual activity in the scientific and engineering sphere, is referred to as know-how as a type of commercial secret. The exclusive know-how right belongs to its holder and is valid as long as the information on it is confidential.

Hence, we considered the allocation of rights to the results of intellectual activity between the scientific organization and outside contractors (the state and/or ordinary customers).

INTELLECTUAL PROPERTY RIGHTS: ALLOCATION WITHIN A SCIENTIFIC ORGANIZATION

Since the human factor is involved in the creation of intellectual property, it is essential to consider the allocation of rights to this property "within an organization"; i.e., between the scientific organization and its employees. Correctly processing the corresponding relationships guarantees the lawful application of the results of intellectual activity for the scientific organization.

The CC RF confers exclusive rights to employment-related work (work for hire) to the employer, unless otherwise established by a labor contract or any other agreement concluded between the employer and the author of the work. However, Part 4 of the CC RF adopted some new requirements for employers who wish to reserve their exclusive rights to their employees' work.

In particular, the employer is obliged to begin using the work for hire three years from the date of creation, transfer the exclusive right to another party, or inform the author about the confidentiality of this work. Otherwise, the exclusive right to the work for hire will be transferred to the author.

The author retains his remuneration right (if the work is used by the employer) if the employer's exclusive right is transferred to another person and if the employer decides to keep the work for hire in secret.

As in case of scientific work made for hire, the exclusive rights to employment-related invention, useful model, and production items are conferred to the employer unless otherwise established by a contract between the employer and the employee.

The employee is obliged to notify the employer about the creation of patentable work. The employer has four months from the date when the notification is received to obtain a patent in his name, transfer the patent's application rights to a third party, or to keep the fact of the work secret.

> The most important point is that labor interaction that is related to creating patentable work requires maximum formalization and detailing

The employee retains the right to remuneration in the amount specified in the contract concluded between the parties. Minimum remuneration rates are specified by the Government of the Russian Federation.

Upon expiration of the time alloted to the employer to make a decision about the use of rights for the work created, the patent application right goes to the employee.

Since the patentable object is created when the author performs his employment duties, it is essential that the employer and author (employee) conclude a labor contract. Questions related to the creation and use of intellectual property, rights allocation, and order of remuneration may be specified in both the labor contract and any other agreement.

If the parties decide to conclude a uniform labor contract containing all

the necessary elements of the author's contract, it is essential to take into consideration the following information:

(1) The labor contract can be fixed-term and expire prior to the expiration of the three-year period alloted by the legislative body to the employer to start using the work made for hire. Therefore, the parties shall specify the sections of the contract which will remain valid after termination of the contract, or they shall sign a new contract to regulate the order of use of the patentable work by the parties.

(2) The author has the right to remuneration if his work is used by the employer or other parties. Labor legislation obliges the employer to pay a salary to his employees. These are two different types of remuneration which can differ in amount, method, form, conditions, and order of payment. Thus, the labor contract shall clearly specify the mechanism of accounting and payment of the remuneration indicated.

(3) Part 4 of the CC RF defines work made for hire as "work created within the framework of duties carried out by the employee (author)." Thus, it is essential to specify the list of duties in the labor contract. It is reasonable to describe duties as accurately as possible (the duties may be listed in a special supplement to the contract such as job description or scientific plan); to indicate the tasks given and those who can assign these tasks; and to determine the form of reports and results, the method of personal documentation storage by the employee (not to be confused with the service instructions), and the limits of use of the employer's material and technical base by the employee. Accuracy in this will allow the parties to avoid possible disputes concerning the rights to and/or obligations to the patentable work.

(4) The labor contract shall provide a mechanism for employee dismissal which will take into consideration the interests of both parties. It is not infrequent that employees with certain ideas, methods, and results are hired and then dismissed. These elements, as such, are not patentable, but they represent the basis of work created in the past or scheduled for the future. Thus, the parties involved in allocating rights to any results of intellectual activity shall take into consideration the generated obligations and degree of responsibility, including the possibility of bringing to responsibility the party in fault.

(5) It is necessary to take into account the problem of coauthorship, because most objects are a result of the joint creative work of several people. The employer shall specify the order of income distribution between the coauthors. The coauthors, in their turn, shall establish the order of further use of the results of intellectual property if they get exclusive rights to them.

However, the above-mentioned information does not include a complete list of "rough waters" that an employer and employee may find themselves sailing in the course of their interaction aimed at creating employment-related work. The most important point is that labor interaction that is related to creating patentable work requires maximum formalization and detailing.

We have considered the receipt of rights to the results of intellectual property by the scientific organization due to interaction with external (state and ordinary customers) and internal (the employer–employee chain) contractors.

> The state, intending to transfer the exclusive rights to the development work to the institutes and involve them in economic activity, shall provide the legal framework for this procedure to the fullest extent

SCIENTIFIC ORGANIZATIONS AND INCOME FROM INTELLECTUAL ACTIVITY: OPPORTUNITIES AND LIMITS

Let's consider now the possibilities of the scientific organization to earn income from its intangible assets.

The degree of freedom that the scientific organization has concerning the disposition of the results of scientific activity depends on factors such as the type of scientific organization, its organizational–legal form, the mode of financing of the work carried out, and the character of the work created.

Currently, most scientific organizations represent state unitary enterprises and state establishments. In spite of the fact that the state allows scientific organizations to exist as business companies, the percentage of such organizations is insignificant.

The adoption of the Federal Law N 174-FL (d/d November 3, 2006) on Autonomous Institutions initiated the creation and transformation of scientific organizations into autonomous institutions. However, in practice, the functioning of such organizations is not yet significant.

What opportunities were given to scientific organizations concerning the use and disposition of their property (including property rights) depending on the organizational-legal form?

There are several forms, depending on the departmental identity of the research teams. Let's consider the departments—which consist of different organizations—and their possibilities.

The State Unitary Enterprise Based on the Rights of Economic Management (Commercial Organization)

(I) is entitled to administer (sell, lease, and pledge) the assigned or acquired property (except for real estate) on its own initiative;

(II) the decision to participate in commercial or noncommercial organizations is taken with the consent of the owner of the unitary enterprise's property; the disposition of investments (assets) in the charter capital of the business company, association, or joint stock company is carried out only by agreement with the owner as well;

State Unitary Enterprise Based on the Rights of Operating Management (Commercial Organization):

(I) the disposition of property is carried out only by agreement with the owner;

(II) is not entitled to establish any legal entities or to join them;

Establishment (Non-Commercial Organization):

(I) the disposition of the owner's property and property purchased using budgetary funds is carried out only by agreement with the owner;

(II) is entitled to administer on its own initiative income from commercial activity in accordance with the charter documents and the property purchased with this income.

(III) is not entitled to establish any legal entities or join them;

Autonomous Institution (Non-Commercial Organization)

(I) is entitled to administer on its own initiative movable property, except for high-value property;

(II) is entitled to bring cash assets or other property in the charter (share) capital of other legal entities or transfer this property to other legal entities as their founder or participant only by agreement with its founder.

Russian Academy of Sciences (Non-Commercial Organization):

(I) is entitled to administer, possess, and use federal property;

(II) is entitled to create, reorganize, and liquidate subordinate organizations;

(III) is entitled to act as the founder or co-founder of organizations which serve the charter purposes and tasks of the Russian Academy of Sciences and to enter into alliances and associations;

Institutes of the Russian Academy of Sciences (Non-Commercial Organizations):

(I) are entitled to own, use, and administer the federal property of the Russian Academy Sciences transferred to these institutes for operating management. Income from the activity approved by their charters and property acquired with this income are at their independent disposal and are put into a separate balance sheet.

Currently, the most widespread form of conducting scientific activity is the Establishment. Most of them are academic structures such as the institutes making up the Russian Academy of Sciences.

The bulk of scientific activity is carried out in accordance with budgeted financing within the terms of the state contract.

Let's consider the creation of patentable work within the scientific organization (using the scientific organization as an example).

The institute can act both as the principal executor of the state contract (with the attraction of coexecutors or without them) and perform the functions of the coexecutor. We are interested in the first case, because the principal executor has priority rights to the result obtained.

Upon executing the state contract, the institute as the principal executor, along with the coexecutor, carries out a range of work, often resulting in the creation of a complex product. The complex object can include combinations of patentable intellectual products and those not subject to legal protection. For this purpose, the intellectual of the results activity of the institute and third parties—both the official coexecutors working under the state contract and any other persons—may be involved.

The patentable products can include products, the rights to which are conferred to the Institute of the Russian Academy of Sciences (i.e., the exclusive rights are assigned to the state), and intellectual activity results, the exclusive rights to which belong to the institute.

The state contract, as a rule, allows the inclusion of third parties' products of intellectual activity in the results obtained if the rights of those who hold the the legal rights are not violated. In other words, relations between the institute and coexecutors shall be finalized by individual contracts (R&D contract, paid service agreements, licensed contract for granting rights to the corresponding results of intellectual activity, etc.).

Within the terms of such contracts between the institute and companies holding the rights to the results of intellectual activity used to execute the state contract, the latter transfer the exclusive rights or right to use the necessary products to the institute. When the issue is the transfer of the right to use objects, it is essential to determine the extent of these rights, because it must be sufficient for executing the state contract by the principal executor, including the further transfer of rights to the state customer. Requirements for the term and territory where to use the rights to be transferred are analogous.

As was said above, the text of the state contract is standard general law. However, the final act of work completion does not contain the following information: the type of results of intellectual activity; the extent of the rights conferred to the executor of the Russian Federation; and a way for further interaction between the executor, state customer, and third parties concerning the use of the results of intellectual activity. The following will help avoid ambiguities:

(I) concluding an agreement between the state customer and the institute (between the institute and coexecutors when needed) about the order of joint usage and disposition of rights to the result created;

(II) the act of the work completed within the terms of the state agreement shall indicate the objects for which the rights are transferred and

the degree of rights subject to the transfer and/or assignment.

It should be noted that the system of creating a package of documentation to accompany the state contract is rather conservative and unadaptable. Introducing alterations and/or additions is either impossible or time-consuming.

SCIENTIFIC INSTITUTES AND THEIR RIGHTS TO THE PRODUCTS CREATED WITHIN THE TERMS OF STATE CONTRACTS

Summarizing the above-mentioned information, we observe the following: upon executing the state contract, the institute

(a) acquires the right to the results of intellectual activity explicitly and (which is reflected in the cost and tax accounting) by obtaining protection and entitlement documents. The institute has ground for the following disposition of the corresponding rights on the basis of the contract concluded with the state customer;

(b) has a product (a result of developed works or a set of components not specified in the state contract) which is by no means discribed in the balance sheet (or is not reflected as intangible assets). The institute has the signed act of the work done within the terms of the state contract, but the contact does not contain detailed information on the allocation of rights.

Authority to dispose of the results of work is required for further commercialization. This authority will be included in the contract on the transfer of exclusive rights to the institute and will be determined with regard to all conditions specified in the state contract; agreements with the coexecutors, rights holders, and institute charter; agreements with the Russian Academy of Sciences on the transfer of property; and to all conditions specified in the registers of the transferred property. The necessity of registering all these documents is justified by the fact that the result of the work is created using the property or/and property rights of third parties, who can impose additional conditions on the application.

The institute is an establishment: i.e., the property assigned to the institute by the owner (represented by the Russian Academy of Sciences), the property purchased using budgetary funds, and products created using the assigned property and/or provided funds cannot be administered by the institute due to the type of management. However, the institute can independently administer certain products created at its own cost when carrying out R&D that is not directly specified in the state contract.

The state intending to transfer the exclusive rights to the development work to the institutes and involve them in economic activity shall provide the legal framework for this procedure to the fullest extent. In particular, the state shall provide a process for reorganizing scientific organizations and consider the possible participation of these scientific organizations in the charter capital of other organizations. As for the scientific sector, the heads of the scientific organizations shall pay more attention to identifying and recognizing intangible assets and providing patentable and entitlement documents in order to increase the investment potential of their intangible assets. ●

Living Systems in Russia are Evaluated in Terms of the Number of Scientific Publications Related to the Subject

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Those fields within science in which there is continuous development need to be objectively evaluated in order to determine the efficiency of work being conducted by those working in that field. Different criteria provide an estimate of this efficiency at both the level of individual scientists as well as that of entire scientific institutions. Moreover, it is possible to obtain an estimate of the rate of progress in research activities within different states.

The assessment of researchers and their respective level of activity is made based on the amount of research publications. This applies both to individual investigators and to scientific centers as a whole. There are some obvious advantages to this approach.

First, this is the most objective approach, because scientific publications are the most widespread and common measurable result of scientific work, being the vehicle by which research results can be distributed and shared with scientists all over the world.

Second, publication activity is an easily measured variable, especially in recent years, following the advent of new, large databases of research papers. It should be noted that different measurement systems register different scientific publications and, as a result, provide slightly different data. However, significant differences in figures and dynamics have not been observed in Russia.

With the aforementioned in mind, the publication activity (PA) of Russian researchers involved in the "Living Systems" priority direction during the time period between 1996 and 2008 was analyzed using the international SCOPUS database. The "Living Systems" direction is second only to nanotechnologies in terms of the amount of financing but is first from the standpoint of resulting improvement in the quality of life in our country. The purpose of this analysis was to identify those specific spheres of the "Living Systems" Program where scientists are most active. The investigation consisted not only in the analysis of the number of publications, but also in the establishment of leading institutes and scientific centers able to become special "crystallization centers" with their work centered on "Living Systems" in Russia.

METHOD

Information from the SCOPUS database was used for this investigation. SCOPUS (www.scopus.com) is the largest abstract database in

the world, containing more than 15,000 names of scientific, technical and medical publications from some 4,000 international publishing houses. Currently, the SCOPUS database includes more than 300 Russian academic periodicals.

Not all the fields of science related to living organisms were subject to analysis, but rather only those whose results could be used in actual practice. Choosing the particular fields to be included did not cause any difficulties: we analyzed the priority directions in science, technology and engineering development in the Russian Federation approved by the President in May of 2006. The priority directions include:

- Security and counter-terrorism
- Living systems
- Industry of nano-systems and nanomaterials
- Information and telecommunication systems
- Strategic weapons, military and special equipment
- Environmental conservation
- Transportation, aviation, and space systems
- Energy production and conservation

On May 21, 2006, the President approved a list of 34 technologies which were deemed critical for the Russian Federation; some of them are referred to in the "Living Systems" section. The list of critical technologies of the Russian Federation in the sphere of "Living Systems" includes the following critical technologies analyzed herein:

- Bio-information technologies
- Bio-catalytic, biosynthetic, and biosensor technologies
- Biomedical and veterinary technologies related to human and animal life support
- Genomics and post-genome drug formulation
- Cellular technologies
- Bioengineering technologies
- Biocompatible material technologies

Hence, these seven critical technologies were chosen for the present investigation. Using the previously determined set of key words for the search, we calculated the total number of articles published annually by Russian authors in either Russian or foreign scientific periodicals included in the SCOPUS database, separately for each of the above-mentioned categories of technologies. Each list of key words was always accompanied by the "Russia" affiliation.

It should be noted that many works are performed not only by one particular scientific team, but also within the framework of interdisciplinary projects realized by scientific consortiums comprising different institutes. Those data were also taken into account in the course of investigation.

Considering biology publications in the world in 1996-2008, the following picture emerges **(Figs. 1a, 1b)**.

By far, the country with the greatest publication activity is the USA, and its PA increased during that period of time from 192,000 to 251,000 articles per year.

Among European countries, the most active in the sphere of science (Great Britain, Germany, France, and Italy) also demonstrated this trend. However, Great Britain and Germany have experienced a downturn in their PA during the last 2 to 3 years. Italy managed the greatest relative increase in the number of publications: in 12 years, the number of publications written by Italian scientists increased 1.6 times and reached a total of 35,000 articles a year. Switzerland demonstrated a non-significant increase in the number of biology-related articles (from 8.8 to 13.7 thousand).

Special attention should be paid to the increase in the number of articles published by scientists from the BRIC countries (Brazil, India, Russia, and China), which are referred to by experts as countries with growing economies. However, from the standpoint of scientific development, it is more reasonable to speak about a group composed of only three countries, with Russia being excluded from this group.

If, in 1996, Russia, China, and Brazil had almost equal starting positions (6,400, 6,200 and 5,000 articles, respectively), the situation changed dramatically afterwards, primarily as a result of an unprecedented increase in the number of articles written by Chinese authors (57,400 articles in 2008). Currently, China is fourth in the world in the number of biological articles, surpassing even Germany, while Brazilian scientists published 19,000 articles in 2008. Over a period of years, India increased its PA from 11,000 articles in 1996 to 26,000 articles in 2008, and it climbed to the eight position in the list of all countries. Although initially insignificant, the increase in the last few years experienced by Brazil has been remarkable, and it has sur-

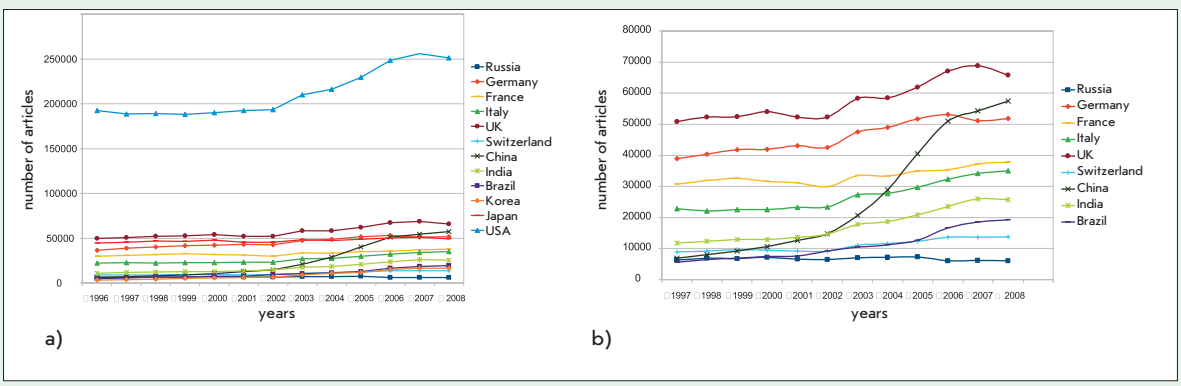


Fig. 1 Russia and the World Leaders in the Sphere of Sciences Related to Living Organisms
a) Total number of publications in biology in 1996-2008
b) Total number of publications in biology in 1996-2008 (USA excluded)

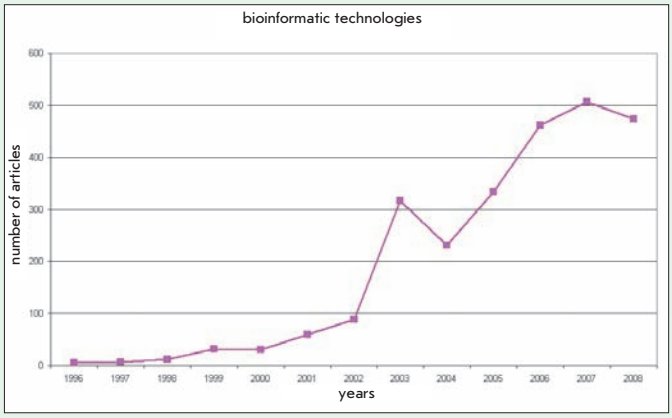


Fig. 2. Number of articles written by Russian researchers on bio-information technology.

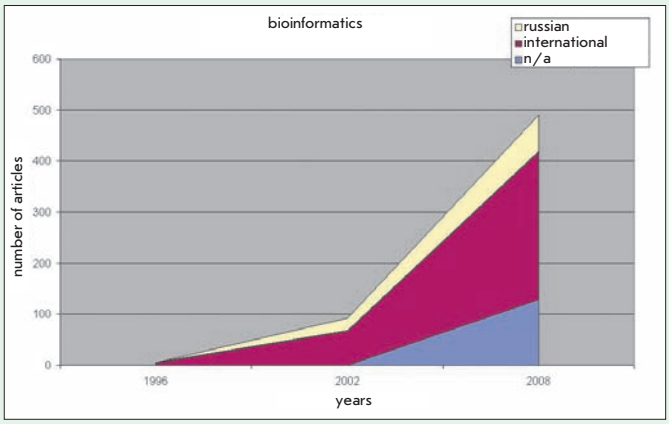


Fig. 3. Relationship between the number of Russian articles on bio-information technology published in Russian journals versus in international journals.

passed Switzerland in the number of publications (13,700 articles in 2008). In 12 years, the number of articles written by South Korean authors has increased 5-fold, from 3,300 to 16,700 articles.

Russia, on the other hand, after an insignificant increase in PA between 2000 and 2003, is now the only country among the BRIC members to actually demonstrate a decrease in its PA (from 6,400 to 5,900 articles a year).

Let's consider in detail the characteristics of the publication activity of Russian authors for each of the spheres of the Living Systems referred to among the critical technologies.

BIO-INFORMATION TECHNOLOGIES

Bio-Information technologies are a relatively new branch within the life sciences characterized by intensive development in the whole world. The significant increase in the volume of investigations carried out in this field is related to the need for processing the large bulk of data generated during the course of conducting biological experiments.

Russian bioinformatics is no exception. Since the end of the 1990s, when work in this field was rather rare, Russian scientists have increased their PA up to 500 articles a year. In spite of the fact that the last year was characterized by a decrease in the growth rate of publication activity, investigations in this sphere reflect rapid progress **(Fig. 2)**. The key words used for this search were: *bioinformatics, comparative genomics,* and *computational.*

The projects in this field are carried out at the international level and are published largely in international English journals, such as *Bioinformatics*, *Nucleic Acid Research*, *The Journal of Bioinformatics and Computational Biology*, *Molecular Phylogenetics and Evolution*, *The Journal of Molecular Biology*, *In Silico Biology*, *BMC Genomics*, *BMC Bioinformatics*, and *BMC Evolutionary Biology*. As for Russian journals, those which commonly publish articles on bio-information technology include: *Biochemistry, Molecular Biology, Biophysics, Genetics, and Reports of the Russian Academy of Sciences*.

According to SCOPUS data, in 2008, the average number of co-authors per published article in this field was 7, and the average number of articles published by each collaborator was 3. Therefore, the approximate number of researchers actively working in this field is 1,000.

The following institutes of the Russian Academy of Sciences (RAS) stand out for a significant number of published articles on bioinformatics: The Institute of Cytology and Genetics, Siberian Branch (84 articles); The Engelhardt Institute of Molecular Biology (82); The Sobolev Institute of Mathematics, Siberian Branch (69); The Institute of Cytology (40); and The Kharkevich Institute for Information Transmission Problems (38).

The institutes of the Russian Academy of Medical Sciences (RAMS) rarely conduct research in this sphere, and most of those studies were conducted at the Orekhovich Institute of Biomedical Chemistry (37 studies); some investigations are being carried out in the Blokhin Russian Cancer Research Center (16 studies), the Gamalei Institute of Epidemiology and Microbiology (13 studies), and the Ivanovsky Institute of Virology (11 studies).

The State Scientific Research Institute for Genetics and the Selection of Industrial Microorganisms is unrivaled among the state scientific centers in terms of the number of articles on bio-information technology (102 articles), surpassing the Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University (42), and the Joint Institute for Nuclear Research in Dubna (27 articles).

The M.V. Lomonosov Moscow State University is the absolute leader among institutions of higher education in terms of the number of publications (448): The St. Petersburg State University (67), Novosibirsk State University (57), and St. Petersburg State Polytechnic University (33) are far behind.

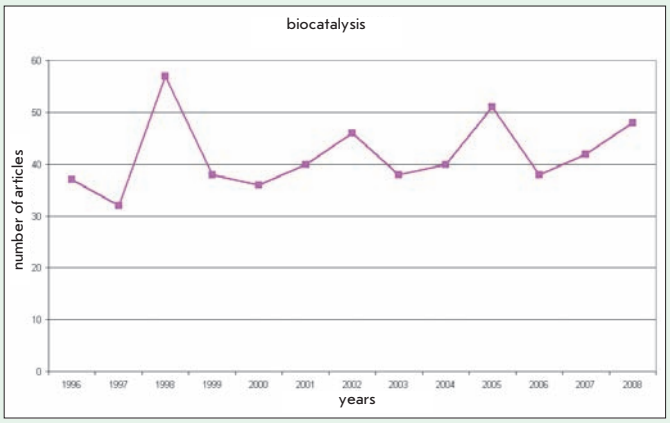


Fig. 4. Number of articles written by Russian researchers on the subject of bio-catalytic technology.

Thus, today, the field of bio-information technology is actively developing. In the period between 2002 and 2008, the number of publications increased almost 5-fold. Research articles in this field are at a very high level, with most of the articles being published in international journals **(Fig. 3)**. This finding can be easily explained: research in bioinformatics does not require any expensive laboratory equipment or reagents. Computers and software are a major work instrument for bio-information scientists. The spheres of living systems studied on laboratory tables are rarely found in scientific periodicals.

BIO-CATALYTIC, BIOSYNTHETIC, AND BIOSENSOR TECHNOLOGIES

This category of critical technologies pertaining to living systems can be divided into three independent subcategories, each of which should be analyzed separately.

BIO-CATALYTIC TECHNOLOGY

Among the Russian Academies of Sciences, bio-catalytic technologies are pursued most actively in the RAS and RAMN institutes. However, the number of publications on this subject included in the SCOPUS database generally ranges between 40 and 50 articles a year, not considering the recent uptrend **(Fig. 4)**. The key words used for this search were *biocatalysis, enzymatic*, and *catalysis*.

Investigations carried out by our scientists in this field are commonly published in such Russian journals as *Biochemistry*, *Microbiology*, *Bioorganic Chemistry*, and *Russian Chemical Reviews*. However, many articles are also published in such international journals as *FEBS Letters*, *Biotechnology and Bioengineering*, *Biocatalysis and Biotransformation*, and *The Journal of Molecular Catalysis B Enzymatic*.

According to the SCOPUS data, in 2008, the average number of co-authors per published article was 5, and the average number of articles published by each collaborator was 1.2. Thus, the estimated number of researchers actively working in this field is 200.

An analysis of those organizations whose research scientists work most actively in this field shows that, among the research institutes and state scientific centers, research in this field is most often carried out in the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms (12 articles) and in the Gauze New Antibiotics Research Institute (8).

Among the RAS institutes, the ones with the highest PA activity are the Boreskov Institute of Catalysis, Siberian Branch (23 articles); the Zelinsky Institute of Organic Chemistry (19); and the Skryabin Institute of Biochemistry and Physiology of Microorganisms (12).

Among the RAMS institutes, the majority of these investigations are conducted at the Institute of Biomedical Chemistry (12 articles) and the All-Russian Cardiological Scientific Center (7).

Among the Institutions of Higher Education, the majority of the studies in question are carried out at M.V. Lomonosov Moscow State University (175 articles), while other institutions have a few publications to their name.

As a whole, the publication activity of Russian scientists in the field of bio-catalytic technology is rather low. However, it should be noted that 60% of all published articles in this field are published in international journals **(Fig. 5),** which is testament to the level of qualification of the scientists who are working in this field.

BIOSYNTHETIC TECHNOLOGY

Biosynthetic technologies are being developed much more rapidly, which is most likely due to the fact that they have been the subject of investigation for a longer period of time in our country. However, the number of publications (which exceeds the number of bio-catalytic related articles) has

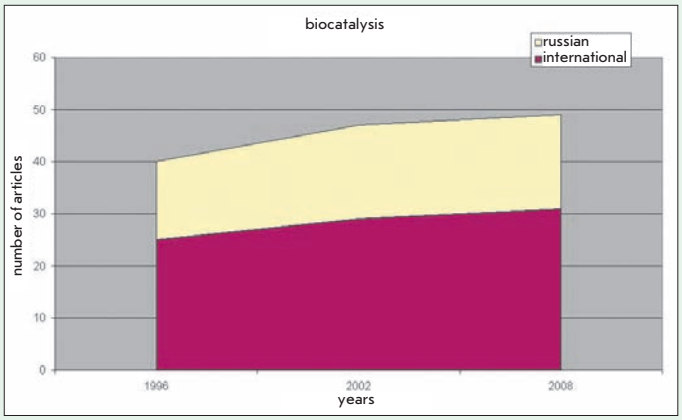


Fig. 5. The Relationship between the number of Russian articles on bio-catalytic technology published in Russian journals versus in international journals.

noticeably decreased over the last 3 years **(Fig. 6)**. The key words used for this search were *biosynthesis* and *synthesis*.

The Investigations by Russian scientists in this field are mostly published in such Russian journals as *Microbiology*, *Biochemistry*, *Bulletin of Experimental Biology and Medicine*, *Genetics*, and *Molecular Biology*. Some of the studies are also published in such international journals as *FEBS Letters*, *Pharmaceutical Chemistry Journal*, *the Journal of Biological Chemistry* and *Biophysics*.

According to SCOPUS data, in 2008 the average number of co-authors cited in each published study was 5, and the average number of articles published by each collaborator was 2.58. Thus, the estimated number of researchers actively working in this field is around 700.

An analysis of those organizations whose research scientists are actively involved in this field shows that among the RAS institutes the highest PA belongs to the Skryabin Institute of Biochemistry and Physiology of Microorganisms (228 articles), followed by the Engelhardt Institute of Molecular Biology (206), the Zelinsky Institute of Organic Chemistry (178), the Timiryazev Institute of Plant Physiology (171), and the Institute of Molecular Genetics (109).

Among the RAMS institutes, the Gamalei Institute of Epidemiology and Microbiology (191 articles) is where investigations in this field are most often carried out.

Among the Institutions of Higher Education, research in biosynthetic technology was mainly carried out at M.V. Lomonosov Moscow State University (997 articles), followed by St. Petersburg State University (129), Sechenov Medical Academy of Moscow (90), Kazan State University (84), and the Moscow Lomonosov State Academy of Fine Chemical Technology (59).

Biosynthetic technology is, perhaps, the only one among the critical technologies that was characterized by such a dramatic decrease in PA during the period between 2002-2008, though in 1996-2002 some increase was noted. In spite of some increase in the number of publications in international journals in 2008 (Fig. 7), the number of articles published in Russian journals in the same year decreased significantly.

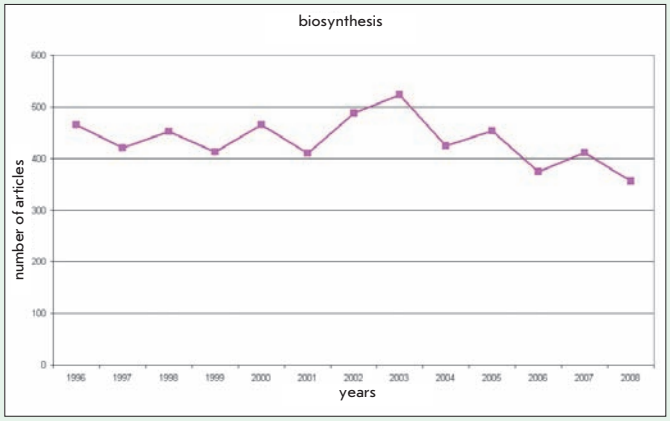


Fig. 6. The number of articles written by Russian researchers in the field of biosynthetic technology.

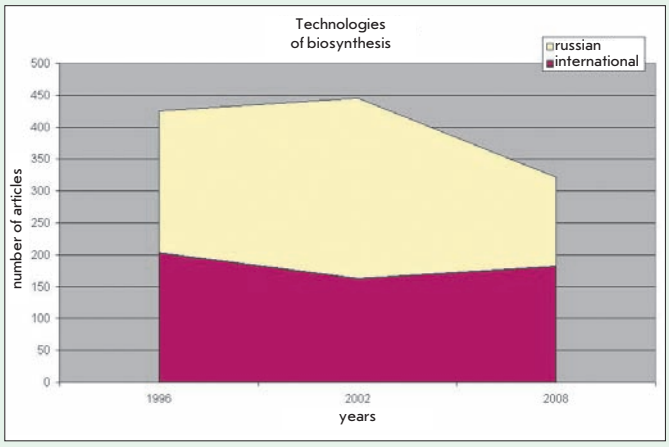


Fig. 7. The Relationship between the number of Russian articles on biosynthetic technology published in Russian journals versus in international journals.

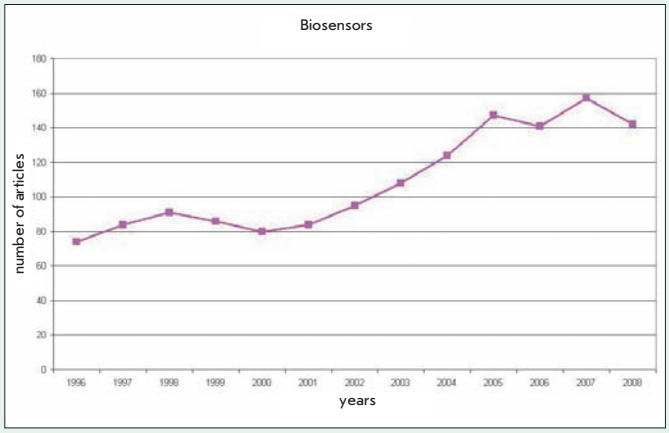


Fig. 8. The number of articles written by Russian researchers on the subject of biosensor technology.

BIOSENSOR TECHNOLOGY

Biosensor technology is a relatively new area of research within the living systems that is characterized by rapid development all over the world. In recent years, the number of Russian publications in the area of biosensor technology included within the SCOPUS database has doubled, from 80 to 160 articles a year **(Fig. 8).** The key words used for this search were *biosensor*, *biochip*, and *biomarker*.

The research conducted by Russian scientists is mostly published in such international journals as *Biosensors and Bioelectronics*, *The Journal of Analytical Chemistry*, *Bio-electrochemis-*

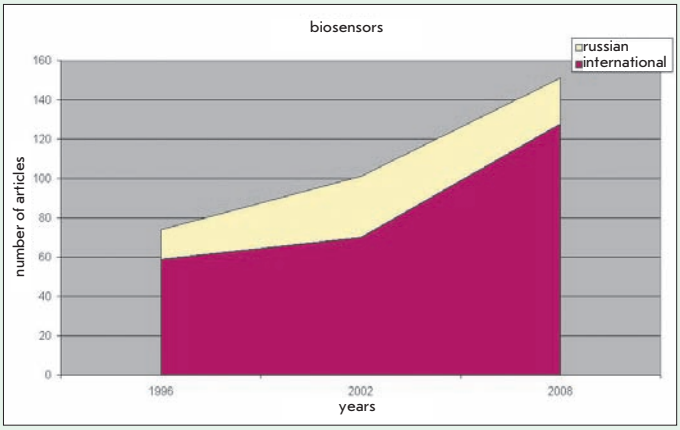


Fig. 9. The relationship between the number of Russian articles on the subject of biosensor technology published in Russian journals versus in international journals.

try, *Analytical Letters*, and *Biomedical Engineering*. Some of the studies are also published in Russian journals such as *Applied Biochemistry and Microbiology* and *Molecular Biology*.

According to SCOPUS data, in 2008 the average number of co-authors cited in each published study was 4.7, and the average number of articles published by each collaborator was 1.6. Thus, the estimated number of researchers actively working in this field is around 400.

An analysis of those organizations whose research scientists are actively involved in this field shows that among the RAS institutes the highest PA is attributable to the Engelhardt Institute of Molecular Biology (49 articles), followed by the Prokhorov Institute of General Physics (33), the Shubnikov Institute of Crystallography (31), the Skryabin Institute of Biochemistry and Physiology of Microorganisms (17), and the Institute of Problems of Chemical Physics (15).

Among the RAMS institutes, research in this field is most often conducted at the Orekhovich Institute of Biomedical Chemistry (74 articles).

Among the Institutions of Higher Education, this research was carried out mainly at the M.V. Lomonosov Moscow State University (362 articles), Kazan State University (87) and St. Petersburg State University (65).

As this analysis suggests, biosensor technology is one of the fastest developing critical technologies. Between 2002 and 2008, the publication activity of those Russian scientists involved in research related to biosensor technology increased by 50%, largely reflecting the increase in the number of publications in international journals **(Fig. 9)**.

Hence, a general analysis of the critical technologies indicates that biosensor technology is characterized by rapid development, whereas bio-catalytic technology remains somewhat stagnant, and biosynthetic technology actually suffered a setback, probably due to insufficient financial support.

BIOMEDICAL AND VETERINARY TECHNOLOGIES RELATED TO HUMAN AND ANIMAL LIFE SUPPORT

Biomedical and veterinary technologies were also analyzed separately, since the SCOPUS database can quite easily select the corresponding journals.

BIOMEDICAL TECHNOLOGIES OF HUMAN LIFE SUPPORT AND DEFENSE

The biomedical technologies pertaining to human life support have been the subject of investigation for a long time. In the last 12 years, the number of publications on the subject of biomedical technology has increased from 500 articles in 1996 to more than 1,000 in 2007. However, 2008 was marked by a decrease in the number of studies published **(Fig. 10).**

The articles by Russian scientists in this field were published mainly in Russian journals, such as *Biomedical Chemistry*, *Biomedical Engineering*, *Medical Engineering*, *Bulletin of Experimental Biology* and *Medicine*, *Problems of Medical Chemistry*, *Medical Radiology*, *Genetics*, *Applied Biochemistry and Microbiology*, etc. Some studies by Russian researchers were also published in such international journals as *The Journal of Biological Chemistry*, *Human Physiology*, *Pharmaceutical Chemistry Journal*, *Biomedica Biochimica Acta*, *Critical Reviews in Biomedical Engineering*, *the Journal of Biomedical Optics*, and many others.

According to the SCOPUS data, in 2008 the average number of co-authors cited in each published study was 5, and the average number of articles published by each collaborator was 3.69. Thus, the estimated number of researchers actively working in this field is around 1,700.

An analysis of those organizations whose research scientists are actively involved in this field shows that among the RAMS institutes the highest PA is attributable to the Orekhovich Institute of Biomedical Chemistry (711 arti-

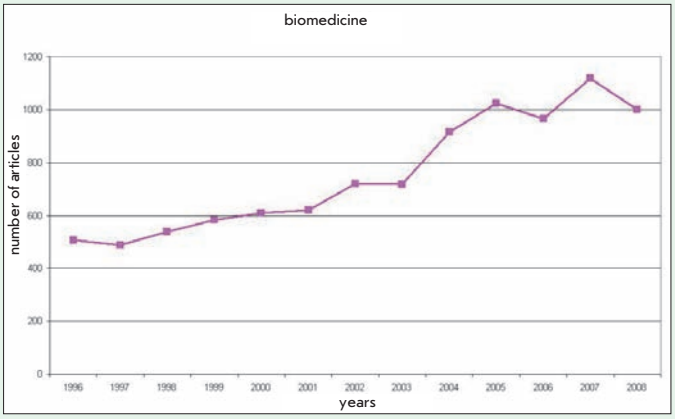


Fig. 10. The number of articles by Russian researchers in the field of biomedical technology related to human life support and defense.

cles), followed by the All-Russian Cardiological Scientific Center (97), and the Blokhin Russian Cancer Research Center (97).

Among the RAS institutes, investigations in this field were carried out most frequently at the Institute of Biomedical Problems (471 articles), followed by the Prokhorov Institute of General Physics Institute (107), the Institute of Applied Physics (100), and the Semenov Institute of Chemical Physics (98).

Among the institutions of higher education, investigations were carried out mainly at the M.V. Lomonosov Moscow State University (1,282 articles), Sechenov Medical Academy of Moscow (166), St. Petersburg State University (159), and Russian State Medical University (136).

Among the scientific organizations conducting research in this field, the most active is the All-Russian Research Institute of Vitamins (118 articles), as well as the Vavilov State Optical Institute (39), the Joint Institute for Nuclear Research (38), and the Research and Practical Center of Medical Radiology (37).

As a whole, biomedical technologies are rapidly developing technologies within the living systems. In the period between 2002 and 2008, the number of articles published on the biomedical technologies increased by more than 30% and amounted to70% of the total number of medical articles registered in the SCOPUS database. During that period, the number of articles published in international journals increased significantly **(Fig. 11)**. This field of research is being actively pursued in the RAMS institutes, the profile RAS institutes, the institutes of the Ministry of Public Health and Social Development, and in medical schools.

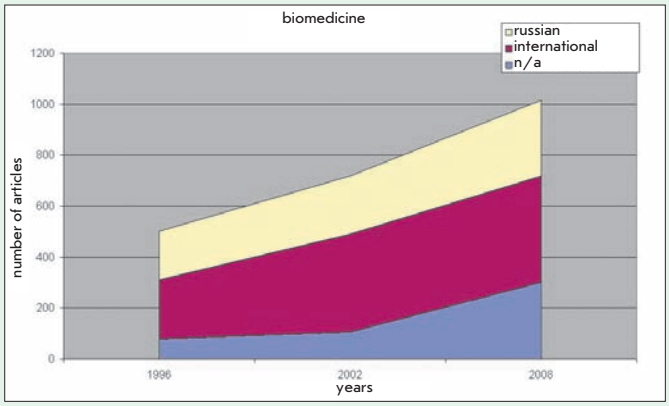


Fig. 11. Relationship between the number of Russian articles on biomedical technology of human life support and defense published in Russian journals versus in international journals.

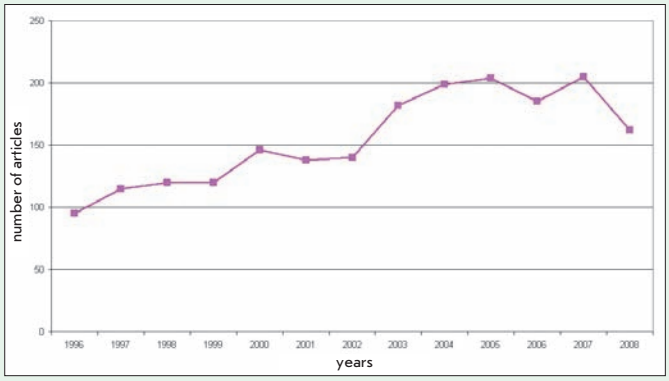


Fig. 12. The number of articles by Russian researchers in the sphere of veterinary technologies of animal life support and defense.

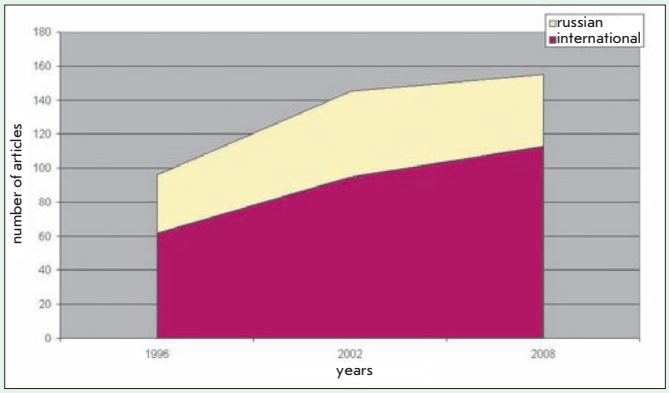


Fig. 13. Relationship between the number of Russian articles on veterinary technologies of animal life support and defense published in Russian and international journals.

VETERINARY TECHNOLOGIES OF ANIMAL LIFE SUPPORT AND DEFENSE

Veterinary technologies have been investigated in Russia for a long period of time as well. During the last 12 years, the number of publications in veterinary technologies included in the SCOPUS database has almost doubled, from 100 to 200 articles per year. However, the last few years have been characterized by a decrease in the number of publications **(Fig. 12).**

Articles by Russian scientists in this field were published largely in Russian journals such as *Radiobiology, Bulletin of Experimental Biology and Medicine, Radiation Biology and Radioecology, Biological Bulletin, Pharmacology and Toxicology, Journal of Microbiology, Epidemiology and Immunobiology*, and *Problems of Virology*. Some investigations by Russian researchers in this field were also published in such international journals as *Pharmaceutical Chemistry Journal, Neuroscience* and *Behavioral Physiology, Vaccine*, and *the Journal of Biological Chemistry*.

According to the SCOPUS data, in 2008 the average number of coauthors cited in each published study was 9, and the average number of articles published by each collaborator was 1.56. Thus, the estimated number of researchers actively working in this field is approximately 1,200.

An analysis of those organizations whose researchers are involved in this field reveals that among the RAMS institutes, the highest PA is characteris-

tic of the Medical Radiological Center (75 articles), as well as the Ivanovsky Institute of Virology (49), and the Gamalei Institute of Epidemiology and Microbiology (21).

Among research institutes and state scientific centers, investigations in this field were performed at the Institute of Biophysics of the Ministry of Public Health and Social Development, (M.p (89 articles), State Research Center of Virology and Biotechnology "Vector" (210), and Petrov Oncology Research Institute (21).

Among the RAS institutes, the highest PA in the considered sphere was noted at the Institute of Cytology (47 articles), Pavlov Institute of Physiology (45), Semenov Institute of Chemical Physics (38), Engelhardt Institute of Molecular Biology (36), and Institute of Biochemical Physics (35).

Among the institutions of higher education, investigations in this field were most often conducted at M.V. Lomonosov Moscow State University (261 articles), the Kirov Military Medical Academy (St. Petersburg, 44), and the Sechenov Medical Academy of Moscow (29).

GENOMICS AND POST-GENOME DRUG FORMULATION

Genomics and post-genome drug formulation represent a fairly new area of pharmacology, which promises to be very important in the future. In recent years, the number of publications included within the SCOPUS database has doubled from 300 to 600 articles per year in the field of genomics, and 100 to 200 articles in post-genomic pharmacology **(Fig. 14)**. The key words used for this search were *genomics, transcriptomics, proteomics, drug, target, drugs delivery,* and *antibiotic*.

Articles by Russian scientists in the fields of genomics and post-genome drug formulation were published largely in Russian journals like *Molecular Biology, Biochemistry, Microbiology, Bulletin of Experimental Biology and Medicine, Problems of Medical Chemistry,* and *Antibiotics and Chemotherapy*. Some studies by Russian researchers were also published in such international journals as *FEBS Letters, Pharmaceutical Chemistry Journal, The Journal of Biological Chemistry and Biophysics.*

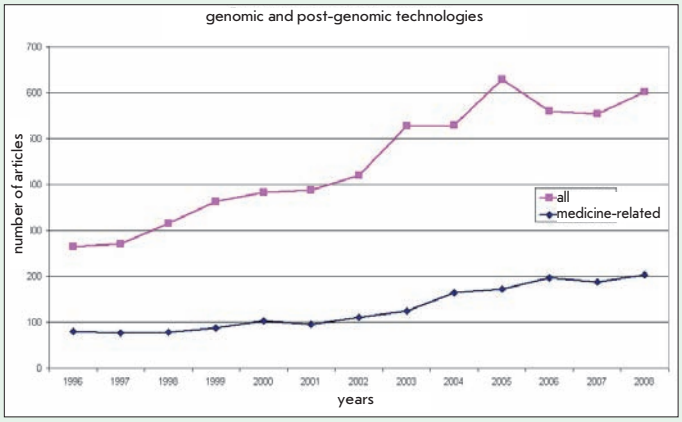


Fig. 14. The number of articles by Russian researchers in the sphere of genomics and post-genome drug formulation.

According to the SCOPUS data, in 2008 the average number of co-authors cited in each published study was 7.15, and the average number of articles published by each collaborator was 3.24. Thus, the estimated number of researchers actively working in this field is around 400.

An analysis of those organizations whose research scientists are actively working in this field shows that among the RAS institutes the highest PA was generated by the Institute of Cytology (67 articles), and significant activity also took place at the Engelhardt Institute of Molecular Biology (66); the Institute of Cytology and Genetics, Siberian Branch (54); the Institute of Molecular Genetics (50); the Institute of Gene Biology (43); the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (26); and the Zelinsky Institute of Organic Chemistry (20).

Among the RAMS institutes, the majority of investigations in this field were conducted at the Blokhin Russian Cancer Research Center (46 articles), followed by the Ivanovsky Virology Institute (35), the Institute of Experimental Medicine (30), and the Orekhovich Institute of Biomedical Chemistry (21).

Among the research institutes and state scientific centers, studies in this area were carried out mostly at the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms (53 articles) and the State Research Center of Virology and Biotechnology Vector (22).

Among the Institutions of Higher Education, investigations in this area were most frequent at the M.V. Lomonosov Moscow State University (194 articles), while a little activity

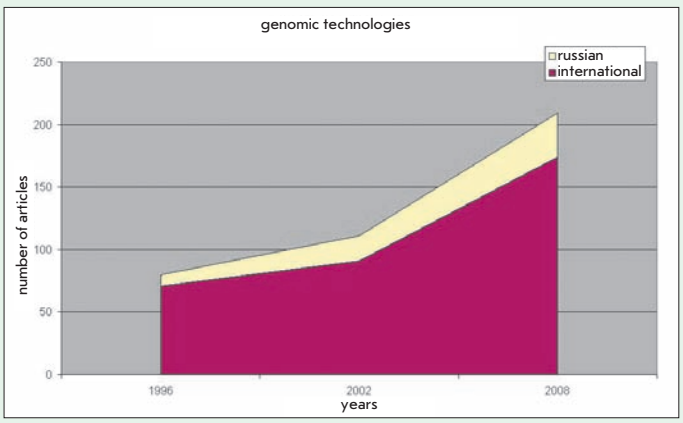


Fig. 15. The Relationship between the number of Russian articles on genomics and post-genome pharmacology published in Russian journals versus in international journals.

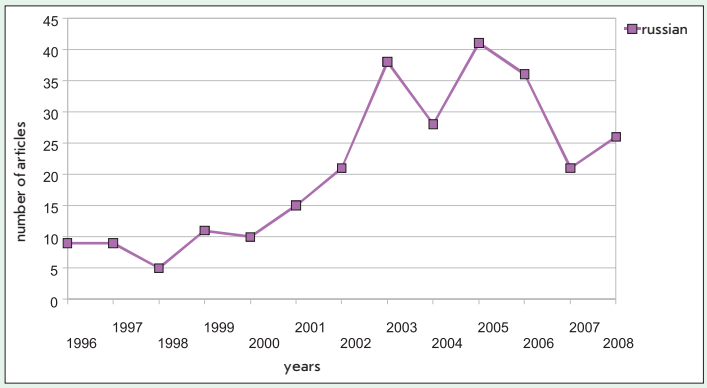
also came out of the St. Petersburg State University, and the Sechenov Medical Academy of Moscow (13).

Research into genomics and post-genome drug formulation is most actively pursued by scientists at the RAS, the RAMN, and the Ministry of Health institutes. Between 2002 and 2008, the number of publications in this field almost doubled. A significant majority of those articles found in the SCOPUS database were published in international journals **(Fig. 15)**.

However, in spite of its relation to biomedicine, this critical technology is a medical component of the living systems which should be analyzed individually and in detail, as one of the factors that exert the greatest influence on the quality of life of the population.

Let's focus our attention on those areas of research that are developing rapidly throughout the world, such as specific drug delivery, the formulation of new medications to be used in the treatment of socially significant diseases, and the creation of new antibiotics and anti-cancer agents. In other words, let us focus on the major and most prominent areas of research.

Fig. 16. The number of articles published by Russian researchers in the field of specific drug delivery.



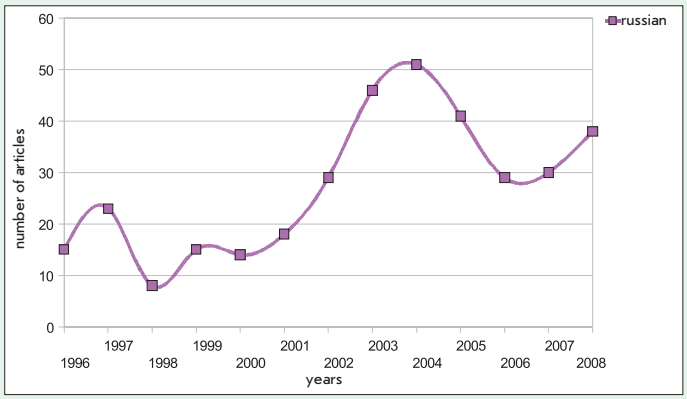
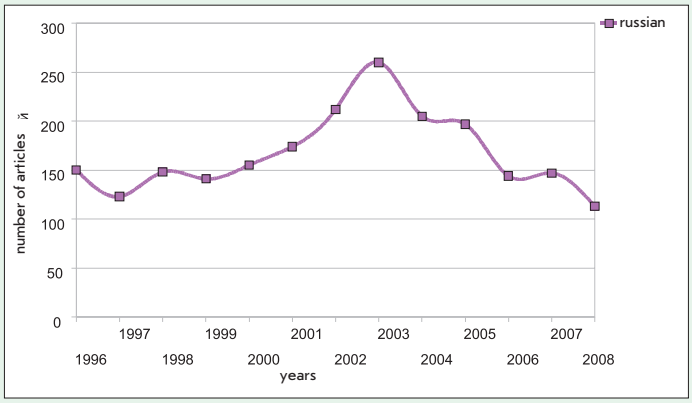


Fig. 17. The number of articles by Russian researchers in the development of cancer diagnostic techniques.

Fig. 18. The number of articles by Russian researchers in the area of new medications and techniques for the treatment of sociallysignificant diseases.



SYSTEMS OF SPECIFIC DRUG DELIVERY

The creation of systems for the specific delivery of a particular drug to pathologically transformed cells and body tissues is one of the major directions of medical biotechnology development in the world. The development of these systems will make medications much more effective.

The Russian scientific organizations most actively involved in pursuing research on this field are the following:

RAS institutes: The Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (34 articles); the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch (26); the Engelhardt Institute of Molecular Biology (82); and the Institute of Gene Biology (15). RAMS institutes: The Blokhin Russian Cancer Research Center (12 articles) and the Orekhovich Institute of Biomedical Chemistry (12).

The institutions of higher education: M.V. Lomonosov Moscow State University (31 articles), and Moscow Lomonosov State Academy of Fine Chemical Technology (13).

Research institutes and federal science centers published only a handful of articles between them.

DEVELOPMENT OF NEW ANTI-TUMOR DRUGS AND TECHNIQUES FOR CANCER DIAGNOSIS

Among the RAS institutes, the theme was investigated the most actively by the Institute of Chemical Biology and Fundamental Medicine, Siberian Branch (16 articles); Engelhardt Institute of Molecular Biology (13); Institute of Applied Physics (Nizhni Novgorod, 11); and Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (10).

Among RAMS institutes, investigations were carried out mainly at the Blokhin Russian Cancer Research Center (64 articles).

Development of New Medications and Treatment Modalities for Socially Significant Diseases.

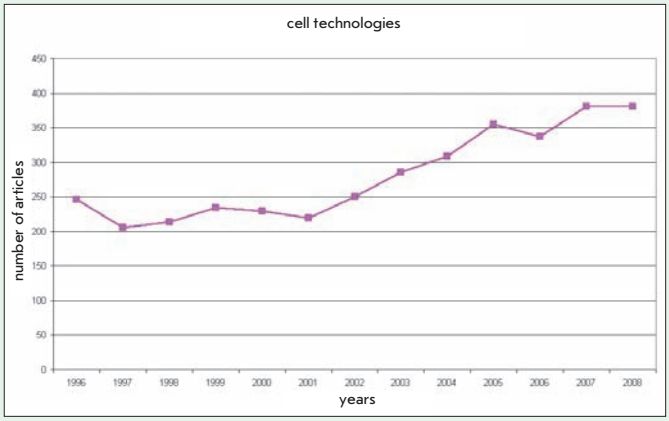


Fig. 19. The number of articles by Russian researchers in the field of cellular technology.

In this area, which is extremely important for our economy and our society due to the high prevalence and widespread distribution of socially significant diseases (for example, tuberculosis, AIDS, viral hepatitis C and B) the highest PA was observed at the following organizations:

RAS institutes: the Zelinsky Institute of Organic Chemistry (37 articles), the Nesmeyanov Institute of Organo-elemental Compounds (30), the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (26), and the Elhardt Institute of Molecular Biology (25).

RAMS institutes: Zakusov Institute of Pharmacology (25 articles), Gauze New Antibiotics Research Institute (23), Institute of Biomedical Chemistry (22), and Orekhovich Institute of Biomedical Chemistry (19).

Institutions of higher education: M.V. Lomonosov Moscow State University (94 articles), Sechenov Medical Academy of Moscow (60), and Moscow Lomonosov State Academy of Fine Chemical Technology (31).

CELLULAR TECHNOLOGIES

Cellular technologies are a relatively new area of research that has been developing quite successfully all over the world. In recent years, the number of publications in the field of cellular technologies included in the SCOPUS database has increased from 200 to almost 400 articles a year **(Fig. 19)**. The key words used for this search were *cell* and *technology*.

The articles written by Russian scientists in the field of cellular technology were published, for the most part, in Russian journals such as *The Bulletin of Experimental Biology and Medicine, Genetics, Molecular Biology, and Cytology*. Some studies carried out by Russian researchers were also published in such international journals as *The Pharmaceutical Chemistry Journal, the Proceedings of the National Academy of Sciences of the United States of America, FEBS Letters, the Journal of Biological Chemistry*, and *The Journal of Molecular Biology*.

According to the SCOPUS data, in 2008 the average number of co-authors cited in each published study was 5.8, and the average number of articles published by each collaborator was 3.19. Thus, the estimated number of researchers actively working in this field is around 700.

An analysis of those organizations whose research scientists are most actively involved in this field shows that among the RAS institutes, the Engelhardt Institute of Molecular Biology has the highest PA (99 articles), followed by the Skryabin Institute of Biochemistry and Physiology of Microorganisms (57), the Timiryazev Institute of Plant Physiology (51), the Institute of Gene Biology (50), the Institute of Molecular Genetics (47), the Institute of Cytology (43), and the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (41).

Among the RAMS institutes, the majority of research studies in this field were conducted at the Orekhovich Institute of Biomedical Chemistry (56 articles), and somewhat less at the Blokhin Russian Cancer Research Center (46), and at the Institute of Experimental Medicine (29).

Among the institutions of higher education, these investigations were carried out mostly at M.V. Lomonosov Moscow State University (526 articles).

In spite of the fact that cellular technologies have been studied for a long time, they are still the fastest developing technologies within the living systems. In the period between 1996 and 2002, the publication activity of the scientists working in this field remained fairly at around 200 to 250 articles a year (almost half of which were published in international journals), but since 2002, the number of publications in this area has increased significantly, in large part due to the increased number of articles published in international journals **(Fig. 20)**. Research into these technologies is conducted most frequently at RAS institutes and at institutions of higher education.

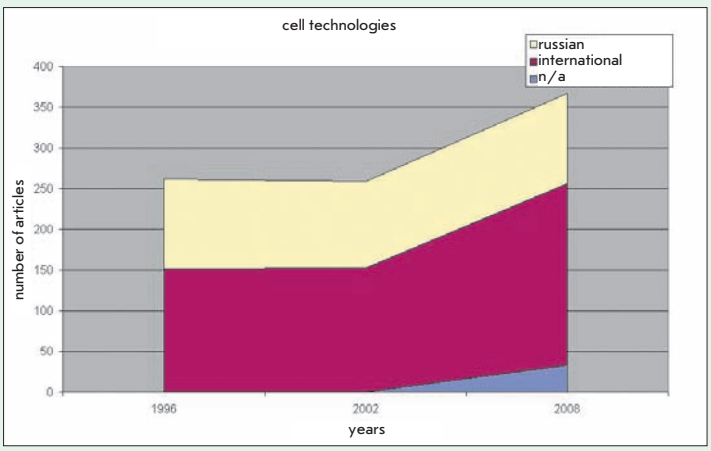


Fig. 20. The relationship between the number of Russian articles on the subject of cellular technologies published in Russian journals versus in international journals.

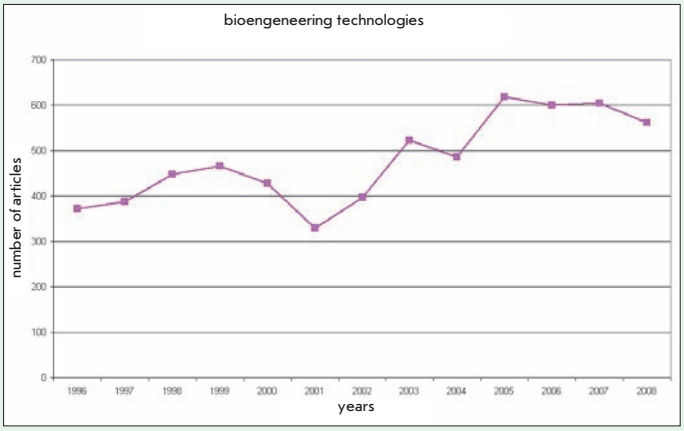


Fig. 21. The number of articles by Russian researchers in the field of bioengineering technologies.

BIOENGINEERING TECHNOLOGY

The bioengineering technologies represent a much older field of research, which has been extensively studied with significant success. The number of Russian publications on bioengineering technologies that are included in the SCOPUS database has increased by 50% (from less than 400 to almost 600 articles a year) **(Fig. 21)**. The key words used for this search were *bioengineering* and *tissue*.

The articles by Russian scientists in the field of bioengineering technologies are published largely in such Russian journals as *Biochemistry*, *Biophysics*, *Bulletin of Experimental Biology and Medicine*, *Applied Biochemistry and Microbiology*, and *Plant Physiology*. However, a significant portion of these studies has also been published in such international journals as *The Proceedings of the National Academy of Sciences of the United States of America*, *FEBS Letters*, *the Journal of Molecular Structure*, *Nucleic Acids Research*, *The Journal of Biological Chemistry*, and *Protein Engineering*.

According to the SCOPUS data, in 2008 the average number of coauthors cited in each published study was 6.4, and the average number of articles published by each collaborator was 2.05. Thus, the estimated number of researchers actively working in this field is around 1,900.

An analysis of those organizations whose research scientists are actively working in this field shows that among the RAS institutes, the Engelhardt Institute of Molecular Biology (256 articles) has the highest PA, followed by the Institute of Molecular Genetics (138), the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (73), and others.

Among the RAMS institutes, research in this field is most often conducted at the Orekhovich Institute of Biomedical Chemistry (74 articles), as well as at the All-Russian Cardiological Scientific Center (62), the Institute of Experimental Medicine (35), and the Blokhin Russian Cancer Research Center (31).

Among the research institutes and state scientific centers, studies in this realm are most often conducted at the State Scientific Research Institute of Genetics and Selection of Industrial Microorganisms (132 articles), and less so at both the Institute of Immunology of Federal Medico-Biologic Agency of Russia (43), and the Joint Institute for Nuclear Research (41).

Among the institutions of higher education, most studies in this field take place at M.V. Lomonosov Moscow State University (1,005 articles), and to a lesser degree also at St. Petersburg State University (122), and at the Moscow Institute of Physics and Engineering (102).

Bioengineering technologies have been successfully developed during the period analyzed (between 1996 and 2002). During that time, the increase in the number of articles published yearly was the result of publications in Russian journals, whereas in the years that followed (between 2002 and 2008), the number of studies printed in international journals has actually surpassed the number of those printed in Russian journals **(Fig. 22)**.

BIOCOMPATIBLE MATERIAL TECHNOLOGIES

The biocompatible material technologies are another new and fast-developing field of research all over the world. The number of Russian publications on the subject of biocompatible material technologies that are included in the SCOPUS database has increased by 50%, from 1 in 1996 to 25 in 2006 **(Fig. 23)**. The key words used for this search were *implant*, *tissue*, and *biocompatible*.

The articles by Russian scientists on biocompatible material technologies have been published in such international journals as *Biomaterials*, *Artificial Organs*, *Biosensors and Bioelectronics*, and in *Biomaterials*, *Artificial Cells and Artificial Organs*.

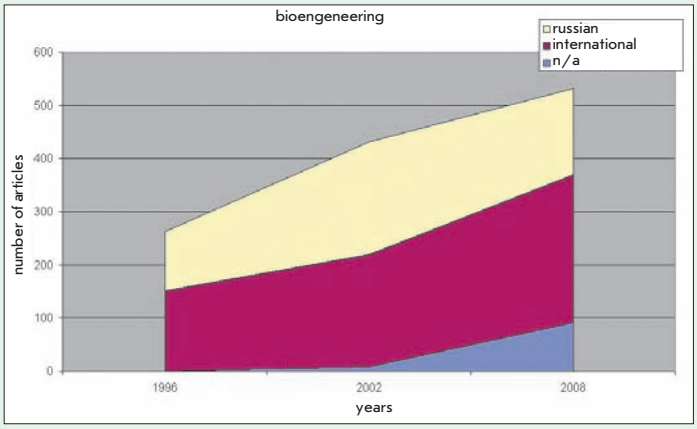


Fig. 22. The relationship between the number of articles by Russian scientists on bioengineering technologies published in Russian journals versus in international journals.

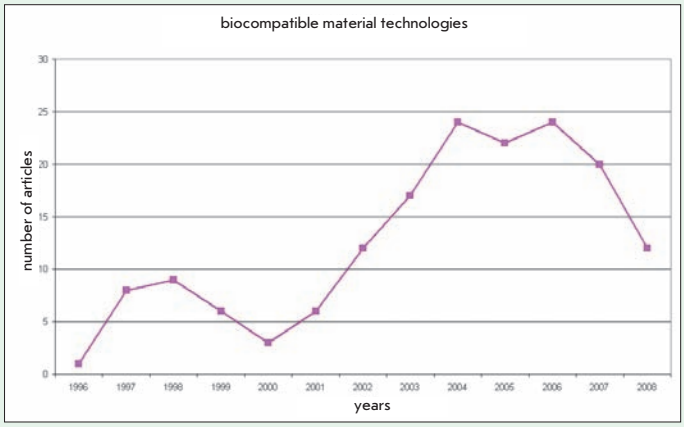


Fig. 23. The number of articles by Russian researchers in the field of biocompatible material technologies.

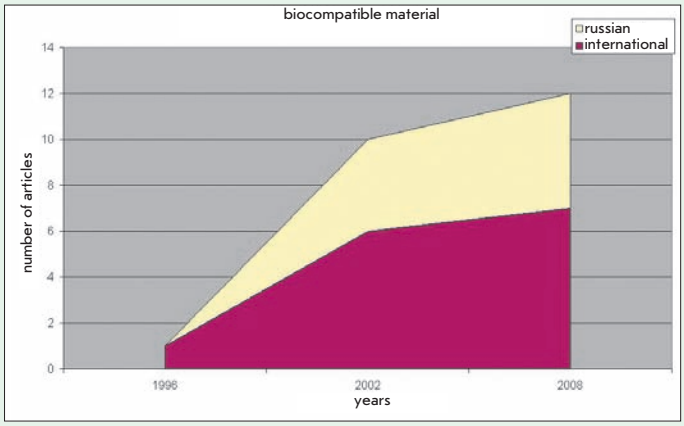


Fig. 24. The relationship between the number of articles by Russian scientists on biocompatible material technologies published in Russian journals versus in international journals.

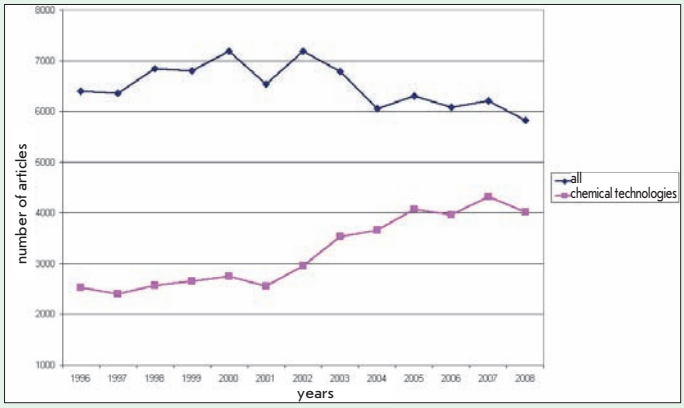


Fig. 25. An overview of the general publication activity of Russian scientists involved in investigations into the critical technologies referring to the sphere of living systems.

Some of the studies conducted by Russian researchers in this field have been published in such Russian journals as *Medical Equipment, Bulletin of Experimental Biology and Medicine, Surgery, Archive of Pathology*, and *Problems of Medical Chemistry*.

According to SCOPUS data, in 2008 the average number of co-authors cited in each published study was 4.83, and the average number of articles published by each collaborator was 1. Thus, the estimated number of researchers actively working in this field is around 100.

However, it should be noted that researchers at RAS and RAMS institutes, state scientific centers, and research institutes have published only a few articles on biocompatible material technologies.

Among institutions of higher education, the majority of investigations in this field were conducted at M.V. Lomonosov Moscow State University (24 articles).

As this analysis of the publication activity indicates, the degree of emphasis placed on biocompatible material technologies by the Russian scientific establishment is not entirely clear. On the one hand, during the period of time analyzed, those fields were the subject of an increased number of publications. On the other hand, the number of publications itself is insignificant, whereas the number of organizations carrying out research is significant enough. Moreover during the last 2 years, the number of publications has even decreased to some extent.

THE LIVING SYSTEMS PRIORITY DIRECTION AND OTHER BIOLOGICAL DISCIPLINES: REDISTRIBUTION OF ROLES

First, it should be noted that the adoption of both the Russian Federation´s List of Critical Technologies and the Federal Target Program Research and Investigation of First-Priority Aspects of Russian Science and Technology had a significant influence on the development of living systems technologies. The number of publications in almost all categories of critical technologies increased. The number of articles on bioinformatics, biomedicine, biosensor and genome technologies accounts for two-thirds of the total number of Russian publications in the sphere of biology and medicine. In spite of the slight decrease in the total number of studies in the realm of biology and medicine, during the period from 2002 to 2008, the number of articles related to critical technologies increased by one-third and in 2008 amounted to 4,000 articles per year. However, it ought to be noted that the publication activity in such critical areas as biocatalytic technologies and veterinary technologies of life support did not change, and publication activity in the sphere of biosynthetic technologies actually decreased.

It is worthy of note that the number of publications on critical technologies increased as a result of a redistribution of the total number of publications in the areas of biology and medicine in favor of critical technologies. At the same time, the decrease in the number of biological publications may be the

result of a reduction in the number of researchers by 2008, and may testify to the so-called over-saturation of researchers in this sphere **(Fig. 26)**. At the same time, the increased publication activity of researchers working in the field of critical technologies of living systems is striking. As a whole, the total number of researchers actively working in this field is approximately 10,000.

THE IMPACT FACTORS (IF) OF RUSSIAN BIOLOGICAL JOURNALS

The impact factor of journals is another indicator that reflects the number of articles published by Russian researchers. The impact factor (IF) is the number of quoted articles from a journal during the two previous years, divided by the total number of articles published in that same journal during those two years. In spite of some problems concerning the correct use of IFs, they are presently considered one of the formal criteria used for comparing investigations conducted between different disciplines. Thus, a change in the IF of Russian biological journals may be indicative of a change in the level of investigations, since this is where the results of those investigations are published.

12 Russian biological journals are registered in the SCOPUS database. Table 1 considers several variations of their IFs.

Table 1. Impact factors of Russian biological journals

Name of journal	Year			
	2004	2005	2006	2007
Biochemistry	1.058	0.858	1.368	1.476
Molecular Biology	0.623	0.435	0.33	0.805
Bioorganic Chemistry	0.358	0.571	0.572	0.63
Microbiology	0.539	0.534	0.543	0.597
Applied Biochemistry and Microbiology	0.381	0.310	0.444	0.51
Plant Physiology	0.16	0.277	0.321	0.439
Biophysics	0.367	0.362	0.435	0.43
Journal of Higher Nervous Activity	0.229	0.368	0.379	0.369
Genetics	0.119	0.240	0.254	0.265
Bulletin of Experimental Biology and Medicine	0.254	0.238	0.19	0.249
Journal of Evolutionary Biochemistry and Physiology	0.178	0.238	0.206	0.199
RAS News. Biological Series	0.037	0.027	0.048	0.098

An increase in the Impact Factor is most notable for such journals as *Molecular Biology*, *Biochemistry*, and *Bioorganic Chemistry* (i.e., the journals which normally publish the articles of scientists from Russia´s leading scientific organizations). During a 3-year period, the total impact factor of the 12 journals listed in Table 1 has increased from 4.303 to 6.076.

CONCLUSIONS

The pattern of investigations observed allows us to make the following conclusions:

First, the lagging publication activity of Russian scientists in the realm of biology, which is now further behind even the developing countries, is striking. To call things as they are, Russia, compared to other developed scientific nations, is moving in the exact opposite direction. This process is likely a reflection of the more general trends occurring in Russian science, namely, the ageing of old cadres, and as a result, a decrease in the number of highly qualified researchers.

Second, we can observe some increase in the number of publications concerning the critical technologies as they relate to the living systems. It is quite possible that this is due to the adoption, in 2002, of the Federal Target Program Research and Investigation of First-Priority Aspects of Russian Science and Technology, as well as several programs from the Russian Academy of Sciences (Fundamental Sciences for Medicine, Molecular and Cell Biology). The increase in the IFs of the leading biological journals testifies to the improved quality of the investigators, whose research is being published by these journals.

Our analysis of the publication activity also shows that the research institutes and the institutions of higher learning working within the framework of the living systems remain segregated in pools, which points to the fact that investigations of the living systems in Russia still have some potential. The state should support and encourage this potential.

The next step in this analysis would naturally be an evaluation of the publication activity generated by different laboratories. This seems only logical, since it is only at the level of individual laboratories that the efficiency of scientific teams may be adequately analyzed. Furthermore, we are planning to examine the joint publication efforts of those Russian scientists working within the Russian scientific establishments and their colleagues in foreign countries, since these joint efforts provide a clear insight into the level and the quality of scientific work.

Finally, the last step involves an individual look at some of those scientists who are leaders in their respective fields, by evaluating the parameters of their publication activity (first of all, we are talking about the citation index). ●

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Modern Technologies for Creating Synthetic Antibodies for Clinical Application

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ABSTRACT Abstract—The modular structure and versatility of antibodies enables one to modify natural immunoglobulins in different ways for various clinical applications. Rational design and molecular engineering make it possible to directionally modify the molecular size, affinity, specificity, and immunogenicity and effector functions of an antibody, as well as to combine them with other functional agents. This review focuses on up-to-date methods of antibody engineering for diagnosing and treating various diseases, particularly on new technologies meant to refine the effector functions of therapeutic antibodies.
Key words: monoclonal antibodies, humanized antibodies, single-chain antibodies, multivalency, bispecificity, target-specific delivery, barnase:barstar module, and immunodibarnase.
Abbreviations: ADCC (antibody-dependent cellular cytotoxicity); CDC (complement-dependent cytotoxicity); MAb (monoclonal antibodies); CH and CL (constant domains of antibody heavy and light chains); CHO cells (Chinese hamster ovary cells); EGFR (HER1) (epidermal growth factor receptor, cancer marker); Fab (antigen-binding fragment of antibody); Fc (constant (crystallizable) antibody fragment); FcγR (cell receptor of antibody Fc-fragments); FcRn (neonatal receptor of antibody Fc-fragments); HER1 and HER2/neu (cancer markers of tyrosine kinase receptor group); IgA, IgG, IgD, IgE, IgM (A, G, D, E, M immunoglobulins (antibodies of the A, G, D, E, M classes)); scFv (single chain fragment variable); PSMA (prostate-specific membrane antigen); VEGF (vascular endothelial growth factor); VH and VL (variable domains of heavy and light antibody chains).

INTRODUCTION

A century ago, antibodies were regarded a "magic bullet" that would selectively strike disease hotbeds in the human organism (see Paul Ehrlich's Nobel Lecture of 1908 [1]). However, Paul Ehrlich's idea was put into practice only in 1975, when Köhler and Milstein's study initiated the development of monoclonal antibody technology. This technology makes it possible to produce not just a set of diverse immunoglobulin molecules (natural antibodies), but also a monospecific antibody focused on one specific antigen (monoclonal antibody, MAb) in response to antigen-driven immunization. This method is still the cornerstone of antibody reshaping. Unfortunately, the first attempts to use mouse MAb for clinical purposes were not successful and revealed the following, virtually insurmountable disadvantages of MAb: in some cases, its antibody affinity is lower than that of polyclonal antiserum; it has a high immunogenicity to humans and, as a consequence, is rapidly eliminated from the body; and it is unable to activate the complement system and cellular mechanisms of the immune response in a foreign environment. Nevertheless, after three decades of battles and defeats, hopes and PR blitzes, MAb proved to be a successful medicinal product from both the clinical and commercial standpoints (Table 1). The unique potential of immunoglobulins characterized by modular structures and functions related to other structural modules was realized, and the antibodies were modified for variable clinical applications thanks to the technologies of genetic engineering and transgenic animals. Depending on the practical task, researchers can

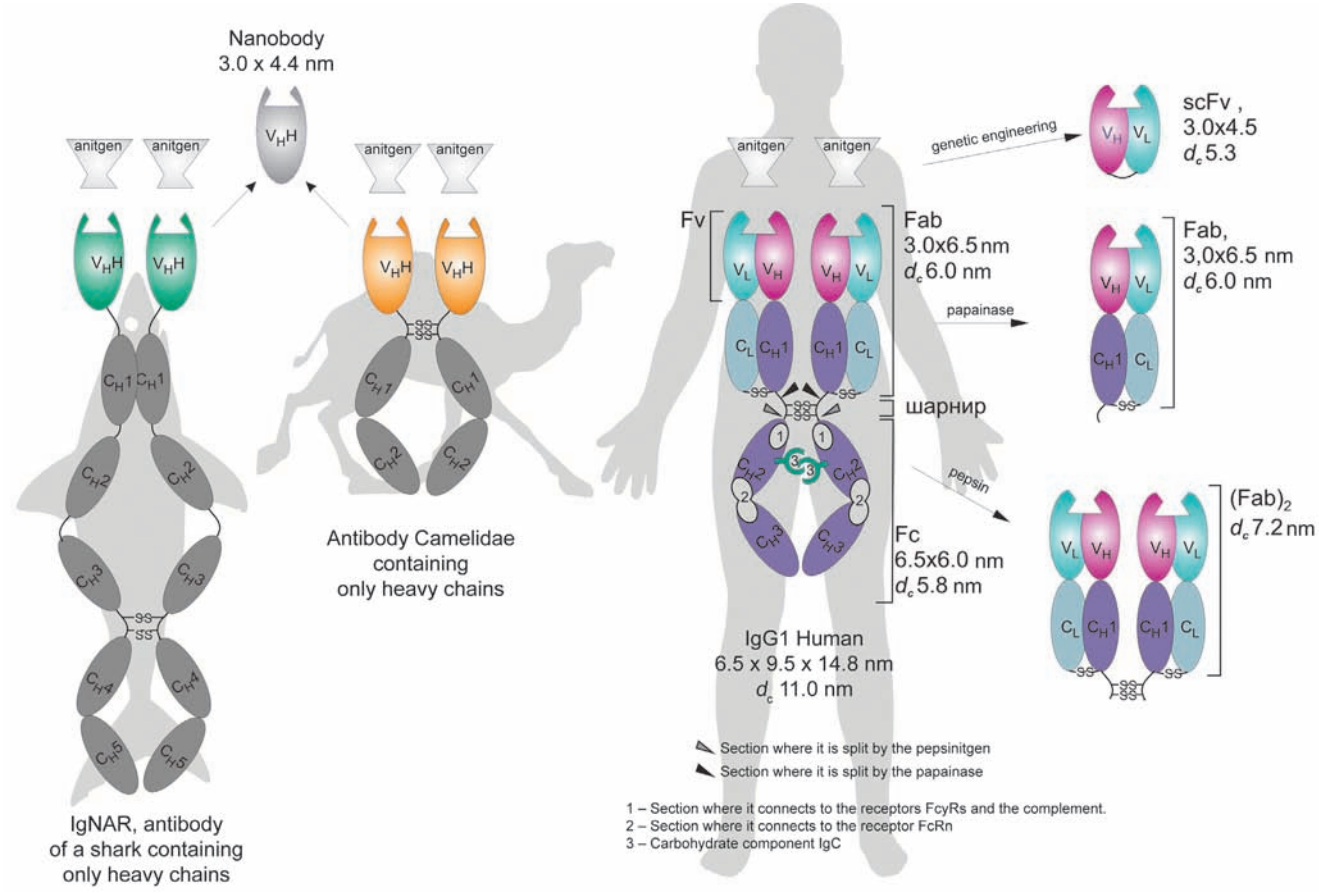

Fig. 1. Natural antibodies and their fragments. Fab and $(Fab)_2$ are antigen-binding IgG fragments produced by papain and pepsin hydrolysis, respectively; Fc is the *C*-end part of IgG composed of CH2 and CH3 constant domains of heavy chains responsible for effector functions; Fv is the variable fragment composed of variable domains of light (VL) and heavy (VH) chains; scFv is the single-chain variable fragment composed of VL and VH (conjugated by the gene engineering method); VHH is the nanoantibody, variable domain of cartilaginous fish and *Camelidae* antibodies containing only heavy chains; -S-S- is the disulfide bond. The indicated linear dimensions of antibodies and their fragments were measured by force microscopy methods [2, 3]; hydrodynamic diameter d_c was calculated by the Stokes-Einstein formula [4, 5].

directionally modify the molecular size, specificity, affinity, and valency; they can decrease immunogenicity and refine pharmacokinetic properties and effector functions. Moreover, antibodies are obtained as recombinant-fused proteins which include other specific antibodies, cytokines, protein toxins, radioisotopes, ferments, and fluorescent proteins. Currently, about 30 antibody medicines are approved for clinical application, 89% of which are used in treating oncological and immunological diseases. Antibodies are also used in treating cardiovascular, autoimmune, and infectious diseases (Table 2). On the pharmaceutical market, antibodies come in second after vaccines in production volume. By 2011, the sales volume of antibody medicines is predicted to increase to $21 billion (Table 1). More than 85% of antibodies approved for clinical application are products of antibody reshaping. The approved antibodies include chimeric, humanized, and human Mab; antibodies obtained using phage display; and genetically engineered antibody conjugates with cytokines and toxins. Hundreds of antibody derivatives are still subject to

Table 1. Commercial success of several MAb used in oncology [Deonarain, 2008].

Commercial/ USAN[1] antibody name	Sales in 2005–2006, US$, mln	Increase in sales relative to previous year, %	Evaluation of sales market in 2011, US$, mln
Rituxan©/ rituximab	3800	16	6300
Herceptin©/ trastuzumab	3100	82	4800
Avastin©/ bevacizumab	2400	77	7800
Erbitux©/ cetuximab	1100	57	2100

[1] Currently, the nomenclature of monoclonal antibodies and their fragments approved in the USA is used around the world (United States Adopted Names (USAN); www.ama-assn.org), see Table 2.

Table 2. MAb medicines approved for clinical use and possible side effects.

Application field	Commercial name	USAN nomenclature name[2]	Antibody format	Target	
Therapy of tumoral diseases	Avastin©	Bevacizumab	Humanized IgG1	VEGF	
	Bexxar©	^{131}I-Tositumomab	Mouse ^{131}I-IgG2a	CD20 (B-cells)	
	Campath©	Alemtuzumab	Humanized IgG1	CD52	
	Erbitux©	Cetuximab	Chimeric IgG1	EGFR	
	Herceptin©	Trastuzumab	IgG1	HER2	
	Mylotarg©	Gentuzumab ozogamicin	Conjugate of humanized IgG4-calicheamicin	CD33	
	Prostascint©	Capromaab pentetate	Mouse ^{111}In-IgG1	PSMA, prostate specific membrane antigen	
	Rituxan©	Rituximab	Chimeric IgG1	CD20 (B-cells)	
	Vectibix©	Panitumumab	Human IgG2	EGFR (HER1)	
	Zevalin©	Ibritumomab tiuxetan	Mouse ^{90}Y-IgG1	CD20 (B-cells)	
Transplantology	Orthoclone OKT3©	Muromonab-CD3	Mouse IgG2a	CD3 (T-lymphocytes)	
	Simulect©	Basiliximab	Chimeric IgG1	CD25	
	Zenapax©	Daclizumab	Humanized IgG1	CD25	
Cardiovascular diseases	ReoPro©	Abciximab	F(ab)-chimeric fragment of IgG1	Complex of IIB-IIIA glycoproteins	
	Monafram®		F(ab')$_2$ fragment of mouse IgG1	Complex of IIB-IIIA glycoproteins thrombocytes	
Therapy of auto-immune diseases	Humira©	Adalimumab	Human IgG1	TNFα	
	Raptiva©	Efalizumab	Humanized IgG1	CD11a	
	Remicade©	Infliximab	Chimeric IgG1	TNFα	
	Tysabri©, Antegren©	Natalizumab	Humanized IgG4	α4 subunit of α4β1 and α4β7 integrins	
	Xolair©	Omalizumab	Humanized IgG1	IgE	
Others	Lucentis©	Ranibizumab	Humanized F(ab) fragment of IgG1	VEGF	
	Synagis©	Palivizumab	Humanized IgG1	F-protein of respiratory syncytial virus	

[1] Currently, the nomenclature of monoclonal antibodies and their fragments approved in the USA is used around the world (United States Adopted Names (USAN); www.ama-assn.org), see Table 2.

[2] Currently, nomenclature of monoclonal antibodies and their fragments approved in the USA is used in the whole world (United States Adopted Names (USAN); www.ama-assn.org), see Table 2.

	Application, action mechanism (↑ increase or ↓ decrease in effect)	Company and registration year	Possible side effects [http://www.i-sis.org.uk/WOFAMAD.php]
	Intestine cancer. Binding with ligand, antagonist. Angiogenesis↓, metastasis↓.	Genentech, 2004	Gastro-intestinal perforations and wound disruption, occasionally with a lethal outcome.
	Non-Hodgkin lymphoma. Radioimmunotherapy, ADCC, CDC.	GlaxoSmithkline, 2003	Hypersensitivity reactions, including anaphylaxis.
	B-cell chronic lymphocyte leukemia.	Genzym/ Schering, 2001	Decrease of blood-forming functions of bone marrow, occasionally serious to lethal outcome.
	Metastatic cancer of intestine, head, and neck. Receptor antagonist. Apoptosis↑, chemo- and radiosensitivity↑, proliferation↓, angiogenesis↓, metastasis↓.	Imclone/Bristol-Myers Squib, 2004	Anaphylactic reactions (3% of cases) (bronchial spasm, hoarse breath, hypotension), rarely lethal outcome (1 case in 1,000).
	HER2-positive metastatic breast cancer. Proliferation↓, angiogenesis↓, chemosensitivity↑.	Genentech, 1997	Cardiomyopathy.
	CD33-positive acute myeloid leukemia. Cell intoxication due to induction of DNA breaks.	Wyeth pharmaceuticals, 2000	Heavy reactions of hypersensitivity, including anaphylaxis, hepatoxicity, and hematologic toxicity.
	Diagnostics of prostate cancer	Cytogen, 1996	Anaphylactic or anaphylactoid shocks at single dosing. Repeated dosing can cause danger to life due to serious systematic reactions of cardiovascular, respiratory, and nervous systems (shock, heart and respiratory arrest, paralysis).
	Non-Hodgkin lymphoma. Chemosensitivity, ADCC, CDC	Genentech/ Biogen, 1997	Reaction to intravenous injection. Anaphylactic shock with lethal outcome.
	Intestine cancer. Receptor antagonist. Apoptosis↑, chemo- and radiosensitivity↑, proliferation↓, angiogenesis↓, metastasis↓.	Amgen, 2006	No data
	Non-Hodgkin lymphoma. Radioimmunotherapy, apoptosis↑	Biogen IDEC, 2002	Scheme of treatment with the medicines includes Rituxan© (see above).
	Prevention of acute rejection at renal transplantation. Blocking of T-killers activity.	Ortho Biootech, 1986	Anaphylactic or anaphylactoid shocks, including: cardiovascular collapse, heart and respiratory arrest, convulsions, brain edema, blindness, and paralysis.
	Prevention of acute rejection at renal transplantation. Blocking of IL-2R.	Novartis, 1998	Heavy anaphylactic reactions (in 24 hours), including: hypotension, bronchial spasm, heart and respiratory arrest, pulmonary edema, fever, and loss of consciousness.
	Prevention of acute rejection at renal transplantation. Blocking of IL-2R.	Roche, 1999	Heavy anaphylactic reactions (in 24 hours), including: hypotension, bronchial spasm, heart and respiratory arrest, arrhythmia, pulmonary edema, fever, and loss of consciousness.
	Thromboprophylaxis. Antagonist of IIB-IIIA glycoproteins.	Centocor/Elli Lilly, 1994	Indigestion, burning sensation, belching, pruritus, numbness, nervous system disorder, mental debility.
	Thromboprophylaxis at coronary angioplasty. Antagonist of IIB-IIIA glycoproteins.	Framon Ltd, Russia, 2002	Bleeding, allergic reactions, thrombocytopenia (few cases of deep thrombocytopenia), immune response in 5–6% of cases
	Rheumatoid arthritis, psoriasis. Blocking of TNFα activity, inflammation↓.	Abbottt Laboratories, 2002	Tuberculosis, fungal invasion and other infections, acute myeloid leukemia.
	Psoriasis. Blocking of T-lymphocytes activity.	Genentech/Xoma, 2003	Hemolytic anemia, serious infections including tuberculosis pneumonia and bacterial sepsis at antimicrobic therapy
	Crohn's disease, rheumatoid arthritis, inflammatory diseases. Blocking of TNFα activity, inflammation↓.	Centocor, 1998	Tuberculosis infection, occasionally with lethal outcome.
	Multiple sclerosis. Inhibition of immune cells migration to inflammatory tissue.	Biogen Idec/Elan, 2004	Sales were stopped in 2005. Progressive leukoencephalopathy in 1 case of extended medication use.
	Allergic asthma. Inhibits egress of IgE from mast cells.	Genentech, 2002	Appearance or recidive of cancer.
	Age-related retina degeneration. Blocking of angiogenesis.	Genentech/ Novartis, 2006	Allergic reactions, intraocular inflammation, retinal hemorrhage.
	Infection prevention with respiratory syncytial virus.	Medimmune, 1998	Rarely acute hypersensitivity; repeated injection can cause (1 case in 100 thousand) anaphylaxis.

clinical testing, including synthetic antibodies produced by gene engineering: bispecific antibodies; single-chain full-sized antibodies; different variants of truncated antibodies, including dimers and monomers of Fab fragments, scFv-fragments (single-chain mini-antibodies), single-domain antibodies (nanoantibodies), etc. Different technologies that make it possible to modify immunoglobulin molecules for certain clinical purposes are considered. This review is focused on antibody reshaping for the treatment and detection of oncological diseases, because this sphere is in particular need of these medicines.

1. THE STRUCTURE OF A NATURAL ANTIBODY AND WHY IT NEEDS MODIFICATION FOR CLINICAL PURPOSES.

Antibodies, also known as immunoglobulins (IgG), are high-avid molecules which can detect and eliminate foreign antigens from the human organism. Most mammal antibodies have similar structures and are bivalent multidomain proteins with two antigen-binding sites (see, for example, human G immunoglobulin (IgG), Fig. 1). This compound Y-shaped molecular complex consists of two identical heavy (~50 kDa) and two identical light (~25 kDa) chains. Four inter-domain disulfide bonds provide stable conditions for the whole molecular complex. Globular structural immunoglobulin domains with a characteristic β-folded structure are stabilized by intradomain disulfide bonds and are subdivided into variable (V) and constant (C) domains. N-tail domains of light and heavy chains (VL and VH variable domains) vary significantly for different antibodies, while the remaining part of the polypeptide chains (CL and CH constant domains) varies for different antibody classes (and within one antibody class, depending on the species). The structural domains of immunoglobulin are isolated in space and perform different functions in the immune response process.

Each antibody is characterized by a private specificity and a high affinity (K_D 10^{-8}–10^{-11} M) to its antigen, which is provided by the complementarity of the antigen-binding site to the definite antigen molecule site (epitope). Each antigen-binding site is composed of two variable domains—VH and VL—which are referred to as heavy and light chains of immunoglobulin, respectively. Each variable domain contains three complementarity determined regions (CDR). The ability of antibodies to bind two antigens simultaneously significantly increases the functional affinity (avidity) of immunoglobulins and the retention time on the surface cell receptors and other polyvalent antigens. In all, humans are known to have five classes of antibodies (IgM, IgG, IgE, IgD, and IgA). Not long ago, some animals (camel, llama, and shark) were revealed to have nonclassic antibodies without any light chains and with two antigen-binding sites, each of which is made up of only one heavy

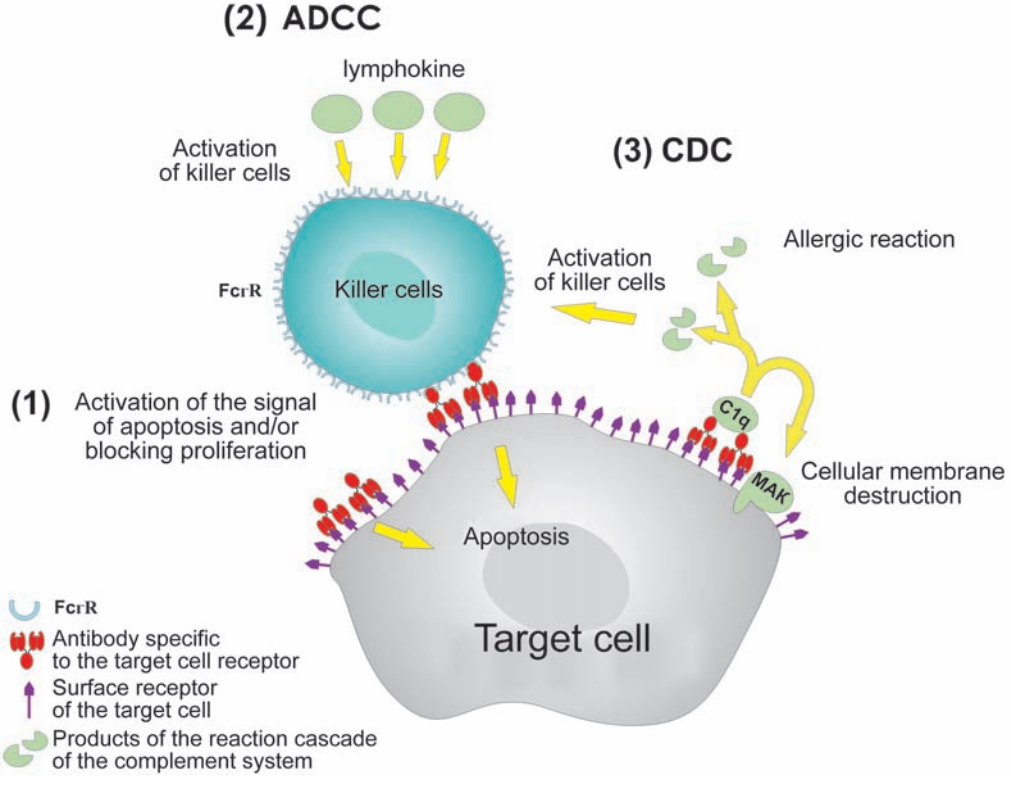

Fig. 2. Interaction Pattern of Unloaded Antibodies and Target Cell. (1) Antibodies can cause apoptosis or block the proliferation of target cells, binding with membrane antigens on their surface (membrane raft mechanism) [6]. (2) Antibody-dependent cellular cytotoxicity (ADCC). Killer cells carrying receptors of IgG FcγRI (CD16), FcγRII (CD32), FcγIII (CD64) constant domains (natural killers, killers activated by lymphokines, macrophages, and phagocytes) and receptors of IgE FcεRI and FcεRII (CD23) constant domains (acidocytes) on their surface attack the target cell, whose surface antigens were bound with antibodies. (3) Complement-dependent cytotoxicity (CDC). Antibodies conjugated in pairs bind to protein C1q complex, causing a cascade of reactions of the complement system, which leads to membrane destruction. Some products of this reaction cascade involve immune cells or cause allergic shock.

chain variable domain. The camel's variable domains were called "nanobodies" (Fig. 1).

The constant part of immunoglobulin consists of one light chain (CL) domain and three or four (depending on the class) heavy chain (CH) domains. Hydrophobic sites on the boundary of CH1 and CH2 domains remain mobile and form a link region, which provides the displacement and rotation of Fb-fragments. The heavy chain constant domains are, as a rule, glycosylated. The type of glycosylation varies significantly for different species and influences the immunoglobulin effector functions. Moreover, heterogeneous glycosylation is characteristic for the antibodies of one isotype within one species.

The IgG constant domain contains binding sites with protein of the C1q complement system and Fc-fragment cell receptors (FcγR), which mediate the effector (secondary) functions of immunoglobulins, i.e., their ability to kill target cells, activating the mechanisms of antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The ability of IgG and IgM to bind to neonatal Fc-receptor (FcRn) plays a very important role in controlling the antibody level in the blood serum. Hence, in the immune response process, an organism produces a complex set of heterogeneous molecules, which can include several cell mechanisms and a series of reactions for eliminating foreign antigens.

Modern antibody reshaping is based on the monoclonal antibody technology put forth by Köhler and Milstein in 1975 [7]. This method involves the fusion (hybridization) of immune lymphocytes (splenicytes) which synthesize antibodies of the required specificity with the immortal myeloma cell line. The cell line obtained (hybridome) secretes monoclonal antibodies (MAb), i.e., antibodies of the same species, specific to the antigen used for immunization. The *in vitro* cultivation of hybridome or the propagation similarly to ascite in a mouse makes it possible to have constant sources of antibodies with definite specificities. The fusion of hybridomes characterized by various specificities enables one to obtain so-called quadroms, which produce—along with the primary MAb—monoclonal bispecific antibodies capable of binding with two different antigens.

The introduction of monoclonal antibody technology led to great progress in using antibodies for both research and practical purposes (*in vitro* diagnostics). However, the hopes for creating specific and highly efficient anticancer drugs on the basis of MAb and their conjugates with active agents and for the rapid replacement of serum-derived vaccines with the MAb medicines, which would eliminate pathogenic microorganisms and toxins, were snuffed out. The idea of using MAb for the delivery of radioactive isotopes to target cells proved imperfect as well. It emerged that MAb have some properties which make them ineffective and dangerous for clinical use. At the first stages, individual MAb produced from a hybridome pool were often specified by a less efficient interaction with antigen than polyclonal antiserum dilution. The reason for this is the interaction between individual MAb and the sole antigenic epitope and, probably, the partial inactivation of antibodies in the release process. Full-size MAb have non-optimal pharmacokinetic properties for application as targeting molecules. As foreign proteins, mouse MAb are subject to rapid catabolism. Moreover, they have almost no interaction with the FcRn receptor, which is responsible for the recirculation of immunoglobulins from lysosomes, which speeds up the MAb removal from the blood flow. On the contrary, full-size MAb circulate in the blood flow for up to 2 weeks. Due to their high molecular weight (150 kDa), immunoglobulins are slowly distributed in the organism, have poor penetration ability, and are not cleansed from the body through the kidneys. The xenogenic nature of MAb impedes the activation of a complement system and the cell mechanisms of foreign antigen elimination (ADCC and CDC) due to the poor identification of the mouse MAb by Fc-receptors of human immune system cells. The high immunogenicity of mouse MAb is dangerous for a human being and cannot be used for clinical purposes due to the risk of hyperimmune reaction formation. Serious side effects related to the nature of the toxin attached were also noted when the first generation of immunotoxins was used. It ought to be noted that the MAb medicines successfully used in medical treatment today have dangerous side effects (Table 2).

The experience and new knowledge about the mechanism of interaction between antibodies and target molecules were gained through trial and error. It appears that some clinical cases require MAb modifications, often mutually antithetical. The technologies of gene engineering and transgenic animals, along with monoclonal antibody technology, made the greatest contribution to antibody reshaping. Let's provide some insight into the major directions of antibody engineering for clinical use. Currently, the antibody medicines approved by the Unites States system (US Adopted Names, www.ama-assn.org) are used (Table 3).

2. TRUNCATED ANTIBODY FRAGMENTS AND ALTERNATIVE FRAMEWORK PROTEINS WITH SPECIFIC ANTIGEN BINDING

Originally, the most attractive property of antibodies in terms of the "magic bullet" construction was their specific binding with the antigen, which was assumed to allow the delivery of an active agent (for instance, a radioactive isotope) to a target molecule. The governing factors which would provide the efficiency of this delivery are the *affinity* and *specificity* of the antibody targeted to the antigen, as well as its physicochemical properties, such as *valency, surface charge,* and *size* [8, 9].

The specificity of an antibody constructed for therapeutic use depends on selecting the appropriate target molecule. The MAb approved for clinical use are directed against 15 targets, most of which are surface cellular antigens, including such widespread cancer markers as HER1, HER2/neu, and PSMA (prostate specific membrane antigen) (Table 2). The last decade was characterized by great progress in understanding the mechanism of antibody action and in developing new target molecules for treating oncological, autoimmune, infection, and cardiovascular deseases.

Five groups of potential target molecules for antibodies in cancer treatment have been pointed out in recent reviews: cancer cells inside solid tumors, which are less treatable; diffusive cancerous cells (leukemia); the stroma

Table 3. USAN nomenclature of therapeutic medicines with antibodies and their fragments (United States Adopted Names; http://www.ama-assn.org/"www.ama-assn.org).

Prefix	Disease or target	Antibody origin	Suffix	Examples
Appropriate syllable(s) different from those used earlier	**vir** - virus **bac** - bacterial **lim** – immune system **les** - infection **cir** – cardiovascular **fung** – fungal **ner** – nervous system **kin** – interleukin **mul** – skeletomuscular system **os** – bones **toxa** – toxins **anibi** – angiogenesis Tumors: **col** – large guts **mel** – melanoma **mar** – lacteal gland **got** – spermary **gov** – ovary **pro** – prostate gland **tum** - different	**u** – human being **o** – mouse **a** – rat **e** – hamster **i** – primate **axo** – rat/mouse **xi** – chimera **zu** – humanized **xizu** – combination of chimeric and humanized chains	mab	
tras	tu	zu	mab	**Trastuzumab** (Herceptin©) – anti-tumor humanized MAb
ab	ci	xi	mab	**Abciximab** (ReoPro©) – chimeric MAb for thromboprophylaxis
r	anibi	zu	mab	**Ranibizumab** (Lucentis©) – humanized MAb to block angiogenesis
To create new designation, it is essential: To choose the appropriate syllable(s) different from any used earlier and to put it (them) at the beginning of a word; To add syllables in the following order: disease class (tumor type) or target, source of origin, suffix "mab"; To simplify pronunciation, the last consonant in the syllable designating the target may be omitted.				

associated with the tumor (fibroblasts); and blood vessels associated with the tumor and vascular endothelial growth factors (VEGF) [10, 11]. Additional target molecules can appear in response to blocking surface receptors [12] or inducing cancer cells under the action of a medical agent [13].

The affinity of antibodies used in therapy today is within the nanomolecular concentrations (from 10^{-8} to 10^{-10} M), which are optimal for solving most problems. Increasing the antibody affinity to 10^{-11} M significantly decreases both its ability to penetrate the tumor and its targeting selectivity [14].

The size of the therapeutic antibody molecules is an important factor, because it determines the possibility and rate of renal excretion. The time of the renal half-excretion of protein correlates with the molecular size: the limit of glomerular filtration is estimated at 60–65 kDa [15]. The full-size IgG molecules (~150 kDa) (Fig. 1) are too large and cannot be excreted through the kidneys. In contrast, the scFv-fragments (~30 kDa) (Fig. 1) are characterized by 0.5–2 hours of half-removal period and readily leave the organism [16]. The monomeric Fab-fragments (48 kDa) hold an intermediate position, with 14–15.5 hours of half-removal time [16, 17].

The optimal surface charge for therapeutic antibodies is the interval of their isoelectric points from 5 to 9. An increase in the positive or negative charge complicates the antibody binding with the target cells [18].

The valency of the recombinant antibody targeted on the antigen plays a very important role in retaining MAb in the target cells. Most natural mammal antibodies are bivalent. The ability to simultaneously bind two antigens significantly increases the functional affinity, or avidity, of immunoglobulins and provides the long-term retention of antibodies on the cell surface receptors or polyvalent antigens. This property is used in the wild: tetra- and decavalent complexes of IgA and IgM are characterized by a higher affinity to the multipoint binding of polyvalent antibodies and can protect against natural polyvalent antigens, such as pathogenic microorganisms, more efficiently.

Hence, using antibodies as targeting components for the delivery of diagnostic and therapeutic agents of, primarily, radioactive isotopes and as specific blockers of pathogenic processes required the elimination of their effector functions and a cardinal modification of their physicochemical properties.

The first truncated fragments of antibodies—monomeric Fab and dimeric $(Fab)_2$ (Fig. 1)—meant for use in radioimmunotherapy were produced by processing intact IgG with such proteolytic enzymes as papain and pepsin (Fig. 1). These fragments are absent in the Fc domain, which mediates the IgG effector properties that are undesirable in the current situation. Moreover, the considered fragments are not capable of recirculating from lysosomes, which causes the dose-limiting myelotoxicity and nonspecific radioactive background to decrease in comparison with the full-size IgG. However, the fragments obtained retain antigen-binding properties that are comparable with those of the parent antibodies. Examples of the therapeutic use of the monomeric Fab-fragment include ReoPro© antiaggregant (Abciximab) and its Russian analogue, Monafram® [19], which are applied in cardiosurgery for thrombosis prevention, as well as an angiogenesis blocker—Lucentis©(Ranibizumab)—used for treating age-related retina degeneration (Table 2). Because of its diminished size, Lucentis©(Ranibizumab) readily penetrates into the inner eye cover, unlike full-size antibodies, making it ideal for clinical application [17]. The main disadvantage of the enzymatic method of antibody fragment production is the complicated purification of the target product. Moreover, further modification of the antibody's physicochemical properties is possible only by chemical methods. Furthermore, the first generation of Fab-fragments was of mouse origin and characterized by high immunogenicity.

Developing genetic engineering methods made it possible to simplify the preparation of truncated antibody derivatives. Recombinant antibodies, so called single-chain variable fragments (scFv) (Fig. 1), are coded by one gene and contain only one antigen-binding site, which consists of variable domains of light (VL) and heavy (VH) chains connected by an elastic peptide linker [20] (Fig. 1). These scFv-fragments are monovalent and are often specified by a lower affinity than the primary one. Their rapid removal from the organism and homogenic focus on the target makes them more appropriate for delivering radioactive isotopes than the parent full-size IgG. Other very important advantages of single-chain antibodies are as follows: their compatibility with bacterial expression systems, their reduced immunogenicity, and their absence of effector functions. Nevertheless, the monovalent scFv-fragments never gained universal currency in medical practice, because even a high-affinity but a monovalent binding with the antigen on the cell's surface does not provide long retention and leads to the rapid dissociation of antibody.

Cloning single-chain antibodies retaining antigen-binding functions had significant consequences. From then on, the technologies for creating synthetic antibodies of almost any specificity were quickly climbing to new heights. Diverse collections (libraries) of recombinant antibody fragments were designed, new methods which could increase their diversity were offered, and technologies which made it possible to increase the affinity of antibodies and efficiently select new variants were developed [21, 22]. The selection platforms for immunoglobulin libraries were developed on the basis of phage, ferment, and ribosomal displays [23]. Nonclassical antibodies of cartilaginous fish and animals of the Camelidae genus (camel and llama), containing only heavy chains, were the perspective source for antigen-binding fragments with uncommon properties (Fig. 1). An antigen-binding site of such antibodies was formed by the sole variable domain, which is characterized by good solubility, high stability, and the ability to bind with difficult-to-access antigens [24, 25].

That period was also marked by the intensive development of molecular constructions—alternatives to the binding domains of antibodies—which were called *scaffolds* in English-language scientific literature. This term was offered by A. Plyuktun [26] for designating a protein carcass or framework bearing altered amino-acid residues or small sequences, which provided different protein variants with various functions, commonly, the possibility of effective binding with specific target molecules. The word *scaffold* is translated into Russian as a construction for capital punishment or as construction trestles. "Construction trestles" is the implied meaning, but it is hardly possible to use this phrase in the context of protein engineering. Therefore, we offer the terms *framework protein* or *framework peptide* along with the English calque *scaffold*.

Typical natural framework proteins or scaffolds are antibodies or T-cell receptors. There are many other natural and synthetic framework proteins, for instance, affibody, peptabody, ankyrin repeats, and lipokalins [26]. Developing alternative framework compounds is a perspective direction in research for creating new compounds to selectively bind to target molecules and cells. New compounds are meant for biomedical use and are appropriate for robotic technologies and the construction of supramolecular nanostructures. These facts are confirmed by the creation of the ProteomeBinders Association in Europe, which is focused on thoroughly studying human proteome using high-affinity reagents [27].

3. GENE ENGINEERING TECHNOLOGIES FOR OPTIMIZING BIODISTRIBUTION AND PHARMACOKINETICS.

A wide range of theoretical and experimental data testifies to the fact that the simplest method to overcome the imperfection of the recombinant single-chain Fv-fragments is a return to multivalency, which is a primary characteristic property of antibodies. Indeed, every effort was spent to construct multimeric formats of truncated antibodies in order to optimize their pharmacokinetic properties and to improve the biodistribution of the active agents delivered by antibodies. Currently, a wide variety of such constructions is subject to clinical testing [28, 29].

In the past decade, a wide range of methods was developed in engineering multivalent antibodies; they are described in detail in our review [9]. The valency of the constructed multivalent derivatives, i.e., the number of antigen-binding units, may vary from 2–10. The offered strategies are based on the chemical conjugation of anti-

body fragments, the use of self-associating peptides, and the phenomenon of domain shuffling when two antibodies of different specificities are mixed. Antibodies, both bivalent tandem and bispecific are obtained by linear gene fusion. Using heterodimerization modules, including the streptavidin-biotin system, the barnase–barstar module [30, 31], and the "knob-and-holes" [32] and "dock and lock" [33, 34] methods, is more universal. Each strategy has advantages and disadvantages, but none of them is universal. The systematization and comparative analysis of the experimental data on the pharmacokinetics of multivalent antibody derivatives of different formats focused on HER2/neu and CEA cancer markers and on angiogenesis markers, such as the ED-B domain of fibronectin, showed that pharmacokinetic characteristics and biodistribution are significantly improved when monovalent antibodies are replaced with bivalent (multivalent) varieties [9].

One more important method of varying the MAb pharmacokinetic characteristics is a delicate site-specific modification of the constant domain depending on certain applications of the immunoglobulin constructed. Neonatal or so-called "salvage" Fc-receptors (FcRn) perform the following important functions in an organism: they transport IgG through epithelial and endothelial barriers, they transmit immunoglobulins from mother to child, and they protect IgG and albumin against catabolism in lysosomes of vascu-

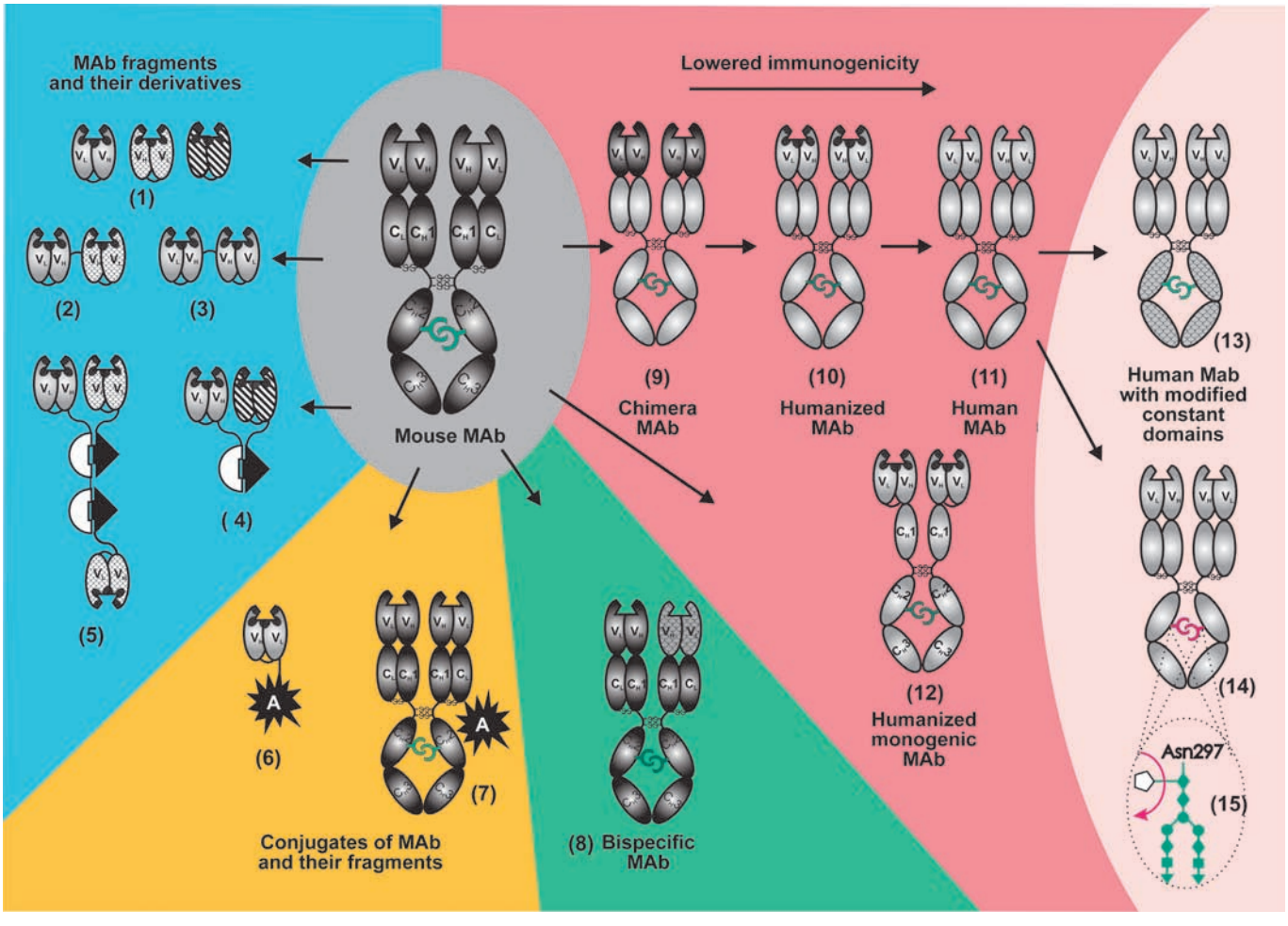

Fig. 3. Modification of mouse monoclonal antibodies (MAb) for clinical application. The following MAb fragments produced by gene engineering are considered: (1) single chain antibodies (scFv or mini-antibodies) of different specificity and composed of VL- and VH-domains bound by peptide linker; (2) bispecific mini-antibody; (3) dimeric mini-antibody; (4, 5) dimmer and trimer of mini-antibody bound by the barnase–barstar module; (6) conjugate of mini-antibody with bioactive agent produced by the gene engineering method; (13) human MAb with modified constant domains; (14) human MAb with modified carbohydrate component (15). Legends of antibodies and their fragments are as in Fig. 1; hypervariable sites of variable domains (CDR) are indicated with black color; mouse antibodies and fragments, with dark gray; human antibodies and fragments, with light grey; variable domains of different specificity (1–5) and modified constant domains (13) are indicated with hatching. White semicircle and black triangle denote barnase and barstar, respectively. A denotes the bioactive component (radioactive isotope, toxin, ferment, fluorescent protein, etc.).

lar endothelial [35]. FcRn is characterized by a high affinity to IgG at pH 6.0 and does not bind with it at pH 7.2. Hence, the mechanism of IgG prevention against catabolism is based on the binding of FcRn with antibodies in lysosomes at pH 6.0 and returning to the blood flow [36]. Directed mutagenesis of the Fc-fragment makes it possible to produce different variants of antibodies, randomly changing the time of their half-life in the blood flow [37]. Replacing key amino-acid residues in the constant domain sites of mouse anticancer antibodies that are involved in the interaction with FcRn allowed several mutants to be prepared with a half-life time from 8 to 80 hours in the mouse's organism. The same indicator is ~12 days for wildlife antibodies [38]. These antibodies are readily removed from the organism, which is of critical importance for radioimmuno therapy and improves the resolution when the scintigraphy methods are used. On the other hand, mutant antibodies with a higher affinity to binding with FcRn at pH 6.0 are specified by a longer half-life in the organism [39]. Increasing the circulation time is important when using therapeutic antibodies to treat infectious diseases or neutralize antibodies for treating acute intoxications. For instance, injecting triple mutation M252Y/S254T/T256E into the MAb constant domain specific to the respiratory syncytial virus increased their binding by ten times at pH6.0 with FcRn of a human being and a crab-eating macaque (Macaca fascicularis). At the same time, mutant MAb are readily separated from antibodies at pH 7.4. Investigating the pharmacokinetic properties of mutant MAb on the basis of a primate model showed that the lengths of both their half-life in the blood flow and accumulation in the lungs increased 4 times [40]. It ought to be noted that mutagenesis of the constant domain amino-acid residues responsible for binding with FcRn commonly does not influence other effector properties of antibodies. Hence, directed alteration of the FcRn-binding sites allows the control of the pharmacokinetic properties of MAb constructed for different purposes. Moreover, creating peptide antagonists, which block the FcRn receptor, may be done to decrease the level of pathogenic IgG in the organism, which is one more method of autoimmune disease treatment [41].

An alternative method to improve the pharmacokinetics and biodistribution of mini-antibody derivatives is adding polyethyleneglycol [42] and albumin [43], which would increase the molecular weight of the compound and, respectively, decrease renal excretion. It is quite possible that when albumin is added, additional recirculation mechanisms mediated by the neonatal receptor are activated [44].

4. THE REDUCTION IN THE IMMUNOGENICITY OF MONOCLONAL ANTIBODIES (HUMANIZATION).

One of the first problems arising in the course of creating antibodies for clinical purposes is the high immunogenicity of MAb, which is xenogenic for a human being. The human anti-mouse antibodies response (HAMA) causes serious systematic reactions in the human organism up to allergic shock (Table 2). Using gene engineering methods allowed the partial replacement of the mouse MAb immunogenic sites with the corresponding fragments of human antibodies.

In **chimeric** MAb [45] (Fig. 3), all constant domains of mouse antibodies are replaced with constant domains of human immunoglobulin. In **humanized** MAb (Fig. 3), only hypervariable sites responsible for complementary interaction with antigen (CDR) are of mouse origin. Monogenic constructions of humanized antibodies are produced by gene engineering methods as well [46]. Currently, pure mouse antibodies are applied in small doses only in radioimmunotherapy, due to the necessity of rapid excretion from the blood flow (Bexxar© and Zevalin© medicines in Table 2).

More complex technologies were developed to obtain pure **human** MAb (Fig. 3). Screening antibody scFv or Fab-fragments of the desired specificity from the combinatorial libraries (phage, ribosome, and ferment displays) with the following reconstruction of full-size human immunoglobulins from them does not require preliminary immunization. The Adalimumab (Humira©) medicine widely used in treating autoimmune diseases was initially obtained as a scFv-fragment by the Cambridge Antibody Technology (MedImmune) and then restored to the formation of an antibody [47]. Pure **human** MAb are also created in transgenic mice with the expressing genes of human immunoglobulins. The technology involves replacing mouse IgG gene locuses with their corresponding human genome sites. One example is Panitumumab (Vectibix©), specific to EGFR, made by HuMouse technology (Abgenix).

Currently, this sphere is being actively developed, and all the many methods used for antibody humanization may be conditionally subdivided into rational and empiric [48]. The rational methods involve a so-called design cycle: generate a few variants based on data on the structure of the antibody and its gene, followed by analyzing the variants, and then selecting the best variant. The rational methods include the following procedures: grafting hypervariable sites of the mouse MAb responsible for the complementarity of interaction (CDR-grafting) or only 20–30% of those responsible for specificity (SDR-grafting) into the framework of the human immunoglobulin [49]; resurfacing mouse MAb to make it similar to the human immunoglobulin surface; and superhumanization based on the detection and elimination of potential epitopes from the mouse immunoglobulin molecule for a major complex of histocompatibility and T-cells, i.e., human string content optimization [50]. Recently, the single-domain antibodies of a camel (nanoantibodies) (Fig. 1), which are characterized by high stability, technological effectiveness, and the ability to bind hard-to-access antigens, have drawn the attention of researchers. The general strategy of humanization was developed to apply those nanoantibodies for clinical purposes [51]. Researchers revealed amino-acid residues which determine the distinctions of nanoantibodies and the corresponding heavy chain variable domain of human IgG. Then, nanoantibodies taken from the representative library were humanized in the course of the sequential replacement of several sites in the molecule. After that procedure, nanoantibodies remained stable enough and kept their primary affinity.

In contrast to the rational methods, empiric methods of humanization are based on the creation of large combina-

torial libraries [21, 22] and selecting the required variants using such processing technologies as phage, ferment or ribosomal displays, or high throughput screening methods [23]. These methods may be applied mostly to single-chain antibodies (scFv), which, when needed, may be transformed into monogenic MAb [46] (Fig. 3, *8*) by fusion with the Fc constant domain and dimerization or they may be reconstructed into a full-sized antibody with a classical structure.

As a rule, both rational and empiric methods complement each other. Currently, about 40 humanized and human MAb obtained by new selection methods for cancer treatment are undergoing the last stages of clinical testing [47]. The humanization of therapeutic antibodies not only weakened the dangerous side effects, but also allowed more complete use of the powerful potential of MAb, i.e., the involvement of effector functions.

5. THE OPTIMIZATION OF ANTIBODY EFFECTOR FUNCTIONS. Antibodies bound to the surface of the target cell can cause its death by triggering the ADCC and CDC mechanisms (see above) (Fig. 2). Far from all clinical applications of Mab find these mechanisms useful. For instance, the MAb effector functions are not required for the directed delivery of radioimmunoisotopes or blocking transplant rejection; moreover, they can cause dangerous complications. In other cases, the action of MAb may be intensified with the aid of killer cells. One such method was creating bispecific MAb, which attracted cytotoxic T-lymphocytes to the target cells (see Section 6). Moreover, in recent years researchers have detected a wide range of immunocyte receptors responsible for the interaction with antibodies and determining the role of IgG sites in this interaction. Recent investigations have showed that the efficiency of a therapeutic antibody depends on its affinity to FcγRIIIa (CD16) and FcγRIIa (CD32), which is characteristic for the activating receptors of most killer cells. It became apparent that the reaction of a patient to treatment by MAb medicines depends on his phenotype. Patients with FcγRIIIa-158 V/V and FcγRIIa-131 H/H phenotypes are at an advantage when being treated for follicular and non-Hodgkin lymphoma with Rituxan© (Rituximab) [52]. Analogous results were obtained when breast cancer was treated by MAb Herceptin© (Trastuzumab) [53]. On the other hand, a therapeutic antibody can bind to a FcR receptor on non-toxic cells (thrombocytes and B-cells), which are able to inhibit the activation of effector killer cells (for instance, the FcγRIIb receptor on macrophages). Experiments on animals show that the Fc RII inhibitor isoform decreases the therapeutic effect of humanized Herceptin© (Trastuzumab) [54]. The presented data testify to the fact that, at the current stage of gene engineering, it is essential to optimize the effector functions of the antibody depending on the sphere of application in order to increase the efficiency of the therapeutic treatment.

It is also necessary to mention the constructions where a constant part of immunoglobulin is used in combination with targeting peptides (instead of the IGG antigen-binding sites) [55] or with monomeric and dimeric cytokines to improve pharmacokinetics and protect against degradation [56].

There are several methods to optimize the antibody's effector functions. To deactivate these functions, scientists use different variants of truncated antibodies depleted in the constant domains responsible for binding to the Clq component and killer cells (see Section 3). Examples of truncated antibodies are ReoPro© (Abciximab) and Monafram® antiaggregants, as well as the antigenesis blocker Lucentis© (Ranibizumab) (Table 2). Another method is replacing IgG1 with IgG2 like in the cancer medicine Vectibix©(Panitumumab) specific to the EGFR marker (HER1) [57] (Table 2). The IgG2 constant domain almost doesn't bind to FcγR receptors of the killer cells at all: therefore, antibodies of this subclass cannot cause ADCC.

A more universal approach involves the directed mutagenesis of constant domain binding sites with C1q and FcγR cell receptors. Directed mutagenesis was made possible due to the accurate mapping of FcγR binding sites on the IgG1 constant domains [58]. Researchers managed to obtain a whole spectrum of mutant MAb from antibodies which barely bound to FcγR and C1q and are therefore not able to cause ADCC and CDC, up to high-affinity antibodies characterized by a whole range of reactions caused by those two mechanisms [59–61]. Researchers also created MAb variants that were not able to bind to C1q and cause reactions of the complement but could retain a high affinity of binding to FcγR receptors and the ability to cause ADCC [50]. The important result of that investigation was the creation of MAb (S239D/I332E/A330L) which could readily bind not only to the rare homozygotic allele of FcγRIII-158V/V (20% of patients), but also to more ubiquitous alleles, such as FcγRIII-158V/F (45% of patients) and FcγRIII-158F/F (35% of patients). The construction of therapeutic antibodies with effector functions independent of patients' polymorphism makes it possible to successfully treat all patients with medicines such as Herceptin© (Trastuzumab) and Rituxan© (Rituximab).

IgG contains only 3% of carbohydrate (Fig. 1); however, in spite of its insignificant content, this component plays a very important role in the performance of antibody effector functions. IgG contains two branched oligosaccharide chains, each of which is joined through a nitrogen atom to Asn297 residue in the constant CH2-domain (Fig. 3). The carbohydrate composition is characterized by high species specificity and varies within one species. The glycosylation of synthetic (or constructed) antibodies depends on the system in which they were prepared, the selected clone, and the separation system. Therefore, the "incorrect" glycosylation of therapeutic antibodies obtained in xenogenic systems (see Section 8) can cause an immune response, allergic reactions, and favors the rapid removal of these antibodies from the human blood flow [62]. Oligosaccharides (glycans) are known to be the key components in the IgG composition, because they provide the optimal binding of IgG constant domains with the FcγR receptors mediating the effector functions. It has been proven recently that the removal of fucose from antibody carbohydrate increases the ACDC by over 50 times [63]. A decrease in the antibody efficiency in the organism (when compared to the *in vitro* experiments) forces the concentrations of the medicine injected to increase. This situation is related to the inhibitory

effect of fucosylated immunoglobulins in the blood plasma [64]. Non-fucosylated therapeutic antibodies are characterized by a higher affinity to FcγR receptors than fucosylated ones, which allows them to withstand the inhibitory effect of IgG circulating in the blood plasma and to be effective at lower concentrations [65].

All therapeutic antibodies approved for clinical application are produced today in the CHO cells, in mouse myeloma cells such as NS0 and SP2/0, and in mouse hybridomes. Almost all of them have fucosylated carbohydrate components and, as a result, non-optimal ACDC [66]. The fucosylation of the IgG carbohydrate component is carried out in mammal cells with α-1,6-fucosyl transferase (FUT8). Some mouse cell lines are characterized by a lower level of this ferment than CHO. However, the most efficient method to eliminate fucose is inactivating the FUT8 gene. CHO lines with inactivated FUT8 genes allow the construction of non-fucosylated therapeutic antibodies with ACDC increased 50–200 times [67]. The explanation of the role of the IgG carbohydrate component gave the impetus to a new direction in antibody engineering. Today, technologies of antibody construction, taking into account the carbohydrate component, are making rapid progress [68]. This direction is considered the most promising for constructing next-generation therapeutic antibodies with improved characteristics.

6. BISPECIFIC ANTIBODIES

Natural antibodies are monospecific, i.e., they bind to antigens of only one species. Antibody engineering technologies allow bispecific antibodies (Fig. 3, *2, 4, 5, 8*) with a specificity to two different antigens to be created [29, 69]. The first bispecific antibodies were produced by fusing two hybridome cell lines. The quadroma created yields a random mixture of a target-oriented bispecific antibody and primary antibodies. Then, the required variant is obtained in the course of a complicated separation. A range of approaches was offered to increase the production of bispecific molecules, for instance, the creation of an interspecific mouse quadrome in the course of pairing the light and heavy chains of one species [70] and delicate "knob-and-holes" technology [32]. Two mutants were injected into the MAb CH3-domains: "the knob" for replacing the small amino-acid residue (threonine) with a bulky amino-acid residue (tyrosine) and "the hole," for reverse replacement.

Fusing the hybridomes that produce two types of antibodies routinely led to the formation of bispecific molecules, which could be clicked-up by "knob-and-hole." Chemical conjugation [71] is still considered the most popular method of bispecific antibody creation, in spite of disadvantages such as the necessity of chemical modifications, which are occasionally harmful to antibodies, and the problem of separating the variants obtained.

The gene engineering methods allow the creation of bispecific MAb and their fragments, avoiding the problem of separating the complex mixture produced [9]. The streptavidin-biotin system, characterized by stable association with a constant of K_D 10^{-15} M dissociation, is traditionally used for these purposes. We offered a barnase–barstar protein pair (Figs. 3, *4, 5*) as a heterodimerization module. This protein pair is characterized by a rather stable association with the K_D 10^{-14} M dissociation, almost just like the streptavidin-biotin system, but makes it possible to produce bispecific antibodies with an exact ratio of 1 : 1 or 1 : 2 (trimer) and does not require any chemical modification of the components [72], which is essential for delicate proteins such as antibodies. Recently, a new sophisticated method called "dock and lock" (the DNL-method) was developed to create bispecific antibodies [33] (see also Section 7). Antibodies produced by this method are distinguished by good pharmacokinetic characteristics and bivalent binding with surface antigen; thus they can be regarded as perspective agents for radioimmunotherapy. However, the major disadvantage of this method is the low affinity of the (K_D ~ 10^{-9}) complex, which requires additional stabilization by disulfide bonds.

Bispecific antibodies were primarily used for retargeting the cytotoxic immune cells on the pathologic targets. Numerous bispecific antibodies were created: one identification domain of an antibody was bound to a characteristic surface marker on the cancer cell, while another domain was bound to an activation receptor of the cytotoxic cells [69, 73]. IgG FcγRI (CD64), characteristic of monocytes, macrophages, and dendritic cells; IgA FcαRI (CD89), expressed largely in neutrocyte, macrophages, and acidocytes; and the CD3 marker of cytotoxic T-lymphocytes were used as receptors which can attract cytotoxic cells to the target. It should be noted that T-lymphocyte, one of the most efficient killer cells, cannot be attracted to antibodies due to a lack of Fcγ receptors. Unfortunately, clinical testing established the shortcomings of that method. Immune cells needed additional activation, an effect that appeared only at a 40- to 100-fold surplus of effector over the number of target cells and high dose of medicine, and, finally, high dose-limiting toxicity in medicines was observed [73, 74]. The use of bispecific antibodies for attracting cytotoxic T-cells to the cancer targets by binding with CD3 was effective only upon local application [75] and on the adaptive transfer of T-cells processed by bispecific antibodies *ex vivo* [76].

Almost all those obstacles were overcome by virtue of a new generation of bispecific T-cell engagers (BiTE), which represented recombinant bispecific single-chain antibodies composed of two scFv-fragments specific to the surface antigen of the target cell and T-cell CD3 antigen. In contrast to the above-mentioned full-size bispecific MAb, BiTE antibodies do not require the additional activation of attracted immune cells and have enough cytotoxicity at much less concentrations [77, 78]. Obviously, bispecific MAb, which retargets immune cells, can be a perspective direction especially for the treatment of cancerous blood diseases. Clinical investigations show that the application of Blinatumomab (anti-CD19/anti-CD3) medicine in a concentration of 0.005 mg/m^2/day leads to the complete elimination of cancer B-lymphocytes from the blood flow of non-Hodgkin lymphoma patients [79].

Analogous methods involving bispecific antibodies were used to retarget stem cells, viruses, and pathogens. Bispecific antibodies, which identify the receptor of the CD45 stem cells, and antigens, which become readily accessible in

case of myocardial infarction [71], were suggested as a way to attract the stem cells to the myocard of an experimental animal suffering from a heart attack and for the regeneration of vascularization. This approach allows the delivery of a greater number of cells than injections of the stem cell suspension directly into the myocard. The researchers also tried to use bispecific antibodies for directed delivery to adenovirus tumors for gene therapy [80] and to eliminate pathogens from the blood flow, retargeting them on the CR1 receptor on erythrocytes [81]. These investigations are far from complete and will be subject to clinical trials.

The second considerable sphere of clinical application of bispecific antibodies is pretargeting with the following directed delivery of toxins, chemotherapeutic agents, and radioactive isotopes to the cancer cells (Fig. 4). The idea of pretargeting with the following elimination "on a tip" is based on the desire to maximally decrease the global toxic effect when using strong toxins and radionuclides at the cost of both less circulation time in the blood and a decrease in the toxin therapeutic concentration. For this purpose, at the first stage, antibodies that are focused on a target—for instance, a tumor—are injected into an organism; then, after the natural removal of antibodies unbound to the target, the second cytotoxic component (toxin, radionuclide) is injected and specifically binds to the injected antibodies (Fig. 4). Initially, unmarked bispecific antibodies were suggested to be used for pretargeting: one valency would be bound to the tumor antigen, while another valency would be bound to the hapten carrying a radioactive isotope [82]. The disadvantage of this method is the monovalent non-optimal binding to the antigen, as well as the relatively low production of bispecific antibodies obtained from the myelome line cells. The gene-engineering methods made it possible to construct a range of truncated antibody fragments for radioimmunotherapy, including multivalent fragments (see Sections 1, 2, and 7), which led to a significant improvement in the pharmacokinetic properties of the targeting antibodies, specificity, and resolution of the method [83].

The third direction, which requires the construction of bispecific antibodies, is the simultaneous binding of two different antigenic determinants on one target. The high-

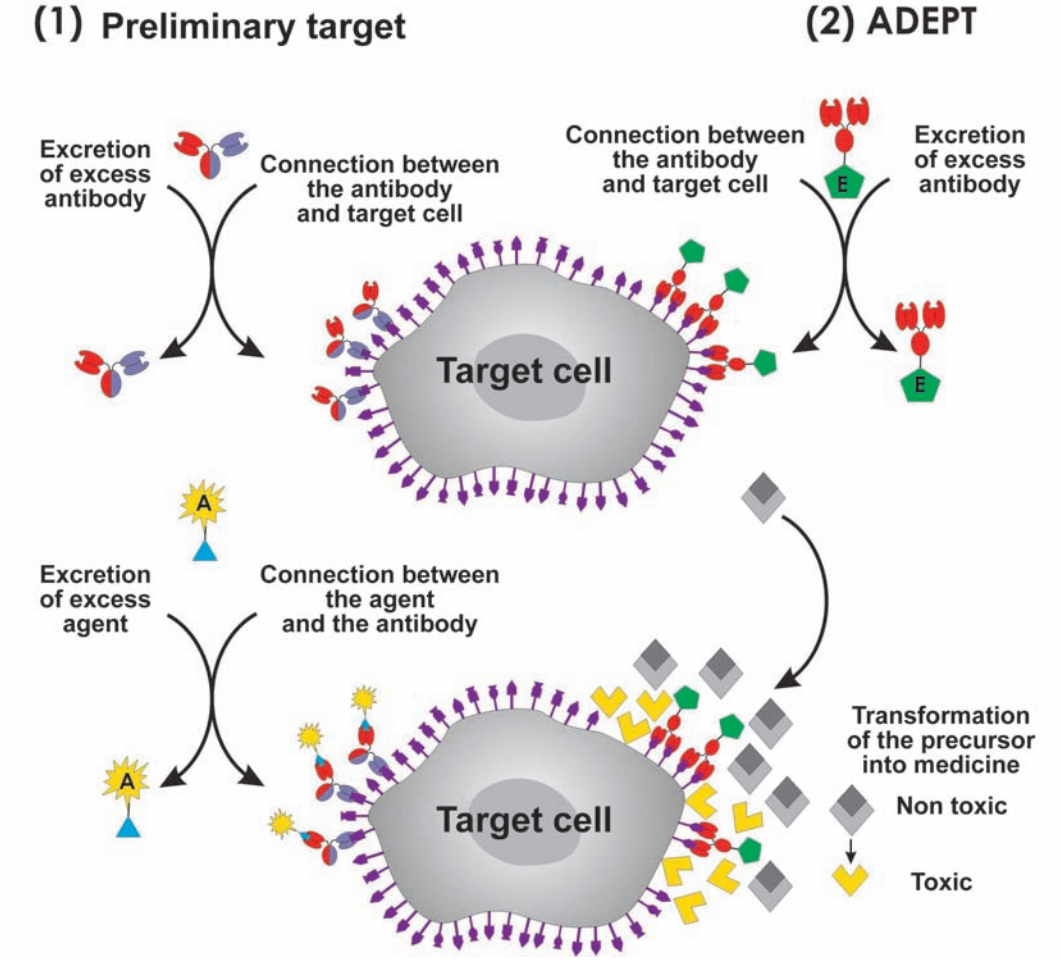

Fig. 4. Scheme of two-stage target-killing technologies. (1) Pretargeting using antibodies with the following target killing by the involved A agent. (2) Preliminary delivery of E ferment to the target. At the second stage, E ferment transforms the nonactive medicine precursor into an active form (ADEPT, antibody-directed enzyme pro-drug therapy).

er density of the surface antigen on the target cells compared to the nonpathogenic cells is an essential condition for using this antigen for targeting; this provides specific action on the target and the safety of the whole organism. The number of molecules on the cell's surface is often insufficient. In this case, one way to increase the specificity of targeting on the cancer cell and the time of antibody retention on the target is to construct bispecific antibodies capable of simultaneous binding with two different tumor-associating antigens expressed on the same cancer cell [72, 84]. This method is important, because additional surface antigens can appear in the treatment process in response to the blocking of EGFR or HER2/neu receptors and cause resistance to medicines such as Trastuzumab, Cetuximab, and Panitumumab. Recently, it was shown that resistance mechanisms may be activated due to a surplus of other tyrosine kinase receptors (IGF-RI, c-MET, Ron, etc.), which activate the same signaling pathways as EGFR and HER2/neu, bypassing the blocked receptors [85]. Using bispecific antibodies for the simultaneous blocking of EGFR and IGF-1R significantly intensified the response of cancer cells when compared with monospecific antibodies [12]. This combined action on several surface antigens can block surplus signaling pathways in the cancer cell and lead to a more successful treatment. A combined effect is also produced by bispecific antibodies such as "intrabodies," which are specific to the VEGF-R2 and Tie-2 angiogenesis receptors. The simultaneous blocking of two angiogenesis pathways by intrabodies intensifies the inhibition of angiogenesis and tumor growth more significantly than the blocking of only one pathway [86].

7. IMMUNOCONJUGATES

The goal of achieving a "magic bullet" and the low efficiency of "unloaded" antibodies relative to the targets induced researchers to conjugate antibodies with other effector molecules (Fig. 3): radioisotopes, toxins, interleukins, and ferments activating medical products. In these immunoconjugates, antibodies usually act as a targeting component which delivers an active (cytotoxic) or diagnostic agent to the target. Chemical conjugation methods are commonly used for the conjugation of low-molecular agents, for instance, radioisotopes or low-molecular fluorescent dyes [11, 87]. Cell-penetrating peptides (CCP), capable of penetrating the membrane and transferring other proteins inside a cell, are conjugated to antibodies in the tumor by gene engineering in order to increase the penetration degree of radioimmunoconjugates. The most efficient cell-penetrating peptides are penetratine, which represents oligopeptide (43–58 a.r.) from the homeodomain of the drosophila Antp protein, and TAT, which is an oligopeptide (49–57 a.r.) from the transactivator of HIV transcription. Mini-antibody radioimmunoconjugates, which are specific to the TAG72 tumor antigen and provided with penetratine or TAT oligopeptide, are accumulated 2.5–3 times better in the tumors of xenographic mice [88].

In oncology, two medical products are approved for the radioimmunotherapy of non-Hodgkin's lymphoma: Bexxar©, which is mouse IgG2a conjugated with β-emmiter of medium energy ^{131}I (the penetration radius is 1 mm), and Zevalin©, which is mouse IgG1 conjugated with β-emmiter of high energy ^{90}Y (the penetration radius is 11 mm) (Table 2). Currently, investigations are focused on replacing mouse antibodies as targeting agents for radioimmunotherapy with less immunogenic fragments (see Section 3), as well as on developing pretargeting technologies to decrease the general radiation load on the organism (see Section 6).

To achieve these goals, the heterodimerization module is required, as with the creation of bispecific antibodies. Today the streptavidin-biotin system [89], which is well-proven for a range of analytical applications, is being used for this purpose. However, using this system in the human organism is limited by its high content of endogenic biotite, which can compete with the biotin-modified components. We have offered a new strategy of two-stage delivery based on ribonuclease, barnase, and its inhibitor, barstar [30, 31]. As was mentioned earlier that, these two proteins (110 and 89 a.r.) form the ($K_D \sim 10^{-14}$ M) complex, which is similar in resistance to the streptavidin-biotin system [90]. The proteins are stable; well-soluble; resistant to proteases; and, thus, compatible to the bacterial expression system. The N- and C-tails of both proteins occur outside the interaction area and are readily accessible for gene engineering fusion with the targeting mini-antibodies and cytotoxic agents [91, 92]. The principal possibility of the two-stage delivery of an active agent to the cancer cells was shown using the example of recombinant mini-antibodies, HER2/neu specific to the cancer surface antigen and conjugated with barstar, and a visualization component such as a recombinant fluorescent protein—EGFP—conjugated to barnase [31]. The essential advantage of the barnase–barstar module is the exact 1 : 1 ratio of components in the complex and the absence of self-aggregation, as well as the high affinity of interaction, which exceeds the values of all other dimerization systems (except for the streptavidin-biotin one). But, in contrast to the streptavidin-biotin system, the use of a heterodimerization module is based on gene engineering technologies and does not require any covalent modifications.

The DNL method is of special interest among other methods developed for pretargeting a target cell [33]. This technology is based on the specific protein–protein interaction of a dimeric regulatory subunit (RII) of cyclic adenosine monophosphate-dependent protein kinase and the anchoring domain (amphipathic helix from 14–18 a.r.) of an A kinase anchor protein. A trivalent bispecific antibody focused on carcinoembryonic antigen (CEA, tumor antigen) and hapten histamine-succinyl-glycine was constructed on the basis of those polypeptides and the Fab-fragments of two different antibodies. At the first stage of directed delivery (Fig. 4), the "landing" component provides the bivalent interaction with the tumor antigen. At the second stage, the "lock," connected by the anchoring domain to the "dock," is bound to the hapten, which is marked by radioactive ^{99m}Tc. The relatively low affinity of the ($K_D \sim 10^{-9}$) complex requires additional stabilization by disulfide bonds. One more disadvantage is the complexity of the trivalent antibody construction composed of five different protein chains. In spite of these problems, pretargeting made it possible to increase the accumulation of the radioactive mark on the

tumors grafted to mice by ~25 times when compared to the one-stage injection [33]. Today, this method can replace the bispecific MAb and streptavidin-biotin system commonly used for pretargeting [89]. Indeed, the trivalent bispecific antibodies created by the "dock and lock" method have a range of undeniable advantages for this application: bivalent binding with a tumor antigen, which intensifies the interaction and increases the time of retention on the pathogenic cells; the absence of the antibody effector functions, which significantly reduces unwanted side effects; the rapid removal of the targeting component from the organism, which decreases the waiting time for the second stage—the injection of the radioactive isotope proper—from 6–7 days to a few hours [93].

Much research is devoted to using different strong toxins for directed action on the target cells; 44% of medicines with anti-tumor antibodies subject to clinical trials are immunoconjugates or recombinant proteins. However, only one medicine, Mylotarg©, which represents a humanized anti- CD33-antibody Gentuzumab chemically conjugated with cytotoxic antibiotic calicheamicin, was approved for clinical application (Table 2). This disproportion reflects all the objective problems appearing in researchers' way when developing such an attractive idea. At the first stage of investigation, immunotoxins constructed on the basis of bacterial toxins or doxorubicin antibiotic were characterized by high systematic toxicity and a great number of side effects. Using antibodies loaded with strong toxins requires a more careful approach to selecting a target, because it is essential to avoid delivering cytotoxic agents to normal cells. It is preferable to target immunotoxins on antigens that are not expressed on normal cells but are readily expressed on the surface of tumorous cells. Unbound immunotoxin should be eliminated from the blood flow as soon as possible. The rapid internalization of antigen after binding with immunotoxin is preferable as well. Moreover, because a medicine's efficiency is related to the compound's toxicity level, it is expeditious to use toxins with a IC_{50} level which ranges within the nano- and picomolar concentrations. To date, researchers have the following range of required toxins: auristatin and calicheamicin, as well as such protein toxins as pseudomonade ricin and diphtherin [94].

All these ideas were taken into consideration in designing Mylotarg© (Table 2). Humanized MAb Gentuzumab is specific to surface CD3 antigen, which is hyperexpressed on the surface of malignant cells caused by acute myeloid leukemia; therefore, a maximum non-toxic dose of this medicine is rather high and attains 9 mg/m2. CD33 is readily internalized, and the therapeutic response depends not on the antigen expression level but on the stage of the cell cycle and multi-drug resistance. The cytotoxic component of this immunotoxin is the antibiotic calicheamicin, which binds to the DNA groove and breaks it. The toxicity of this antibiotic is characterized by the IC_{50} value, which is within the low nM concentration.

The cytotoxicity of antibiotics from the auristatin and maytansinoid group has another nature. These antibiotics bind to α-tubulin and break the polymerization of microtubules, which causes cell-cycle block and cell death. CD20, CD30, CD70, PSMA (prostate-specific membrane antigen), HER2/neu, E-selectin, and LewisY cell markers are used as the targets for immunoconjugates. As in the case with radioimmunotherapy, immunoconjugates are more efficient for treating hematologic types of cancer and are inefficient for the treatment of solid tumors.

Currently, immunotoxins derived from truncated pseudomonade A exotoxin and diphtherin are subject to clinical testing. The action mechanism of these immunotoxins is based on the catalytic ADP-ribosilation of factor 2, inhibiting the translation. The first and the second generations of those multidomain proteins were characterized by high systematic toxicity, which significantly decreased after the elimination of the domains responsible for binding with the cells. The major advantage of these proteins is their high toxicity in the pM concentrations: only a few molecules are needed to kill a cell. The disadvantage is the immunogenicity and presence of side effects such as the capillary leak syndrome. To inhibit immunogenicity, A. Pastan, a pioneer researcher in immunotoxins, carried out hard work focused on the deimmunization of truncated pseudomonade PE38 toxin with the complete retention of its toxicity [95]. The high intensity of his investigations aimed at creating medicines on the basis of immunotoxins will likely make them available for clinical use very soon.

Attempts to use RNAase as toxins were made much later, at the end of the 1990s. The researchers obtained conjugates of bull pancreatic RNAase and anti-EGFR mouse antibodies [96], as well as a recombinant protein on the basis of human pancreatic RNAase and single chain anti-transferrin receptor mini-antibodies [97]. Human pancreatic RNAase was conjugated with a humanized mini-antibody to decrease immunogenicity [98]. The same authors were the first to offer the term "ImmunoRNAase." Today, immunoRNAase is of great interest [99–102]. RNAase draws the attention of researchers due to its accessibility, low immunogenicity, and absence of toxicity outside the cell. Thus, it is quite possible that RNAase-based medicines will not be characterized by systematic toxicity, but the problem of internalization into the target cells remains unsolved. Using human RNAase is significantly limited by inhibition by a natural inhibitor (RI) present in the cells. Researchers produced mutants of human RNAase resistant to the inhibitor [103] by directed mutagenesis and offered a search for the appropriate RNAase of other origins [104]. Currently, the clinical testing of onconase, the first RNAase used for cancer therapy [105], is drawing towards a successful completion. This positively charged protein (104 a.r) from frogs belongs to the amphibian A ribonuclease superfamily and has cytostatic and cytotoxic action largely on cancer cells. This selectiveness is likely related to the strong positive charge of onconase and the fast metabolism of the cancer cells [105].

In our laboratory we used the bacterial ribonuclease of barnase to create immunotoxin [30, 45, 106, 107]. This small, stable, and well-soluble protein is resistant to the natural inhibitor of human RNAase. Earlier, we established that the barnase of multidomain recombinant proteins plays the role of internal chaperone, provides the correct domain coiling, and favors the stability and good solubility of protein [108]. Barnase, which is toxic for cells, has been used

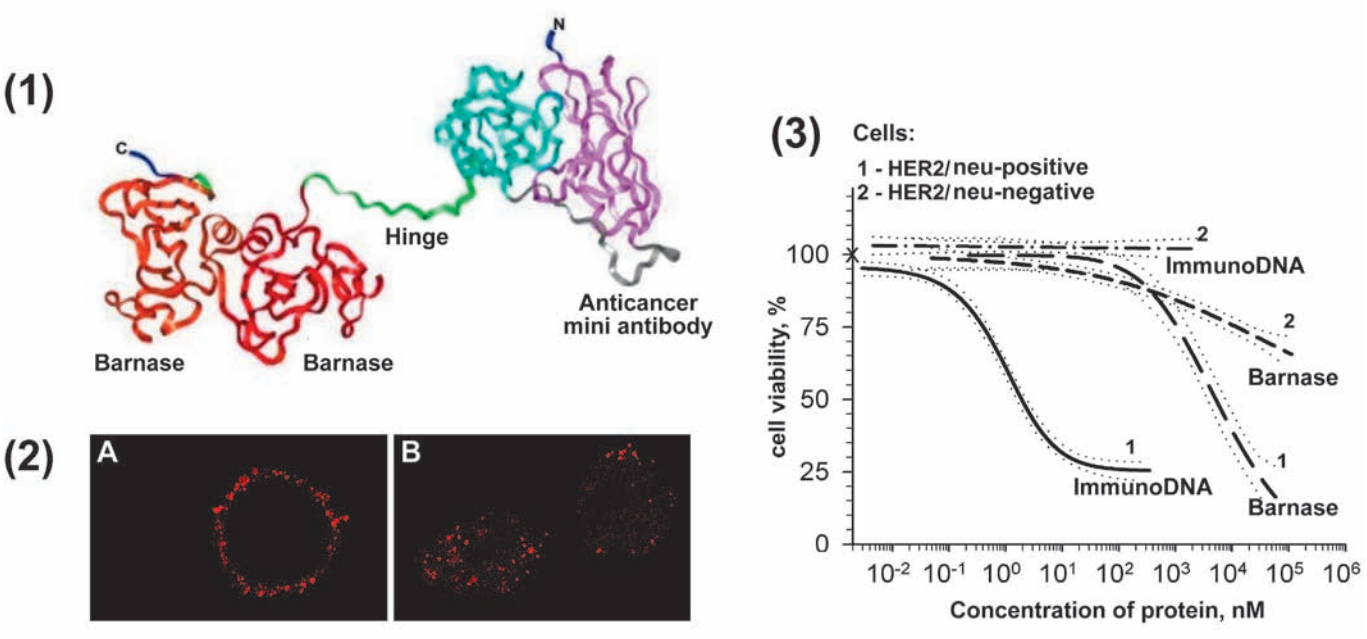

Fig. 5. Immunodibarnase as a perspective agent for treating malignant neoplasms [105]. (1) Scheme of immunodibarnase structure. (2) Interaction of immunodibarnase with the cells expressing the HER2/neu cancer marker: surface binding at 4°C (A) and internalization at 37°C (B). (3) Cytotoxicity of immunodibarnase for the cells with hyperexpression of the HER2/neu cancer marker.

to create a zero-background vector [90]. The constructed immunodibarnase contains two barnase molecules and a humanized mini-antibody specific to the internalized cancer HER2/neu marker (Fig. 5, *1*). The *in vitro* experiments show that providing a toxic barnase agent that addresses the mini-antibody significantly increases the efficiency of action on the cancer cells. The anti-cancer mini-antibody delivers barnase on the surface of cancer cells (Fig. 5, *2(A)*) and provides for its penetration into the cell (Fig. 5, *2(B)*), where it exposes its toxic nature. Immunodibarnase (1.8 nM) causes the apoptosis of 50% of HER2/neu-positive cells, whereas HER2/neu-negative cells require a concentration 250–300 times higher (Fig. 5, *3*). The cytotoxic effect of barnase depleted in mini-antibodies is non-specific and ~1000 times less acute than the cytotoxic effect of immunodibarnase (Fig. 5, *3*) [107]. Immunodibarnase can be a perspective agent for treating tumors thanks to its specific action on the cancer cell and low active concentration.

RNAase circulating in the blood stream is nontoxic to the organism and becomes toxic only after penetrating into the cancer cell, where it plays the role of a so-called protoxin [99]. A similar idea would be to create nonactive pre-medicine which would be delivered to the target and then transformed into active form; this is being developed in different variants on the basis of ADEPT technology (antibody-directed enzyme pro-drug therapy) (Fig. 4). The technology is based on the preliminary directed delivery of a ferment conjugated with the antibody to the target cells. Targeting the delivery is provided by the especificity of antibody to the cell antigen. At the next stage, in the microenvironment of the target, the injected non-active precursor of the medicine is transformed into an active form by a ferment delivered earlier and kills the surrounding cells. β-lactamase, G2 carboxypeptidase, and others are used as ferments at this stage [86, 109, 110].

Creating anti-idiotypic antibodies with definite catalytic functions—so-called abzymes—which can perform the functions of antibodies and are characterized by fermentation activity is a new prospective scientific direction of gene engineering. Modifying the anti-idiotypic antibody scFv-fragments, combined with the use of phage libraries, makes it possible to select abzymes with preprogrammed properties [111, 112].

Some cytotoxins (for instance, IL-2, IL-12, TNFα, IFNγ, GM-CSF) have immunomodulatory and antitumoral value. Unfortunately, using these proteins as medicines is hardly possible due to their high systemic toxicity in active concentrations, fast decomposition and removal from the organism, and the undirected and nonspecific action on the tumor cells. The conjugation of antibodies with cytotoxins and the creation of immunocytotoxins makes it possible to overcome the limits pointed out. Investigating interleukin-2 therapeutic and adjuvant properties brought about the greatest step forward. For instance, we have obtained encouraging results in the course of clinical trials of recombinant immunocytokines constructed on the basis of interleukin-2, anti-cancer humanized antibodies (GD2 disialoganglizid and the epithelial cell adhesion molecule

EpCAM), and anti-angiogenesis antibodies (EDB and the extra domain B of fibronectin) for treating melanoma, neuroblastoma, and non-Hodgkin's lymphoma [113, 114] and on the basis of interleukin-2 conjugated with anti-CD20 antibody for treating B-cell malignancy [115]. Using immunocytokines helps increase the effectiveness of chemotherapy as well [116].

Conjugates of antibodies with different fluorescent probes are often used in immunology for *in vitro* investigations. Introducing a powerful instrument such as fluorescent proteins by molecular biology (2008 Nobel Prize, Dr. Osamu Shimomura, Dr. Martin Chalfie, and Dr. Roger Y. Tsien) allows us to observe the vital activity of the cells on a real-time basis. Japanese researchers created a system for visualizing living cancer cells in the organism based on the pH-activation fluorescent agent directed to the target cell by an anti-cancer internalized antibody [117]. Fluorescence is observed only if the agent occurs in the lysosomes of the living cancer cell and disappears when it dies. The method's specificity is 99%. These investigations are far from being used in therapy, but they enable us to discover the new abilities of immunoglobulin conjugates.

8. RECOMBINANT ANTIBODY CONSTRUCTION SYSTEM.

Today, producing stable and high-affinity MAb in a sufficient amount for pre-clinical and clinical investigations is a "bottleneck" for the wider application of these therapeutic compounds. The rapid growth in demand for antibodies and their quality has led to the development of numerous systems for producing antibodies and their fragments on the basis of Gram-positive (Bacillus) and Gram-negative (Escherichia) bacteria, ferments, filamentous fungi, and the cell lines of insects and mammals [118–120]. High-technological systems such as bacterial and ferment producers, which favor the overgrowth of biomass in fermenters and the production of highly efficient recombinant proteins, can solve the problem of creating truncated MAb fragments, non-glycosylated Fab-fragments, and scFv provided, as a rule, with special tail peptides for fast activation on affine sorbent. Currently, antibodies are created in transgenic plants and animals which are appropriate for the production of full-size antibodies [72, 121]. The expression system is created on the surface of bacterial cells (so called "E-clonal" antibodies) used for both full-size and divalent formats [122] for the fast production and selection of full-size antibodies. The glycosylation of antibodies in ferments, plants, and insects differs from that in a human being: therefore, antibodies obtained in those systems are applicable only for experimental investigations. Today, therapeutic antibodies are produced in transgenic mice, whose immunoglobulic locuses are inactivated and replaced with the genes of human immunoglobulins [10, 123]. The most well-known mice transgenic lines are as follows: Xenomouse (Abgenix, www.abgenix.com), HuMab mouse (GenPharm, www.genpharm.ca), and TC mouse and KM mouse (Kirin Brewery Company, www.medarex.com). Medicines with MAb created in these producers are characterized by low immunogenicity due to xenogenic post-translation modifications and, primarily, to glycosylation specifics. Moreover, the researchers are developing new technologies which would allow the production of MAb with a human profile of glycosylation in ferments, insects, and transgenic plants [124, 125].

9. CONCLUSIONS AND PROSPECTS.

In the early 21st century, one hundred years after Ilya Mechnikov and Paul Ehrlich, the founders of the current immunity theory (1908), received the Nobel Prize, knowledge about the delicate molecular mechanisms of antibody functioning and interaction with the organism's protective systems has made the greatest step forward. This progress is provided by the innovative technologies used in the course of scientific investigations and the accumulation and systematization of large amounts of information. The quick development of bioinformatics allows us to model the compounds with preprogrammed properties, while revolutionary progress in genetic and cell engineering technologies makes it possible to create biotechnological producers of therapeutic medicines.

Modern technologies make it possible to modify the antibody properties depending on the situation and use all their functions. Thanks to gene engineering, researchers manage to decompose antibodies and compose new constructions. Truncated antibodies, focused on binding with a target and compatible with a bacterial expression, are used for producing a wide range of compounds meant for the highly efficient delivery of active agents. Researchers have created clone libraries of immunoglobulin fragments and highly efficient screening systems. Moreover, a new scientific direction has appeared; it is focused on constructing framework molecules, also called scaffolds (as an alternative to antibodies), which are characterized by an analogous ability to specifically bind. Undoubtedly, this sphere will develop in both directions: the construction of the next generation of recombinant truncated humanized antibodies with multivalent binding characterized by improved pharmacokinetic properties [34, 126] and the creation of alternative framework constructions [127]. Truncated antibodies that are depleted in constant domains are widely used in radioimmunotherapy for the directed delivery of radioactive isotopes as immunotoxin components in oncology and as specific blockers of targets in cardiosurgery.

The bispecific antibodies in both truncated and full-sized formats have great potential. These antibodies have been used in radioimmunotherapy for a rather long time, and new technologies can only modify and improve the currently available and well-established methods of one- and two-stage delivery of radioisotopes to a target [93]. On the contrary, using bispecific antibodies in the sphere of cell immunotherapy has just begun [128], and researchers have a wide field of experimental and clinical investigations. The high variability of the cancer cells and the problem of resistance to therapy generate a need for complex treatments and the use of several medicines with different mechanisms of action. Bispecific antibodies, which can strike two targets simultaneously and block two metabolic pathways, are irreplaceable in this case as well.

New technologies for controlling the functions of the antibody constant domain are reaching new heights. The development of constant domain engineering and, in particular, carbohydrate MAb engineering testifies to a return

to "traditions" and growth in interest in the antibody effector functions and full-size MAb, because they attract all the protection systems of the organism to the pathological center [129].

One scientific direction which is quite promising from the technological standpoint is the creation of hybrid biocompatible nanoparticles from organic and inorganic material. Providing these nanoparticles with antibody particles will ensure high-accuracy targeting. Semiconductive fluorescent crystals ("quantum dots"), magnetic nanoparticles, gold colloids, and fullerene derivative delivered with their help will carry medicinal compounds and allow additional external action on the targets by means of laser, acoustic, and microwave radiation [130, 131]. This new generation of multifunctional nanoconstructions should have complex properties that cannot be used separately. This combined action on the tumors will help achieve a whole that is truly more than the sum of its parts.

Today, only 15 out of 266 applied medical targets found in the human genome are used as antigens in making therapeutic antibodies to treat different diseases, and almost all of them are surface cell antigens [132]. Accumulating knowledge about cancer biology will make it possible to reveal new cell chains and directionally create multifunctional constructions for accurate action against the targets responsible for the profileration, conglutination, metabolism, distribution, and other mechanisms of malignant neoplasms (see, for instance, [133]).

It can be expected that the future success of the clinical application of antibodies will depend to a great extent on the development of new targets for them in the course of accumulating new knowledge about the mechanisms and molecular participants of the pathological processes. ●

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The Regulation of Telomerase in Oncogenesis

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ABSTRACT Telomerase is a complex ribonucleoprotein that completes the telomeres' ends in eukaryotic cells which shorten due to DNA underreplication. The core enzyme consists of a protein catalytic subunit—Telomerase Reverse Transcriptase (TERT)—and telomeric RNA (Telomerase RNA (TR)); a small region of this RNA serves as a template for the telomeric repeats synthesis. Apart from rare exceptions, telomerase is not active in the somatic cells and tissues of the human body. However, the activation of telomerase activity in cancer cells was shown for certain in 80–90 % of cases. Understanding the mechanism of telomerase functioning and the mechanisms of its regulation could be used in oncodiagnostics. Telomerase itself and its regulators could be important targets for anticancer therapy. The activity of telomerase in a cell is affected by proteins with multiple functions, and this influence is not necessarily specific. There are also cases when telomerase regulators act together or when several regulators are organised in the cascade. The aim of this review is to generalize and systemize data about the regulation of telomerase in ontogenesis.

Key words: telomerase, telomerase reverse transcriptase, telomerase RNA, regulation, cancer.

Abbreviations: 5azadC (5-aza-2'-deoxicitidine), AV (adenoviruses), HBV (Hepatitis B Virus) HIV (Human Immunodeficiency Virus), HPV (Human papillomavirus), EBV (Epstein–Barr Virus), np (nucleotide pair), OB-motive (oligonecleotide/oligosaccharide binding motive), RT (reverse transcription), PCR (polymerase chain reacion), RNAse (Ribonuclease), TERT (Telomerase Reverse Transcriptase), hTERT (human TERT), mTERT (mouse TERT), DN-hTERT (dominant-negative mutated hTERT), ERβ (Estrogen β receptor), HTLV-I (Human T-lymphotropic virus), TR (Telomerase RNA), TRAP (Telomeric Repeat Amplification Protocol), PHA (phytohemagglutinin), HRE (Hypoxia Response Element), NES (Nuclear export signal).

In 1961 Hayflick and Moorhead showed that a somatic cell culture has a limited life span [1]. In 1973 Olovnikov suggested that shortening the chromosomal ends (telomeres) determines the potential number of cell divisions. [2]. Telomeres protect the cellular genome from degradation; they participate in the chromosomal pairing during meiosis and in the gene expression regulation in the telomeres region [3]. In immortal cells that can divide infinitely, this should be the mechanism for compensating the chromosomal shortening. In 1975 Blackburn and Greider discovered the enzyme telomerase that elongates chromosomes [4].

Telomerase is a ribonucleoprotein complex that consists of components that are absolutely required for its activity: the RNA molecule and Telomerase Reverse Transcriptase TERT [5]; also, optionally several telomerase-associated proteins could be included in the telomerase complex. TR is also a template for TERT when telomerase elongates telomeres. Telomerase exists in human cells as dimers and contains two subunits of reverse transcriptase and two RNA molecules [6]. In human telomerase, p23/p90-shaperone, which is responsible for the complex assembling/configuration, binds 14-3-3, which is responsible for nuclear localization, and TP1 with an unknown function. Proteins hGAR1, Dyskerin/NAP57, hNHP2, and C1/C2, which are responsible for the stability, maturation, and localisation of RNA, bind to the hTR; La and hStau, which are supposedly responsible for the binding to telomeres; L22, which acts in processing and nuclear localisation; and hNOP10, A1/UP1, and TP1 with an unknown function [7]; TCAB1, which is responsible for the localisation of hTR in Cajal bodies and binding with telomers [8]. The enzymatic activity of human telomerase in the rabbit reticulocytes lysate is detected by adding hTR and hTERT [9, 10]. Note that telomerase functioning *in vivo* is not always consistent with the telomerase activity that was measured *in vitro*. For example, adding the Hemagglutinin epitope to the C-end of hTERT stops telomere alongation but does not suppress telomerase activity [11].

Telomerase activity detected *in vitro* appears in leucocytes in the G1 phase (Fig. 1) [12]. On the other hand, telomeres are replicated *in vivo* during the S-phase (Fig. 1) [13, 14]. During most of the cell cycle, TCAB1 helps the hTR accumulation in Cajal bodies [8], and in S-phase it is combined with telomeres in a cell. During the S-phase of the cell cycle, hTERT also moves to telomeres [15, 16]. This means that there is regulation at the level of the spatial localisation of active telomerase (enzyme) and telomere (substrate). A correlation between the telomerase activity and the length of telomeres is not always possible to obtain. For example, there is no dependency between the length of telomeres and telomerase activity in leukaemia [17].

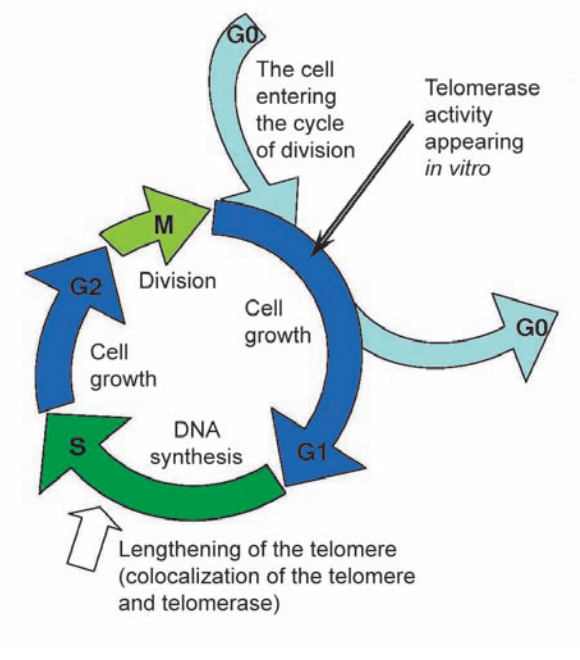

Fig. 1. Cell cycle scheme. The appearance of telomerase activity *in vitro* happens in the G1-phase, but it works at the S-phase.

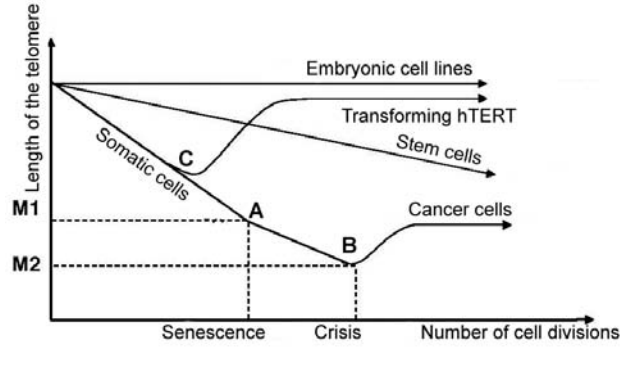

Fig. 2. Dependence of telomeres length on the cell cycle number in different types of cells: embryonic cell lines, somatic cells, and hTERT-transfected cells. (A) achieving the Hayflick limit by cells, (B) crisis with the following cell death and transformation of surviving cells into cancer cells, (C) the transfection of cells by the hTERT gene.

The two-step hypothesis of cell aging and immortalisation M1/M2 theory describes the activity of telomerase dependency on the number of cell divisions very well (Fig. 2). In embryonic cell lines, telomerase is active and the length of telomeres is constant. In stem cells, the activity of polymerase is lower and it only partially compensates the telomeres shortening. In somatic cells, telomerase in not active. The shortening of telomeres leads to the moment of M1, i.e., the achievement of the Hayflick limit (point A on Fig 1) and the transition of cells to the senescence (aging) condition, which could be rescued by the inactivation or deletion of pRB/p16 or p53. Cells that pass through M1 continue their cell division and achieve the condition of crisis M2 (point B on Fig 2), which leads to massive cellular death. Cells that survive begin their transformation into cancer cells. Cancer cells have the ability for unlimited cell division and support the length of telomeres (usually due to telomerase activity). In the case of the transfection of hTR-expressing somatic cells by the hTERT gene (point C on Fig. 2) before the moment of M2, they, similarly to cancer cells, demonstrate elongation and stabilization of telomeres [7].

Telomerase activity, with rare exceptions, does not occur in human somatic cells and tissues. Its activity was shown in reproductive tissues, as well as in intensively renewing tissues, such as some types of blood cells, the intestinal epithelium, and the layer of skin cells [18]; however, the level of telomerase activity in the somatic cells with active polymerase is lower than in cancer cells [19].

THE FREQUENCY OF TELOMERASE ACTIVITY DETECTION IN DIFFERENT TYPES OF TUMORS

Telomerase is active in most (80–90%) tumor cells (Table 1), and this activity is the main instrument for supporting the telomeres length. There are non-malignant types of tumors and other types of non-cancer lesions that demonstrate telomerase activity almost 100% of the time, but there are others without any activity [19–21]. Tumor cell of some cell types can use an alternative mechanism of telomeres length support that is based on recombination [22]. In case of the transaction of cells with an alternative telomere-supporting mechanism by the gene hTERT, both mechanisms are active; however, in the case of the hybridization of cell lines with different mechanisms, telomerase is present in hybrids, and indications of an alternative mechanism of telomere lengthening disappear [23]. It should be noted that telomerase itself is not oncogene. Cell lines that were tranfected by the hTERT gene do not demonstrate indications of malignant transformation for a long time [24, 25].

Telomerase activity could appear as a result of clone selection in the situation of critical shortening of telomeres [26] (Fig. 2, position B). Firstly, cells start to divide intensively, and the telomeres shorten; however, only cells with telomerase activation survive. In this case telomerase activity could be a marker of malignant progression and negative prognosis. For instance, the main increase of telomerase activity during lymphogranulomatosis appears at the transition between the first and second stages [27]. In other possible scenarios, telomerase activity appears at the same time as

Table 1. Frequency of telomerase activity in different types of tumors.

Type of tumor	Frequency of telomerase activity, %	Number of researched samples	[Reference]
MALIGNANT TUMORS:			
small-cell lung cancer	100	15	[21]
	90	10	[30]
Non-small-cell lung cancer	78	125	[21]
	83	68	[31]
	84	32	[30]
phlegm from lung cancer patients	67,6	34	[30]
Stomach cancer	72	85	[21]
Colon cancer	89	138	[21]
Pancreas cancer	95	43	[21]
Liver cancer	86	173	[21]
	79	24	[32]
Mammary gland cancer	88	339	[21]
	59	44	[33]
Cervical cancer	100	16	[21]
Ovarian cancer	91	23	[21]
Prostate cancer	90	58	[21]
Kidney cancer	83	115	[21]
William cancer	100	6	[21]
	95,7	164	[34]
Retinoblastoma	50	34	[21]
Neuroepithelial tumors	62	107	[35]
Glioblastoma	75	60	[21]
	72	47	[35]
	28	25	[36]
	26	38	[37]
Astrocytoma, II stage	20	15	[35]
Oligodenryogliomas	100	19	[21]
	100	4	[35]
Oligodenryoglioma, II stage	14	14	[37]
Anaplastic oligodenrioglioma	43	7	[37]
Anaplastic astrocytoma	10	20	[21]
	40	15	[35]
	23	13	[37]
Neurobalstoma	94	100	[38]
Melanoma	86	7	[21]
Squamous cell carcinoma	83	18	[21]
Basalioma	95	77	[21]
HEMOBLASTOSES:			
Lymphima, low level of malignancy	86	14	[21]
Lymphima, high level of malignancy	100	16	[21]
Malignant lymphoma of CNS	83	12	[39]
Myelodisplastic syndrome	67	6	[21]
Cgronic myeloleucosis	71	42	[21]
Cgronic myeloleucosism, acceleration phase	100	21	[21]
Cgronic myeloleucosis, early stage	14	14	[21]
Cgronic myeloleucosis, late stage	57	7	[21]
Acute myeloblastic leucosis	73	64	[21]
Acute lymphoblastic leucosis	80	5	[21]
Lymphogranulematosis (lymphoid predominance)	63,6	33	[40]
Lymphogranulematosis (nodular sclerosis)	89,7	39	[40]
Lymphogranulematosis (mixed-cells variant)	96,1	26	[40]
Lymphogranulematosis (lymphoid exhaustion)	100	7	[40]
NON-MALIGNANT DISEASES:			
Adenoma of colon	45	44	[21]
Hepatitis/liver cirrhosis (activity is weaker than in case of liver cancer)	29	148	[21]
	8/24	24/34	[32]
	25/45,9	80/37	[41]
Mammary gland fibroadenoma	75	12	[21]
Leiomyoma	0	14	[21]
Meningomyoma	0	25	[36]
Non-malignant lesion of lymphatic vessel	33	15	[21]
Non-malignant lesion of amygdalae	100	23	[21]

other cancer-leading metabolic abnormalities as a result of original cell damage. In this case telomerase activity appears just at the beginning of a disease, and it could be a good marker of the beginning of oncological processes. For example, there is no dependency between telomerase activity and the stage of cancer during cervical carcinoma; telomerase is active even at the first stage, and its activation occurs in pre-tumor illnesses [28]. Telomerase could be active originally in the cell types of interest, and this activity just becomes stronger during the transition to cancer; e.g., as happens during hemoblastosis [26]. Telomerase also could be originally active in the case of the transformation of stem cells [29]. In this case telomerase activity will be obtained at the beginning of tumor growth, because the method of its detection does not allow the activity from the single cell from the surrounding tissue to be seen, but even a small pool of telomerase-positive cells will be detected.

Unfortunately, most publications provide information only about the presence of telomerase activity in certain types of cancer. The mechanisms of telomerase activation are usually investigated in a cell culture, and it is not often possible to conclude by which mechanism and how often they are met in the cancer under study *in vivo*.

THE AMPLIFICATIONS OF HTR AND HTERT GENES

The hTR gene has one copy and is located in the Chromosome 3 at the position 3q26.3. This chromosomal region is amplified, for example, in the case of cervical cancer, lung cancer, and squamous cell carcinoma of the head and neck. The number of copies of the hTR gene increases in tumor cells more than in normal cells, and, correspondingly, the hTR expression increases in the case of cervical cancer, lung cancer, and squamous cell carcinoma of the head and neck [42].

The hTERT gene is located in Chromosome 5 at the position 5q15.33 in the region that is also amplified in some types of cancer [42]. Because the amplification of telomerase genes occurred during the amplification of chromosomes with those genes but not in the locus-specific amplification, it is possible to conclude that this process is nonspecific. In the case of cervical squamous cell carcinoma, the expression of hTERT is not related to the amplification of hTERT [43]. On the other hand, this amplification could possibly occur as a result of the chromosomal instability and aneuploidy that happens during the critical shortening of telomeres.

REGULATION OF TERT TRANSCRIPTION

hTERT transcription is very low or not detected at all in most human tissues, but it often appears in these tissues after neoplastic transformation [44].

STRUCTURE OF THE TELOMERASE REVERSE TRANSCRIPTASE PROMOTER

The hTERT promoter does not contain TATA or CAAT, which are typical for the binding of PNA-polymerase II, and it is GC-rich. There are different data on the position of the region of the transcription's initiation. Note that, now and further in this paper, we use the numeration of nucleotides (point + 1 bp (nucleotide base pairs)) from point A in the triplet ATG, which is the beginning of translation. It was shown [45] (using the method of anti-RNAse protection) that there are several protected regions in different hTERT-positive cell lines, which indicates the possibility of several sites of transcription initiation (in the region of from −40 to 100 bp). The most commonly protected region in the region of transcription initiation is at the position −55 G from the beginning of translation. It was shown [46] by analyzing caped mRNA from the HeLa cell line that the site of transcription start is at position −77 bp. Recently, most authors have accepted this view.

Sites that are responsible for the regulation of hTERT transcription are located in the region of 2000 bp before the sites of translation and transcription initiation [47, 48]. The most important site for activation is the region from 250−300 bp before ATG to tens of bp after it [46]. Apart from this, the GC-rich region of the promoter forms CpG-islands near the ATG, which indicates that methylation could take part in the regulation hTERT expression [7].

The activity of the promoter is related to the distribution of its regions for the binding of regulatory proteins, which do not interfere with the promoter, but also the transmitters of the effects of other regulators. Figure 3 shows the scheme of the influence in different effectors on the hTERT promoter; this will be considered further in detail.

THE METHYLATION OF THE HTERT PROMOTER

In tumor cells, the mutilation profile is different from the mutilation profile of normal cells. An analysis of the telomerase promoter has shown two CpG-islands; one of them is −900 bp from the start codon ATG [49].

In the case of cancer cell lines and intestinal cancer tissues, as has already been shown, there is a hTERT expression and CpG-islands of the hTERT promoter are fully or partially mutilated [50]. After extended treatment with the demethylation agent 5-aza-2'-deoxicitidine (5azadC) of cell lines Lan-1, HeLa, and Co115, in which telomerase is active and the hTERT promoter is hyperventilated, a 95% decrease in the level of hTERT-promoter methylation, and a decrease of the level of the hTERT mRNA expression were shown. Telomerase activity strongly decreases after 2−4 passages of cells in the presence of 5azadC [51].

The markers of active chromatin and telomerase expression are detected when the region of hTERT-promoter located from −73 to +227 bp around the transcription site is not methylated [52]. In normal cells, the hTERT-promoter hypermethylation suppresses telomerase activity and hTERT-mRNA expression, and, after treatment by 5azadC, telomerase starts to be activated [53]. Analysis of hTERT-promoter methylation in patients with chronic B-cells lymphoid leucosis has shown that they have high telomerase activity but a low level of methylation [49]. There is no direct correlation between telomerase activity and the hTERT-promoter methylation status for cell lines of ostecarcoma, ovarian cancer tissue samples, cervical cancer, and normal tissues [54, 55].

This data variation indicates that DNA methylation is not a critical factor for telomerase expression regulation in can-

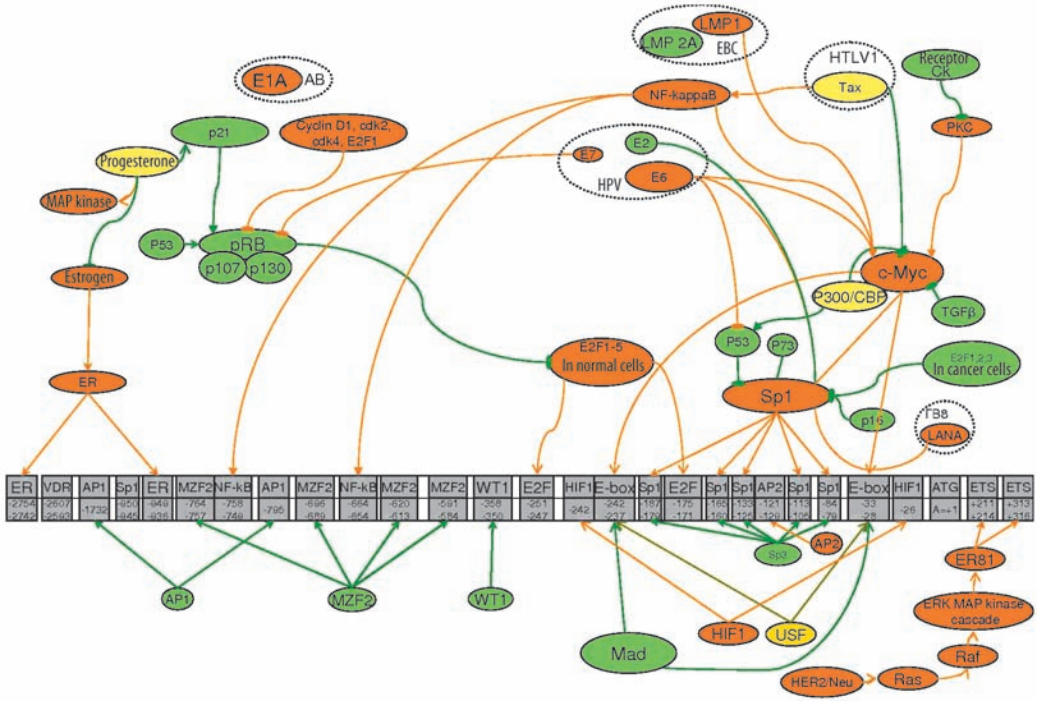

Fig. 3. The scheme of the effect of hTERT- promoter's transregulators. The inhibitors of the hTERT promoter are green. Activators are orange. Double-action effectors are yellow. → points to the activation of the following player of cascade, ⊣ is the inhibition of the following cascade player, and the absence of an arrow means co-action.

cer. It occurs together with other disturbances of the system of hTERT-promoter regulation. Apart from this, both methylation and demethylation could affect telomerase transregulators, but not the promoter itself.

THE METHYLATION OF HISTONES OF THE HTERT PROMOTER

Histone methylation, which is provided by methyltransferases and demethylases, plays an important role in the regulation of the chromatin structure and transcription. Methyltransferase SMYD3, which is involved in oncogenesis, specifically activates hTERT. This methyltransferase binds to its specific binding region on the hTERT promoter and the thrice methylated H3-K4 histone. Suppressing SMYD3 in cancer cells stops H3-K4 threemethylation, makes the binding between promoter and trans-activators Sp1 and c-Myc weaker, and leads to the decreased acetylation of H3 histone in the hTERT-promoter, which leads to a decrease in hTERT-mRNA and a decrease of telomerase activity [56].

THE ACETYLATION AND DEACETYLATION OF HTERT-PROMOTER'S HISTONES

Acetyltransferases GCN5 and Tip60 acetylate histones H3 and H4, which leads to the activation of hTERT-trancription. The same acetyltransferases acetylate Myc, which leads to a decrease of its degradation [57] and other proteins that are involved in gene transcription, which could nondirectly regulate the hTERT promoter. Acetyltransferase p300 and the accompanying CBP are transcription coactivators which interact with a lot of sequence-specific transcription factors, and they are coactivators of onco-suppressor p53 [58]. p300 is a coactivator of hTERT transcription, and the c-Myc protein brings this protein to the promoter. Even p300 and CBP can stabilize Myc in an acetylation-independent manner; acethylation by p300, however, decreases the binding level of Myc with the promoter. Thus, p300 can participate in both activation and the inhibition of the hTERT promoter. The Myc/Max complex is acetylated differently, by p300 and GCN5, and is not acetylated by Tip60 *in vitro*, which means that the mechanisms of the action of these two transferases are different [59].

Histone deacetylation leads to a decrease in the expression of hTERT [60, 61]. The superexpression of histone deacetylases (HDAC1) leads to the suppression of the hTERT-promoter activity. HDAC1 binds to the telomerase promoter, inhibits telomerase activity, and interacts with Sp1 [62]. Histone deacetylases can interact with the hTERT promoter through the Mad1 protein, which binds to E-blocks [60, 62, 63]. Inhibiting histone deacetylation by Trichostatin A (TSA) leads to the activation of the hTERT expression; this effect depends on Sp1, but not on c-Myc. In the case of Sp1 superexpression, the effect of TSA is stronger; in the case of mutations in the Sp1-binding regions (but not with c-Myc), this effect does not occur [63]. Inhibiting histone deacetylation leads to an increase in the expression and activity of telomerase in normal cells—but not cancer cells (lung cancer cell lines)—that already have active telomerase [63, 64]. It is reasonable to suggest that, in the case of cancer, deacetylation is already switched off.

CASCADE MAPK

The signaling cascade of Mitogen-activated protein kinase MAPK, which is an effector of the extracellular growth and stress signals, can regulate the transcriptional activity of many promoters by the direct phosphorylation of Sp1 or through other mechanisms. The MAPK-signaling Pathway is important for hTERT transcription regulation through the number of effectors which bind to the regions in its main promoter, including the transcription factors c-Myc, AP-1 and Ets [42]. The inhibition of the MAPK-cascade leads to a weakening of the phosphorylation of the estrogen receptor β (Erβ), to a decrease of the binding of ERβ to the hTERT promoters, and to the respective decrease in the level of hTERT expression [65].

ONCOGENE MYC AND ITS ANTIPODE MAD

E-blocks are located in positions −242 and −34 np of the hTERT gene, with whose oncoprotein c-Myc interacts, which is one of the main hTERT transcription activators [48, 66]. This protein is also a transcriptional activator of a number of promoters of other genes, and it is an inhibitor of the transcription of genes that are involved in the ceasing of cell growth [67].

It was shown that c-Myc expression suppression by anti-c-Myc antisense-oligonucleotides c-Myc, which was done on three leukaemia cell lines, also decreases telomerase activity [68]. c-Myc induces hTERT transcription and telomerase activity in normal cells of the epithelium mammary glands and primary human fibroblasts [69]. A high level of c-Myc leads to the activation of the hTERT promoter, but this effect disappears in the absence of E-blocks [66, 67, 70]. Blocking c-Myc bindings has a different effect in different cell lines. Thus, introducing mutations to the E-block (in position −242 np in the cells of lines C33A and ME180) leads to a fall in the hTERT promoter activity by 70%; however, in the cells of cell line SiHa, the same mutations have a very small effect. Introducing mutations into the E-block at position −34 np provokes a decrease of the hTERT promoter activity by 60% in cells ME180; however, hTERT promoter activity changes insignificantly in the cells C33A and SiHa [71]. c-Myc-induced hTERT promoter activation occurs quickly and independently of cell proliferation and protein synthesis [67, 70]. Heterodimers c-Myc/Max interact directly with the hTERT promoter [61, 66].

It is possible that N-Myc is another telomerase activator. This gene could be amplified in neurobalstoma at the same time as the hTERT promoter activation [44]. Binding N-Myc with the hTERT promoter has already been shown [72].

The Mad protein is an antipode of c-Myc; it binds to the E-blocks as Mad/Max heterodimers and makes the hTERT promoter activity weaker. The suppression of hTERT promoter by Mad requires the activity of hitones deacylases. However, the inhibition of histone acetylases by TSA is not dependent on the presence of E-blocks in the hTERT promoter [60].

TRANSCRIPTION FACTORS SP1AND SP3

Protein Sp1 regulates several specific promoters which initiate transcription by RNA-polymerase II in vertebrates. Sp1 binds to the sequence GGGGCGGGGC and similar sequenc-

es that are called GC-boxes. It regulates both hTERT and hTR promoters.

There is a set of regions for Sp1 binding in the hTERT that are required for promoter activity [46–48, 71, 73]. Similar clusters often exist in other promoters without the TATA-box, and they are required for its complete activation [74]. There are five known regions for Sp1 binding; they are located mainly between the promoter and E-blocks [46]. Two regions for Sp1 binding are identical for humans and mice [67]. It is very likely, judging by the active promoter regions, that sites for the Sp1 between E-blocks are involved in this regulation; introducing mutations into those sites leads to a decrease of promoter activity of different extents, depending on the mutated site and cell line [71]. In the case when those five binding regions are switched off, a 90% decrease of the hTERT promoter activity occurs, which means that Sp1 is absolutely necessary for its activity [60, 71]. Interestingly, that introduction of c-Myc activates the hTERT promoter in the presence of Sp1-binding regions; however, in the absence of those regions, the hTERT promoter activation by c-Myc is insignificant [71]. Two regions for Sp1 binding located in the region from −320 to −350np before the translation site and which are not yet well investigated and, possibly, two regions from −800 to −1000 np are probably involved in this regulation [47]. It is unlikely that other Sp1-binding sites that are further away from the transcription beginning site [47] are involved in hTERT transcription regulation.

Another ubiquitously expressed protein from the Sp protein family is protein Sp3, which often acts as a concurrent inhibitor of Sp1 [75]. Changing the ratio of those two proteins in favour of Sp£ leads to the inhibition of hTERT transcription [76].

Both Sp1 and Sp3 are necessary for repressing the hTERT promoter by histone deacetylases; they probably bind them to the promoter of TERT [77]. Apart from the above stated, Sp1 is also a transmitter of both activators and inhibitors of hTERT transcription (Fig 3).

NUCLEAR NF-KB

NF-kB controls the expression and function of several genes involved in cancerogenesis [78] and, in particular c-Myc, which is a trans-activator of telomerase. When the protex Tax of the human Lymphotrophic virus type 1 (HTLV-I, Human T-lymphotropic virus I) activates NF-kB, the cascade increase of c-Myc and hTERT promoters occurs [79]. Chromatin immunoprecipitation showed that there is an increase in the binding of c-Myc and Sp1 to the hTERT promoter during its activation by NF-kB [80].

The expression of NF-kB and hTERT genes happens at the same time and increases at the early stages of stomach cancer formation [81]. The synthesis of hTERT and potential sites for the NF-kB binding in the hTERT promoter (regions −758--749 np and −664--654 np) at the same time allow us to suggest that NF-kB participates in telomerase activation [82]. The activation of the mTERT promoter (as a result of binding with NF-kB) was shown for mice [82].

NF-kB (p65 subunit) can interact with histone acetylases (HDAC1): because of this, it plays a role in the negative regulation of gene expression [83]. Histone deacetylases inhibit the hTERT promoter and bind to the promoter with the help of Sp1. NF-kB, in the case of binding with the promoter, can be used for binding HDAC1.

TRANSCRIPTION FACTORS AP1 AND AP2

The transcription factor AP1 (activator protein 1) participates in the processes of cell proliferation, differentiation, cancerogenesis and apoptosis; it is expressed both in cancer and normal cells. It is a heterodimer of Jun (c-Jun, JunB or JunD) and Fos (c-Fos, FosB, Fra-1 or Fra2) [84].

The super-expression of AP-1 leads to the suppression of hTERT transcription in the HeLa cell line. The combination of c-Fos and c-Jun or c-Fos and JunD decreases the activity of the hTERT promoter by 80% in experiments with a short time expression. The region of the hTERT promoter between nucleotides −2077 and −455 np participates in this process. JunD and c-Jun bind with both potential binding sites of AP-1 at positions −1732 and −795 np. Introducing mutations in the AP-1- binding sites at the hTERT promoter compensates AP-1-induced inhibition [84]. The correlation between the c-Fos expression, which is one of the AP-1 subunits, and the hTERT mRNA expression was shown [85].

The hTERT promoter also contains the potential region for the AP-2- binding [48, 86]. AP-2 binds with the region from −121 to 129 of the hTERT promoter. In rhabdomyosarcoma cells, the mutation in the AP-2 binding region leads to a decrease of promoter activity. At the same time, the super-expression of AP-2 does not lead to an increase in the hTERT promoter's activity [86].

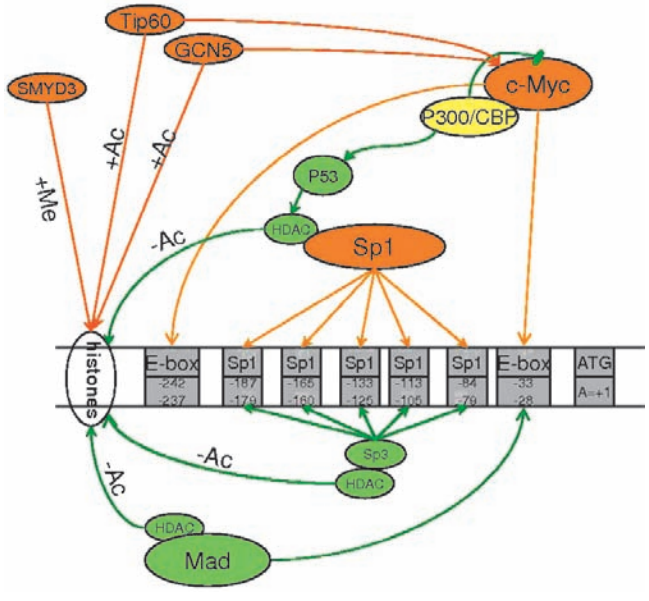

Fig. 4. Methylation (+Me), Acetylation (+Ac) and De-acetylation (-Ac) of histons of hTERT promoter. The inhibitors of the hTERT promoter are green, activators are orange, and the effectors of double action are yellow. → marks the activation of the following player of the cascade, ⊣ is the inhibition of the following cascade player.

ONCO-SUPPRESSORS P53 AND P73

Protein p53 regulates plenty of genes that play a role in the control of the cell cycle and oncogenesis (p21, MDM-2, Bax, c-Fos/Jun, pRB, 14-3-3σ, Bcl2 and others) [87–89]. p53 suppresses oncogenesis by switching on the mechanisms of cell cycle arrest and apoptosis as a response to different cell damages [87]. This protein is not active in more that half of human tumors [90]. Recovering the functional p53 in the case of cervical cancer, Burkitt's lymphoma, cancer of mammary glands, and pancreas leads to the inhibition of telomerase activity through the inhibition of the hTERT expression [91–94]. This effect appears several hours after the induction of p53, before the beginning of cell cycle abnormalities and apoptosis.

Mutations in the domains of p53, which are responsible for histones acetylases and co-repressor Sin3A, do not affect hTERT inhibition; even deacytilases participate in the inhibition of hTERT transcription by protein p53. p 53 mutations in the domain of DNA-binding, oligomerisation domain, or domain of transcription activation lead to the deactivation of p53 in relation to the telomerase. However, p53 does not bind with hTERT promoter *in vivo*, which means that its action on the promoter is non-direct. p53 can use proteins p21, E2F and proteins of group pRB [95].

Sp1 is necessary for the inhibition of p53 [92, 94]. Mutations in the regions of Sp1 binding inhibit the hTERT promoter activity by p53 [92]. Experiments on the cells of Drosophila Schneider SL2 have shown that the Sp1-ectopic expression-dependent hTERT-promoter activation is eliminated by wild-type p53. p53 interacts with Sp1 and blocks its binding to the hTERT promoter *in vitro* [94]. It is possible that p53 uses protein p21 as a messenger [95]: however, in previous similar experiments it was found that p21 is not a messenger between p53 and telomerase [94].

Protein p73 has an onco-supressing function similar to p53. Research on cells without p53 has shown that the super-expression of C-end isoforms p73 (α, β, γ, δ) leads to a decrease of hTERT-promoter activity. The supression of hTERT expression happens with the mediation of the endogenous p73 after the activation of E2F1 in cells. Mutations in the regions of Sp1 binding in the mane region of the hTERT-promoter rescue the repression of the hTERT-promoter by p73, which means that Sp1 acts as a messenger in this process. Apart from this, p73 binds Sp1, which proves the participation of Sp1 in the p73-dependent suppression of the hTERT expression [96].

PROTEINS pRB, E2F, p21, AND p16

Proteins E2F can both suppress and activate oncotransformation on model systems [97]. The super expression of E2F-3 correlates with the worst prognosis in the case of prostate cancer, ovarian cancer, and nonsmall cell lung cancer; a high level of E2F-1 exists in case of lung, mammary, and pancreas cancer. A low expression of E2F-1 is obtained in case of a more aggressive illness during colon cancer and urinary bladder cancer. E2F-1, E2F-2 and E2F-3 are also regulated by protein pRB, but E2F-4 and E2F-5 are mainly regulated by proteins p107 and p130. E2F-1, E2F-2, and E2F-3 can bind Sp1, but E2F-4 and E2F-5 do not interact with it [98].

Protein E2F-1 binds to the telomerase promoter at twp specific regions (at the regions −251 and −175 np). Apart from this, there is a nonclassical E2F-1-binding region at from −67 to 61 np [99]. E2F-1 decreases the expression of hTERT m RNA and telomerase activity in the cell lines of squamous cell carcinoma [99, 100]. The ectopic expression of E2F-1, E2F-2, and E2F-3 leads to a decrease in the activity of the promoter of telomerase reverse transcriptase in cell lines HeLa, U2OS, and 273 with the mediation of Sp1; E2F-4 and E2F-5 do not inhibit telomerase. However, in the hTERT cells that are not transformed, the activation of endogenous telomerase occurs as an effect of E2F-1,2,3,4,5 [98].

Other results were obtained when researching [101] the cells of squamous cell carcinoma. It was shown that the activity of the hTERT promoter and telomerase activity are decreased only by the super-expression of pRB: however, the super-expression of E2F-1 restores the hTERT promoter acivity. In research on glioblastoma, a correlation between the expression of E2F1 and hTERT was found; patients with a low level of E2F-1 expression had a much better prognosis [102]. The ectopic expression of exogenous E2F-1 increases the activity of the hTERT promoter in cell lines Saos-2, HeLa, and U-251 MG [102]. The variation in the data could be related to the heterogeneity of the cell lines (for example, cell line HeLa has been cultured for decades in different laboratories, and could be very different depending on the source). Another reason for this could be the importance of not only the presence of E2F proteins for the inhibition activation of the hTERT promoter, by also of their posttranslational modifications and of modifications of pRB, which also participates in this cascade.

E2F-1 could be one of the p53 messengers. The mutations of the noncanonical region for the binding of E2F-1 and super-expression of the mutant E2F-1, which binds to DNA but without the domains of transactivation and binding for the pRB, lead to the complete compensation of the p53 effect. The same effect occurs after the inhibition of proteins pRB, p107, and p130 [95].

The super expression of p21 and pRB completely suppresses the hTERT expression and stops the cell cycle in the cell line U-251 MG [102]. The appearance of protein p21, a cycline-dependent kinase inhibitor, leads to the accumulation of the hyperphosphorilated active form of pRB, p130, and p107. Those proteins bind to the proteins of the E2F family and transform them from activators of transcription to repressors [103]. Recovering the expression of pRB in pRB- and p53-negative cancer cells leads to the suppression of telomerase activity and stops the cell cycle [104]. The superexpression of the cyclin-dependent kinases cdk2 or cdk4, or of cycline D1 or E2F-1, leads to the restoration of pRB-supressed telomerase activity. For the functioning pRB as an inhibitor of the hTERT promoter, the phosphorilation of pRB is critically important [101]. The inhibition effect of pRB can be explained as binding to E2F-1 with further binding to the hTERT promoter [101] and particularly with bringing additional inhibitors, for example, histone deacrtylases. Also, protein pRB can disturb the binding of E2F-1 with the hTERT promoter. pRB and E2F-1 can also regulate an expression independently.

Onco-suppressor protein p16 acts in the regulation of the pRB/E2F system. Its expression significantly decreases the level of telomerase activity in glioma cell lines. p16 inhibits the binding of Sp1 with its binding regions in the promoter [105]. It was shown [100] that p16 insignificantly inhibits telomerase in the cell lines of head and neck epidermoid cancer; in [101] it was shown that telomerase activity disappears completely in the cell line SSC25, which was transfected by the p16-containing vector.

Bcl2

Bcl2 is one of the apoptotic factors [106].

The superexpression of Bcl2 in human cancer cells with a low endogenous level of expression of this protein causes an increase in the level of telomerase activity. When the Bcl2 expression was switched off and activated after that of the cell line CTLL-2, telomerase activity also decreased and increased correspondingly and reversibly [107]. When researching the number of mammary gland samples, no correlation between Bcl2 expression and telomerase activity was found. Also, after the inhibition of the Bcl2 expression in the leukemia cell line HL-60, no changes in hTERT mRNA expression were obtained [109].

Possibly, the regulation of the expression of hTERT by the protein Bcl2 is not used or used very rarely during natural ontogenesis; the hTERT activation by Bcl2 that was found is not direct. Another explanation could be that this process is tissue-specific.

ONCO-SUPRESSOR WT1

Onco-supressor WT1 participates in the inhibition of telomerase activity [110]. In the hTERT promoter at the position -352 np, there is a WT1-binding site, the mutations in which can increase the activity of the hTERT promoter (it can increase it in cell line 293 but not in cell line HeLa). The superexpression of WT1 suppresses the expression of hTERT, mRNA, and telomerase activity in cells 293 [110]. Because gene WT1 is expressed in certain types of cells during differentiation (kidney, reproductive organs, spleen) [111], the role of WT1 in the telomerase inhibition is probably tissue-specific.

MYELOID CELL-SPECIFIC PROTEIN MZF-2

In the hTERT promoter, there are four possible regions for the binding of transcription factor MZF-2 at the positions −687, −619, −543 and −514. They are responsible for suppressing the activity of the hTERT promoter, and MZF-2 specifically binds with those regions. The superexpression of MZF-2 suppresses the activity of the hTERT promoter [112].

MZF-2 is expressed in cancer telomerase-positive cell lines, and it seems that this protein does not play a major role in telomerase inhibition [112].

REGULATOR PROTEIN OF THE USF GROUP

The hTERT promoter contains E-blocks with which not only dimmers Myc/Max and Mad/Max can bind, but regulator factors USF as well.

In the model system with a reporter vector, the expression of USF1 or USF2 inhibits the activity of the promoter. These proteins do not interact with c-Myc or Mad and do not influence their expression in the cell: however, they directly bind with E-blocks in the hTERT promoter. Analyzing the clinical cancer and normal samples from the mouth has shown that the level of expression of USF1 and USF2 is lower in the cancer samples, but the hTERT expression and telomerase activity is higher in the cancer samples [113].

According to other data, USF1 and USF2 as heterodimers act with both the binding regions in the hTERT promoter and do not affect the hTERT transcription in hTERT-negative somatic cells. In hTERT-expressing cells, these proteins activate transcription and participate in the appearance and support of the cell immortality [114].

TRANSCRIPTION FACTORS ETS

ETS is a family of transcription factors; these proteins contain a conservative DNA-binding domain which specifically acts with GGA(A/T) sequences.

MAP-kinases can phosphorilate proteins ETS1 and ETS2 after activation by the Epidermal Growth Factor (EGF) and its analogue HER2/Neu. The phosphorilated form of ETS is active in transcription. Culturing cancer cells A-431, ME180, and MCF-7 c EGF makes the hTERT promoter stronger. The effect of EGF is compensated after the addition of the MAP-kinase inhibitor or after removing two supposedly closely located regions of ETS binding factors with the promoter in the region c from −22 to −14 np. EGF can lead to the phosphorilation of c-Myc, and, as a result of this, c-Myc activates transcription: however, mutations in the regions for c-Myc binding in the hTERT promoter do not affect the ability of EGF for transcription activation [115]. ETS interacts with the hTERT promoter DNA at the −36 np-position with activation, but it interacts at the −293-np position with the inhibition of hTERT expression, forming the complex Ets-Id2-DNA (Id is a family of regulator proteins of cell growth and differentiation inhibitors) [116]. The superexpression of ETS1 and ETS2 leads to a decrease and the superexpression of Id2 leads to an increase of telomerase activity in the K562 cell line [117].

Onco-protein HER2/Neu activates the transcription of hTERT; it uses the transcription factor ER81, a member of the ETS family, as a messenger. An expression of ER81 only or HER2/Neu in the cell line BJ does not stimulate an expression of hTERT mRNA. An expression of both of these genes induces the transcription of hTERT, mRNA, and telomerase activity. In the model system, the expression of ER81 and HER2/Neu increases the promoter activity 3 and 9 times, correspondingly, and their combined expression increases the promoter activity 37 times (in the cell line 293T). By now, five possible regions for binding have been found, and for two of those regions (at the positions +211 − +214 np and +313 − +316 np) the ability to bind with ER81 has been shown. Mutations of only those two regions lead to a cooperative decrease of the activation of the hTERT promoter by ER81 and HER2/Neu [118]. ERK MAP-kinases are mediators between HER2/Neu and ER81. It was also shown that Ras and Raf, which are regulators of ERK MAP kinases, stimulate the transcription of hTERT [118].

PEA3 and ERM, two other proteins of the ETS group that are from the same subfamily as ER81, in collaboration with HER2/Neu, activate the hTERT promoter, but

to a lower degree than ER81. On the other hand, four transcriptional factors of the ETS group from other subfamilies (Elk1, Sap1a, Elf1, and ER71) practically do not activate the hTERT promoter [118].

SIGNALING THE PATHWAY OF RECEPTOR CK

A disturbance of the signaling pathway of the cholesterol-specific Ck receptor was obtained in leukemia patients, in the leukemia cell lines, and in the Central Neural System Cancer.

The Ck active receptor decreases the expression of hTERT mRNA by inhibiting proteinkinase C. Proteinkinase C activates the transcription of PPARγ (peroxisome proliferated receptor γ), which inhibits the expression of c-Myc and hTERT and decreases telomerase activity. Also, PPARγ can interact with transcription factor Sp1, which is an activator of the transcription of hTERT. Apart from this, PPARγ is an antagonist of NF-kB, which is also an activator of the transcription of hTERT [119].

STEROID HORMONES

In many cases the probability of oncogenesis can be increased as a result of disturbances in the hormone-mediated regulation of gene expression. Several hormones that participate in cancerogenesis could affect the expression of hTERT.

ESTROGENS.

Estrogen (17β-estradiol) activates hTERT transcription in hormone-sensitive tissues. After the treatment of mammary gland cancer cells or normal cells of ovarian epithelium by estrogen, the level of hTERT, mRNA, and telomerase activity increases within a couple of hours [120, 121]. An analysis of the hTERT promoter has shown that there are two regions for esrogen-receptor binding [122]. The region for binding the estrogen receptor at the position −2754 np increases the activity of the promoter by five times as an effect of the hormone. After removing this region, the activation hTERT promoter by estrogen decreases by 70%. The second region, at the position −949 np, probably works in cooperation with the Sp1 site that is located nearby [123]. It was shown using the foot printing approach that protection of the region at −949 np is effected in the presence of estrogen. Mutations of this region strongly decrease the activation of the hTERT promoter by estrogen in the reperter construction [121]. In another work it was found that the estrogen receptor is bound only with the −2754-np region, but not with the −949-np region; it was also found that removing the −949-np region from the hTERT promoter does not affect the activity of the promoter [120]. Both receptors α and β bind to the hTERT promoter. The low telomerase activity in the mammary cancer correlates with the absence of the estrogen receptor β [33]. The activation of the hTERT promoter in the cell line NIH_3T_3 depends on the presence of the α estrogen receptor, but not on β [121].

The scheme of the multi-level activation of telomerase is achieved during estrogen regulation. Estrogen activates telomerase not only as a direct regulator, but also through the induction of c-Myc, which is another activator of the hTERT promoter [120].

Estrogen also activates hTERT expression through the PI3K/Akt/NF-kB cascade. Estrogen also induces the phosphorilation of hTERT, binding proteins 14-3-3 and NF-kB with the hTERT, and the Akt-kinase mediated accumulation of hTERT in the nuclei [124].

ANDROGENS

Normal prostate tissues and epithelial cell lines usually do not have telomerase activity in the presence of androgens, but the absence of androgens leads to telomerase activation in normal rat prostate tissues and does not produce significant changes of telomerase activity in the heart, kidney, liver, and lung tissues [125]. However, most types of prostate cancer have strongly expressed telomerase activity at the normal level of androgens. In the cell lines of the prostate cancer, telomerase activity is suppressed in the absence of androgens [126]. Antibody staining has shown a significant suppression of telomerase in a set of clinical samples of a prostate without androgens [127].

In the cell lines of the mammary gland and uterus cancer, progesterone increases the mRNA of the Telomerase Reverse Transcriptase within three hours of treatment. After 12 hours, its amount peaks and starts to decrease; after 48 hours progesterone opposes estrogen and inhibits the estrogen-induced expression of hTERT mRNA. The activating effect of progesterone happens through a cascade of MAP-kinases, and inhibition happens by p21 [128]. Combining estrogen and progesterone (which models a decrease in the risk of mammary gland cancer during pregnancy) leads to the suppression of telomerase activity and increases the expression of its inhibitors p53 and p21 in the cell line of the mammary gland epithelium 76N TERT [129].

During the growth of normal and androgen-independent prostate cell lines in the presence of dihydrotestosterone, no changes in telomerase activity were obtained. In the case of an androgen-dependent cell line of LNCaP prostate cancer, the absence of androgen in the media leads to a decrease in telomerase activity. Dihydrotestosterone activates telomerase activity in the G1-phase of the cell cycle. However, there was no increase of promoter activity obtained in the experiments with reporter construction [130, 131]. The action of androgen is non-direct, and it is consistent with the absence of elements of the response to androgen in the promoter of hTERT [126].

THE VIRAL REGULATION OF HTERT EXPRESSION

HUMAN PAPILLOMAVIRUS

Human papillomaviruses (HPV) are divided into 3 groups: non-oncogenic, low risk, and high risk by the probability of neoplastic transformation of contaminated cells. Proteins E6 and E7 of HPV from the high-risk group participate in oncogenesis by inactivating cancer suppressors p53 (E6, together with E6AP ubiquitin protein ligase), pRB and pRB-associated proteins, p130 and p107 (E7), and some other proteins [132].

After the transfection of telomerase-negative cells (primary keratinocytes) by genes of E6 and E7, only E6 (and not E7) activates telomerase [133, 134]. After the transfection of cells by both genes (E6 + E7), the data is contradictory: the

activation of telomerase can be the same as at transfection only with genes E6 [134], somewhat lower [133], or somwhat higher [73]. In the cell line of cervical cancer C33A (with active telomerase, but without HPV), an expression of E6 activates an expression of hTERT by 3 times and E7 by 1.5 times [73]. In the line of mammary gland epithelium, E6 activates telomerase almost immediately but E7 accelerates the process of telomerase activity appearance in the cell population gradually (high telomerase activity occurs after 20–25 passages with the expression of E7) [135]. Shortly, E6 is a direct telomerase activator, and the possible activation of telomerase by the protein E7 is a mediated and weak effect.

E6 activates telomerase transcription through the promoter region from –260 np until the site of translation initiation (from –15 to –266 np [134], –258 np [73]), in which there are 2 regions of c-Myc binding; when any of these two regions are removed, the activity falls by 60% [134]. E6 is coprecipitated with c-Myc by the immunoprecipitation method [136]. No changes in the c-Myc expression level after the transfection of cells by the gene of E6 appeared. After the superexpression of the gene that encodes Max (protein-antagonist of c Myc), the E6-mediated suppression of hTERT occurs [134]. According to the data of other authors, introducing mutations into both regions of c-Myc binding leads only to a minor decrease of the promoter's activity. However, mutations in Sp1-binding sites lead to a decrease of E6-induced promoter activity by 50%. Mutations in the regions of c-Myc and Sp1 binding at the same time lead to the almost complete disappearance of the telomerase transcription activation by protein E6 [73].

In keratinocytes and mammary gland epithelium cells, E6 from the HPV of a high-risk group (HPV 16, HPV 18, HPV 31, and HPV 54) has a strong telomerase activation effect; however, in the case of HPV from the low-risk group (HPV 11, APV 6), hTERT transcription activation is not strong [73]. Only the protein E6 of papillomaviruses from the high-risk group binds to the minimal promoter of hTERT (–300 to +1 n.p.) [136]. The activation of telomerase by the protein E6 is cell-type specific. The transfection of cells by gene E6 (which, in the case of the cell of uterine cervix epithelium, leads to the telomerase activation in the case of foreskin fibroblasts [137] or cells IMR90 [69]) does not give a similar effect.

Protein E2 HPV can inhibit the hTERT promoter. This protein can bind with the hTERT promoter. For the promoter inhibition, the interaction with Sp1 (the binding regions which are located between E-blocks) is important. E2 can inhibit the growth of HPV-infected cells and leads HeLa cells to apoptosis [138].

HEPATITIS B VIRUS

Protein X of the Hepatitis B virus (HBV) is a transactivator whose targets are the genes c-Myc, AP1, AP2, and NF-kB [19], which are activators of telomerase.

The frequency of telomerase activity appearance increases from the normal tissue to the cancer tissue: in 79% of cancer cases, 24% of cirrhosis, and 8 % of chronic hepatitis [32]; in 85,2% of cancer cases, 45,9% of cirrhosis, 25% of chronic hepatitis, and 15,7% of normal tissues [41].

After the transfection of cell lines by the gene of the protein X HB, an increase in the hTERT mRNA was shown (cell lines of hepatic carcinoma and cholangiocarcinoma) [139]. Western Blot has demonstrated that the amount of Telomerase Reversed Transcriptase increases in the hepatoma cell line after the superexpression of the protein X HBV [140].

A slight increase in c-Myc, together with hTERT, after the superexpression of protein X in the hepatoma cell line was also found [140]. Because c-Myc is activated by protein X and activates the expression of hTERT, it is, possibly, one of the mediators in the activation of telomerase after contamination by HBV. However, the relationship between the hTERT mRNa expression and the level of c-Myc was not found by *in situ* hybridisation in clinical hepatic cancer samles. Mutations were found in the regions of the c-Myc binding to the hTERT in clinical samples of hepatocellular carcinoma [141].

The regions of possible binding of the nuclear hepatocellular factors HNF-3b and HNF-5 were found in the promoters of TERT in both humans and mice. They are conservative, but their functional significance has still not been defined [67].

HERPES VIRUSES (EPSTEIN–BARR VIRUS, KAPOSI'S SARCOMA)

Both herpes viruses, which are oncogenic for human, can participate in the regulation of the transcription of hTERT.

The Epstein–Barr virus (EBV) is a causative agent of infectious mononucleosis and is related to cancerogenesis, for example, Burkitt lymphoma [19, 142]. The latent membrane protein 1 (LMP1) of EBV induces the specific binding of hTERT with the p65 subunit of NF-kB and the transfer of both proteins from the cytoplasm to the nuclei [143]. Another mechanism of hTERT expression activation by the protein LMP1 occurs through its c-Myc [144]. The latent membrane protein 2A LMP 2A suppresses the transcriptional activity of the hTERT gene [145].

Herpes virus type 8 is identified as a causative agent of multiple pigmented sarcoma (Kaposi) [19]. The nuclear antigen LANA of this virus is an activator of hTERT transcription. This protein can bind with Sp1, and the activation of the telomerase promoter probably occurs as a result of this interaction [146].

T-LYMPHOTROPIC VIRUSES

T-lymphotropic viruses of the 1st and 2nd types (HTLV-I and HTLV-II) activate telomerase [80, 147].

All adult patients with acute or chronic T-cell leukemia demonstrate a high activity of telomerase; however, nonsymptomatic HTLV-I carriers show telomerase activity only in 29% of cases. Two out of seven patients with active telomerase transformed to the acute form in one month [148]. However, the lack of data (there were only 24 patients) does not allow one to draw a definite conclusion about a direct relationship between the increase of telomerase activity and disease progression. Telomerase activity is high in both HTLV-I transformed cells and in lymphocytes from leukemia/lymphoma patients when compared with nontransformed or normal cells [149].

In the regulation of hTERT transcription, the protein Tax of the HTLV-I virus participates; even this protein is oncogene: it can suppress the expression of the gene hTERT twice in three days [80, 150] after the stimulation of cell

division by phytohaemagglutinin. In the absence of phytohaemagglutinin and in the presence of a Tax, there is an increase of telomerase activity by 25% in the same time span. The activation of the expression of NF-kB happens during this process, which activates the hTERT promoter [80]. The suppression of the hTERT promoter activity happens as a result of the competition between Tax and c-Myc for the canonic region of c-Myc binding in the hTERT promoter [150].

SV 40 AND ADENOVIRUSES.

Neither SV 40 nor adenoviruses (AV) are related to the ethiology of natural types of human cancers, but in the model systems they can act in the transformation.

AV are not oncogenic for human, and gene therapy uses them for the creation of genetic material delivery vectors. However, the expression of adenoviral protein E1A is enough for the immortalisation of rodent primary cells, and, in the presence of a second oncogene E2A or ras, their transformation is possible. Protein E1A 243R and, particullary, second exon E1A, activates the promoters of hTERT and hTR. This activation could be suppressed by the repressor CtBP (C terminal binding protein), which does not influence the basic level of hTERT expression by its own. The activation of hTERT by protein E1A probably occurs through the regions of Sp1 binding [151]. These data show that it is very important to be extremely careful with AV and with the nonocogenicity–safety of their use in human therapy.

Virus SV 40, which has long been a suspected oncogene, is not considered dangerous for humans [152]. In human cell lines, big and small SV40 antigens can lead to transformation [153]. There is data that virus SV40T accelerates the appearance of telomerase activity in human mammary gland epithelial cells. But this activity is dependent on the number of passages, which means that telomerase activation may happen indirectly and as a result of an increase in the frequency of some random event that happens as a result of cell metabolism abnormality [135].

THE EFFECT OF HYPOXIA ON THE HTERT EXPRESSION

The regions of cancer hypoxia are characterized by resistance to therapy, genetic instability, and by increasing malignancy. Hypoxia can lead to the intensification of telomerase activity, for example, in the cell lines of cervical cancer [154]. In its main part, the hTERT promoter contains two regions of a hypoxia responsible element (HRE) for binding hypoxia-inducible factor-1 (hypoxia-inducible factor-1 and HIF-1) in the regions −242 and −26 np. It was found that these regions are necessary for the activation of hTERT by HIF-1 [155]. The incubation of cancer cell lines in the lack of oxygen leads to the assembling of a transcriptional complex that includes HIF-1, p300/CBP, RNA-polymerase II, and TFIIB on the hTERT promoter in the region of HRE. The superexpression of HIF-1 in the ovarian cell line leads to the growth of hTERT promoter activity by almost two times. During the growth of a cancer cell line in the condition of hypoxia without the superexpression of HIF-1, the redistribution of splice forms of hTERT mRNA takes place (see in detail in the section “Regulation of hTERT splicing”) with a very insignificant increase in the total hTERT mRNA [156].

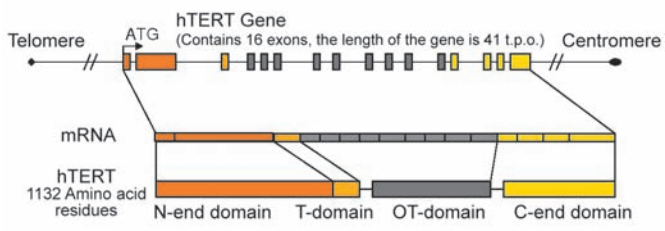

Fig. 5. Gene of telomerase reversed transcriptase. Regions that encode N- and C- ends, reverse-transcription (RT), and domains typical for telomerases (T) are shown [48].

POSTTRANSCRIPTIONAL REGULATION OF HTERT

Cancer needs the support of the telomeres' length, and the activity of telomerase that supports the telomeres' length may not be in correlation with the transcription of hTR and/or hTERT mRNA [32].

REGULATION OF HTERT SPLICING

The telomerase reversed transcriptase gene consists of 16 exons and makes up ~37,000 base pairs of genomic DNA, in which introns make up ~33,000 base pairs and ~4,000 are related to the transcript [48] (Fig. 5).

NATURAL SPLICING OF HTERT MRNA

Only full-sized hTERT mRNA provides telomerase activity. Thirteen alternative splicing variants of hTERT mRNA are known [48, 157–159]. Isoform with α-deletion (the deletion of 36 nucleotides in the reverse-transcription domain) act, after superexpression, as the dominant inhibitors of telomerase activity [160, 161]. This variant of hTERT mRNA is translated, and the appearing protein can be included in the telomerase dimer complex (Fig. 6).

Deletions β and α+β do not lead to the formation of the active telomerase, but neither do they inhibit it [161]. However, after the treatment of immortalized cell lines with the TGF β1 (transforming growth factor β1), the β-variant of hTERT mRNA that is formed as a result of alternative splicing and telomerase activity drops [162]. The opposite situation could exist at the regions of cancer hypoxia: the redistribution from the spliced β-variant to the active transcript [156] (Fig. 6).

Telomerase activity in the cells of osteosarcoma, which express only full-sized mRNA, is higher than in the case of cells with a set of different splicing forms of hTERT mRNA [54]. Telomerase activity increases in the stomach adenocarcinoma tissue, the total amount of hTERT mRNa is increased compared to the surrounding tissue, and the ratio of the amount of α, β and α+β forms is the same, which indicates the absence of regulation of the splicing of hTERT [163].

The variant with the deletion γ is expressed at a low degree and does not affect telomerase activity in the cell lines that were derived from hepatic cancer [159]. Deletions α+β, β and 4 insertions INS 1-4 [48, 157] cause the early termination of hTERT translation [161] similarly to variants with deletions β+γ, α+β+γ [159]. Part of the transcripts that were

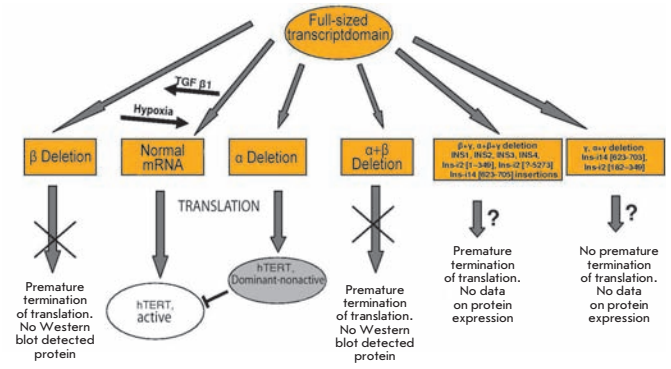

Fig. 6. Different variants of splicing of the hTERT mRNA.

found recently [158] have still not been investigated in relation to telomerase activity.

Possibly, splicing telomerase regulation is tissue-specific. An analysis of embryonic tissue has shown that telomerase activity in the heart and liver correlates with the hTERT gene expression: in a kidney, this activity disappears on the 15th weak of development, and hTERT transcripts could be found at the 21st week [164].

ARTIFICIAL SPLICING

Intron of the first group from Tetrahymena can induce new RNA, which substitutes hTERT mRNA with a high accuracy and specificity as a result of trance splicing [165]. In the cell line of prostate cancer 2'-O-methyl-RNA phosphothioate oligonucleotides, which are specific to the splicing region between the 5th intron and 6th exon in hTERT pre-mRNA, there is a decrease in the amount of full-size transcripts and, at the same time, an increase in the amount of alternatively spliced transcripts, which leads to a decrease in telomerase activity. The growth rate decreases in this process, and cells start apoptosis within two days [166].

CELLULAR LOCALIZATION

Even telomerase activity needs the localization of telomerase in the nuclei; near the telomeres, a high activity of telomerase *in vitro* occurs in the cytoplasmatic extract, but not in the nuclear extract [167]. However, *in vivo* GFP-containing telomerase is located in the nuclei [168].

When the binding of NF-kB (p65 subunit) with the protein hTERT occurs, the tumor necrosis factor α (TNF α) induces the transfer of NF-kB-bound hTERT from the cytoplasm to the nuclei [169].

Protein 14-3-3, which is responsible for nuclear localization, binds with telomerase. Dominant-negative 14-3-3 directs hTERT, which is normally localized in the nuclei, into the cytoplasm. Mutant hTERT, which is incapable of binding with 14-3-3, localizes in the cytoplasm. 14-3-3 disturbs protein CRM1 binding with the c NES-motif (nuclear export signal). Inhibiting the CRM1/exportine1-pathway of the nuclear transport, as well as damaging the NES-motif, leads to a decrease in the localization of hTERT in the cytoplasm [170].

During most of the cell cycle, hTERT is not localized in the nucleoli, Cajal bodies, or telomeres. At the S-phase of the cell cycle, hTERT moves to the nucleoli, then to the Cajal bodies, and then to the telomeres [15, 16].

THE PHOSPHORILATION AND DEPHOSPHORILATION OF HTERT

The phosphorilation of the telomerase reversed transcriptase by the proteinkinase Cα (PKCα) is required for the telomerase activity in mammary-gland cancer cells [171]. Another proteinkinase from this group, proteinkinase Cz (PKC zeta), controls the telomerase activity in the cell of the cancer of the nasopharynx without any effect on hTERT expression. Switching off the PKC-activator zeta Cdc42/Rac1 leads to a decrease of telomerase activity [172] (Fig. 6).

Phosphatase PP2A inhibits telomerase activity in mammary-gland cancer cells [173] (Fig. 7).

The kinase Akt increases telomerase activity by phosphorilation hTERT in the melanoma cell line [174]. The dominant-negative mutant Akt-kinase significantly decreases the level of telomerase activity in the endothelial cells. Also, it suppresses telomerase activity by inhibiting kinase PI3K (phosphoinositol 3-kinase), which phosphorilates and activates Akt-kinase [175] (Fig. 7).

Phosphorilation not only directly affects the activity of hTERT, but it also affects the transcription of this gene. The treatment of cells by the PI3K inhibitor or the expression of the dominant-negative Akt-kinase in the cells makes the estrogen-activated hTERT activity weaker in the human ovarian carcinoma cell lines [124].

Two-strand DNA gaps, activated Tyrosin-kinase c-Abl, bind and phosphorilate hTERT, which inhibits its activity. The irradiation of cells by ionizing radiation induces the phosphorilation of hTERT by the c-Abl-dependent mechanism [176].

Under oxidative stress conditions, GTP-ase Ran provides the export of hTERT. hTERT-phosphorilation by kinase Scr is required for this export. [177]. The superexpression of phosphates Shp-2 blocks this mechanism of exporting hTERT [178].

THE REGULATION OF TELOMERASE RNA

In adult human tissues, a high level of telomerase RNA was found in primary spermatocytes and in the Sertoli cells, a middle level of expression was found in lymphatic follicles, and a low level of expression was obtained in the epithelium; hTR expression is absent in the neuronal system and in

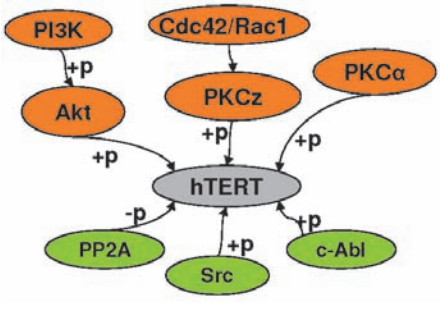

Fig 7. Regulating the activity of the protein hTERT by phosphorilation–dephosphorilation. Activators are shown in orange, inhibitors are shown in green. "+p" means phosphorilation, and "-p" means dephosphorilation.

mesenchyme-derived tissues [179]. A significant level of expression was found in the small intestine, thymus, kidneys, and prostate. A low level of hTR expression is obtained in the brain, liver, stomach, pancreas, lungs, and heart [180].

In telomerase-positive samples of tumor tissues, a strong expression of hTR was demonstrated, but only half of telomerase-negative soft tissue sarcomas expresses hTR to a different degree. In telomerase-negative tumors, there is no relation between the expression of hTR and the proliferating status, telomeres length, and expression of hTERT [181]. The high expression of hTR is not related to the telomerase activity, in lung cancer, for instance [30].

Using *in situ* hybridization, it was demonstrated that, in the case of Barrett's esophagus and of early stages of esophageal dysplasia, hTR is absent or expressed at a middle level; however, the effectiveness of hTR expression is high at late developmental stages of dysplasia or cancer [182]. By using RT-PCR (reverse transcription and PCR), hTR is detected in 90–100% of the samples of lung cancer, both in cancer and normal tissues [30, 183]. Using *in situ* hybridization, hTR is detected only in 26% of cancer tissues that are defined by histological analysis; for some samples, a difference between cancer and normal tissues was obtained. The same approach detected an expression of hTR in 41% of squamous cell carcinoma, 13% and 17% of adenocarcinoma of mammary gland and ovary, 43% of cancer, and 40% of cervix uteri precancerous lesions [34]. Unfortunately, the further fate of patients with precancerous lesions is unknown, so it is impossible to discuss the expression of hTR as a marker of oncogenic process development. In order to use hTR expression as a cancer marker, it is necessary to do a precise quantitative analysis, unlike the difference in expression of hTERT or determination of telomerase activity.

An analysis of nueroblastoma samples has shown high or middle level hTR expression in 9 out of 12 samples of the middle stages of cancer and only in 2 out of 8 samples of the early stages. The illness did not progress in patients with a low level or without the expression of hTR. In case of eight samples of skin cancer taken from seven patients with a middle or high level of hTR expression, the development of diseases was not good.

It should be mentioned that, in late stages of diseases with large metastases (four samples), the expression of hTR was weak. The expression of hTR in the ganglyoneuroblastomas and gangloneuromas is located only in neural cells, and it is absent in lemmocytes. Therefore, hTR seems to be a good prognostic factor in non-metastasis neuroblastomas [184].

For Williams cancer, hTR (but not hTERT) is a predictive factor of further development. In 30% of patients with the highest expression of hTR (quantitation was done by Real-Time PCR), the probability of relapse was twice as high as in patients with the lowest level of hTR expression [185]. Also, the high level of expression of hTR correlates with the bad prognosis in patients with lyposarcoma [42].

Increasing hTR expression does not always correlate with the appearance of telomerase activity [30]. hTR inhibits the proteinkinase of the ATR checkpoint. Suppressing the level of hTR expression stops the cell cycle at the G1- and G2-phases as a result of the activation of p53 and of the proteinkinase of CHK1 checkpoints. This effect is not dependent on telomerase activity. Increasing the hTR expression as a response to ultraviolet irradiation stops the activation of p53 and CHK1 as a result of the inhibition of ATR activity; because of this, it makes the cell response to the DNA damage weaker and allows cells to pass the checkpoint G2/M. No interaction between hTR and ATM was found, and the mechanism of this inhibition is still unknown [186].

REGULATION OF TRANSCRIPTION OF TELOMERASE RNA

hTR promoter contains CCAAT- and TATA-blocks near the region of the transcription start and several binding sites for the receptors of glucocorticoids, progesterone, androgen, and transcription factors AP1 and ETS [187]. The minimal region of the human telomerase RNA promoter is from –272 to –42 np prior to the start of transcription. The activity of the promoter is maximal when the region before –463 np is used; if the size of the region used is larger, the level of transcription decreases [187].

Investigating the methylation status of the hTR promoter has shown that three out of eight telomerase-positive cell lines and both telomerase-negative cell lines are hypermethylated; however, at the same time, no methylation of the hTR promoter was obtained either in cancer or in normal tissues. So, it is very likely that the methylation of the promoter is not related to the regulation of the hTR expression [181].

hTR promoter contains four regions for the binding of proteins Sp1/Sp3 (Fig. 8). Sp1-binding activates the promoter, but Sp3 inhibits it [188]. Mutation analysis has shown that the region before the CCAAT-block is required for the activation of the hTR promoter, as well as the fact that three regions after CCAAT are responsible for promoter inhibition. The region immediately after CCAAT has an inhibition effect at the strongest level but the lowest affinity Sp1. This can be explained because it is close to the region of the binding of transcription factor NF-Y. Two regions just after the start of transcription are under complicated regulation;

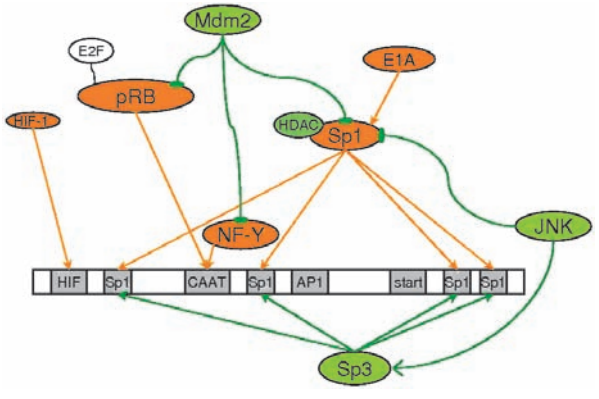

Fig. 8. Scheme of the effect of transactivators of hTR promoter. Inhibitors of the hTR promoter are green, activators are orange, and cofactors are colorless. → means activation of the following cascade player, ⊣ means inhibition of the following cascade player, and the absence of an arrow means co-action.

the introduction of mutations into both regions results in a strong activation effect that is not just the sum of the effects of single mutations [189]. On the other hand, research [151] has shown that the introduction of mutations into four regions of Sp1-binding at the same time does not affect the base level of hTR promoter activity, although it disturbs its transregulation.

The transcription factor NF-Y, which is capable of recruiting several components of the RNA-polymerase II complex to the promoter [190], is the main activator of the telomerase-reversed transcriptase promoter. NF-Y binds to the region CCAAT of the hTR promoter, and the disturbances of this binding lead to the almost complete disappearance of promoter activity [189].

pRB is also an activator of the hTR promoter. The CCAAT region is required for its action. The activation of the hTR promoter by the pRB protein decreases in case of mutant forms of pRB, which are not able to bind E2F, and it disappears in the case of the mutant Δ657 form, which is not able to bind E2F or activate gene transcription [188].

Mytogene-activated proteinkinase kinase 1 – (MEKK1)/c-Jun-NH(2)-kinase (JNK) suppresses the expression of hTR. The transfection of the permanently active kinase domain of MEKK1, the main MAP3K in the JNK-pathway, leads to the strong inhibition of the hTR promoter in some cancer cell lines. The suppression of the hTR promoter by kinase MEKK1 could be blocked by the SP600125 inhibitor of JNK. The effect of hTR-promoter inhibition using kinase MEKK1 can be intensified by the co-expression of wild types of JNK, but not by the co-expression of the mutant form of JNK, which cannot be phosphorylated. The cotransfection of Sp3 and MEKK1 gives an additive effect of hTR inhibition. According to immunoprecipitation data, treating cells with SP600125 leads to a change in the ratio of Sp1/Sp3 on the promoter, increasing Sp1. Thus, this kinase helps change the Sp1/Sp3 balance on the promoter, increasing Sp3 without changes in the level of expression of Sp1 and Sp3 or the inhibition of the hTR promoter [191].

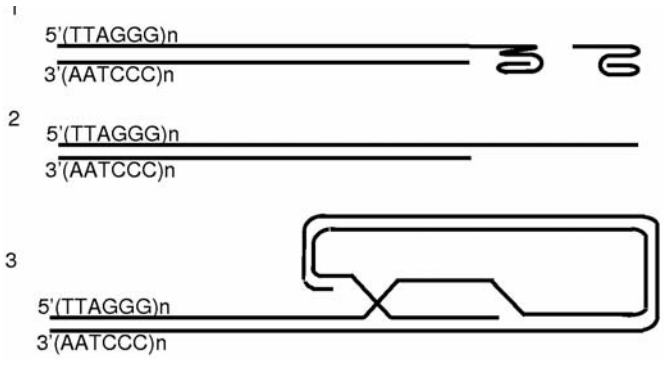

Fig. 9. Possible organization of telomere ends: (1) examples of G-quadruduplexes, (2) protracting 3'-end (substrate for telomerase), and (3) T-loop.

Ubiquitin lygase Mdm2 decreases the stability of p53 and regulates the pRb/E2F complex [192]. Mdm2 reacts with Sp1 *in vitro* and *in vivo* and inhibits the transcativation of Sp1-activated promoters. Mdm2 interacts with the promoter of hTR *in vivo* and inhibits it; however, regions for Sp1 binding do not act in this process. Mdm2 suppresses activation by pRb, NF-Y, and Sp1. Mdm2 and pRb, as well as NF-Y, can interact with the complex of RNA-polymerase II and, as a result, they can affect the expression of hTR [190].

The telomerase RNA gene contains the HRE region, and HIF-1 binds to this region. Superexpression HIF-1 in the cancer cells leads to an almost twofold growth in the activity of the hTR promoter in 6 h of incubation in hypoxia conditions, and then to a decrease back to the normal level in 4 hours. The transcription complex (including HIF-1, p300, RNA-polymerase II, and TFIIB), which was assembled on the hTR promoter in the cancer cell line in hypoxia conditions, was shown [156].

Adenoviral protein E1A increases the expression of the reporter vector under the control of the hTR promoter by 2.5 times. This activation likely occurs through the regions of Sp1-binding, because their mutations lead to the disappearance of the E1A effect. The activation of the hTR promoter by the E1A protein is also inhibited by the protein CtBP, but it does not influence the basic level of the hTR expression [151]

THE POST-TRANSCRIPTIONAL REGULATION OF TELOMERASE RNA

TR is accumulated in the Cajal bodies in the cancer cell lines, but not in the cell lines of the normal cells. It is possible to provoke the accumulation of hTR in the Cajal bodies by the expression of the hTERT in the cells [193]. It is hTERT that is the most important factor of the hTR localization in both the Cajal bodies and on the telomeres [194]. The previous 3'-end of the hTR processing is also required for accumulation in the Cajal bodies [195].

The stability of telomerase RNA in cells can increase at oncogenesis. Upon expression of hTERT in the hTERT-negative cells, the period of hTR half-decay increases by 1.6 times. This may happen due to binding and stabilization by the TR catalic subunit [196].

CONTROL OF TELOMERASE ACCESS TO THE TELOMERS

Telomers have a projecting 3'-end which can form several structures: T-loop [197] and G-quadruplexes [198, 199] (Fig. 9). In the T-loop, the projecting 3'-end is a joint with the internal region of the telomere and is stabilized by proteins [197]. G- are formed by the projecting 3'-end because of the formation of Hughstein's pairs [198, 199]. In humans, six proteins (TRF1, TRF2, hRap1, TIN2, TPP1, and POT1) form a Shelterin complex that is a permanent component of human telomeres [200].

Changing the level of expression of the Shelterin complex strongly affects the telomerase length. For instance, inhibiting TRF1 leads to the telomeres lengthening in human cancer cells, and its superexpression leads to the shortening of telomeres without any changing of the telomerase activity *in vitro*. Decreasing the amount of protein TIN2 or the superexpression of its mutant alleles, which disturbs TIN2

binding to TRF1 and TRF2, leads to telomeres elongation. The superexpression of TRF2 causes telomere shortening; this happens not only because of the *in vivo* inhibition of telomerase, but also because of the increase in the velocity of the shortening. Suppressing TPP1 by RNA interference or disturbing the TPP1–POT1 binding also leads to telomere elongation, with the following loss of the protein POT1 by the telomeres [200]. According to the data of other authors, TPP1 and POT1 form a complex with telomeric DNA, which increases the activity and processivity of human telomerase. It was suggested that TPP1-POT1 switches from inhibiting telomerase access to the telomere as a component of Shelterin to working as a factor of telomerase processivity during the telomere elongation [201].

POT1 binds sing-strand telomeric DNA with a high specificity using two OB-motifs (oligonucleotide/oligosaccharide-binding folds). Without the 1st motif, the superexpression of POT1 leads to the quick elongation of telomeres [200]. Decreasing the hPOT1 expression can also lead to telomere elongation [202]. Another group of researchers has found that expressing the full-length protein leads to telomere elongation [203]. The binding of the recombinant POT1 with the telomere oligonucleotide inhibits the binding of telomerase. On the other hand, POT1 *in vitro* can destroy qudriplex structures, which are formed because of Hughstein pairing of nucleotides in telomeric DNA. This can explain how POT1 brings positive participation into the telomerase-dependent elongation of telomeres, because G-quadruduplexes seem like a very bad substrate for telomerase. The destruction of G-quadriduplexes could be also done by helicases WRN or BLM from the family RecQ, which react with POT1 [200]. In humans, mutations in genes that code helicases WRN or BLM lead to the development of the Blum and Werner syndromes, which are characterised by genomic instability [204].

Longer telomeres contain more Shelterin complexes, which could be a detector of the telomere length. Binding the protein POT1 with Shelterin can affect the binding of POT1 to the single-strand region of telomere DNA. Also, Shelterin can inhibit telomerase and facilitate T-loop formation, in which one of the 3-ends is not accessible [200].

The amount of POT1 mRNA in the case of stomach cancer often decreases in the early stages and increases at more advanced stages. The level of POT1 expression decreases according to the telomere shortening. Apart from this, the inhibition of POT1 in the stomach cancer cell lines by the anti-sense oligonucleotides, similarly to the inhibition of telomerase activity, also leads to telomeres shortening [205].

The binding of telomerase to the telomeres can be regulated by the formation/degradation of G-quadruduplexes. Single-stand DNA-binding protein RPA can untwist the telomeres G-quadruduplexes in the model systems [206]. On the other hand, RPA is able to inhibit telomerase activity in the model systems by binding with the telomere-imitating oligonucleotie [207]. The inhibition of telomerase *in vitro* occurs both after removing RPA from the mix and with its huge excess [208].

Unfortunately, the methods for analyzing telomerase activity use model systems with an artificial substrate without estimating access to the telomeres.

REGULATION DURING THE RESPONSE TO THE IONISING IRRADIATION

When investigating the processes that happen in oncogenesis, it is impossible to ignore the consequences of the effect of radiation from different sources on the cells. On the one hand, it can provoke oncogenesis (UV irradiation provokes skin cancer, penetrating ionising radiation can cause different types of cancers, etc.); on the other hand, different types of radiations are used in cancer therapy (β-particles, neutrons, γ and X rays, etc). Because the activity of telomerase is associated with a lot of different types of cancers, the following question occurs: what happens with this activity upon radiation? Which leads to another interesting question: could irradiation provoke telomerase activity?

While investigating the effect of ionizing irradiation on the HeLa cell line, it was found that telomerase activity and the amount of hTERT mRNA increase during the first 24 hours, until the twofold increase in comparison with the non-irradiated samples; however, after that they return to their original level within 72 hours [209].

After the application of γ and neutron/γ irradiation to the hemopoetic cell line, the increase in telomerase activity and in the amount of hTERT mRNA occurs in a dose- and intensity-dependent manner. In the case of neutron/γ irradiation, a decrease in telomerase activity occurs first, but it is followed by an increase. The influence of irradiation with different energies is different in scale and kinetics, but it is similar in the mechanism of its action on cells. Changes in telomerase activity are not related to either the changes in the cell cycle or the induction of cell death; they are a result of the specific regulator responses to ionizing irradiation [210].

HeLa cells with shortened telomeres—after transfection by DN-hTERT (dominant-negative mutant hTERT)—become more sensitive to the effect of several chemotherapeutic agents and irradiation. Cells transfected by the wild type of hTERT with longer telomeres show higher resistance to the chemotherapeutic agents and irradiation [211].

UV irradiation provokes telomerase activity in different types of cells, including skin cells. Apart from the skin, eyes are also permanently under UV irradiation. It was shown that the level of telomerase activity and amount of hTERT mRNA and hTR increase only after receiving a certain amount of UV-irradiation energy by crystalline lens [212].

CONCLUSIONS

Knowing about the system of telomerase regulation allows us to create methods and agents for suppressing telomerase activity in cancer cells more effectively. Unfortunately, many mechanisms of telomerase regulation are tissue-specific. Understanding the interrelationship between the telomerase regulation system and other oncogenes can help in developing complex cancer diagnostics, which allows one to identify diseases and define the tactics for fighting them at the least aggressive stage.

Telomerase activity is a marker of actively dividing cells and one of the most universal markers of cancer. ●

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Catalytic Bioscavengers Against Toxic Esters, an Alternative Approach for Prophylaxis and Treatments of Poisonings

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ABSTRACT Bioscavengers are biopharmaceuticals that specifically react with toxicants. Thus, enzymes reacting with poisonous esters can be used as bioscavengers for neutralization of toxic molecules before they reach physiological targets. Parenteral administration of bioscavengers is, therefore, intended for prophylaxis or pre-treatments, emergency and post-exposure treatments of intoxications. These enzymes can also be used for application on skin, mucosa and wounds as active components of topical skin protectants and decontamination solutions.

Human butyrylcholinesterase is the first stoichiometric bioscavenger for safe and efficient prophylaxis of organophosphate poisoning. However, huge amounts of a costly enzyme are needed for protection. Thus, the bioscavenger approach will be greatly improved by the use of catalytic bioscavengers. Catalytic bioscavengers are enzymes capable of degrading toxic esters with a turnover.

Suitable catalytic bioscavengers are engineered mutants of human enzymes. Efficient mutants of human butyrylcholinesterase have been made that hydrolyze cocaine at a high rate. Mutants of human cholinesterases capable of hydrolyzing OPs have been made, but so far their activity is too low to be of medical interest. Human paraoxonase a promiscuous plasma enzyme is certainly the most promising phosphotriesterase. However, its biotechnology is still in its infancy. Other enzymes and proteins from blood and organs, and secondary biological targets of OPs and carbamates are potential bioscavengers, in particular serum albumin that reacts with OPs and self-reactivates. Lastly, non-human enzymes, phosphotriesterases and oxidases from various bacterial and eukaryotic sources could be used for external use against OP poisoning and for internal use after modifications for immunological compatibility.

Key-words: bioscavengers, carbamates, cocaine, enzyme engineering, enzymotherapy, organophosphorus compounds

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CaE, carboxylesterase; ChE, cholinesterase; DFPase, diisopropylfluorophosphate hydrolase; GMP, good manufacturing practice; GMP, good manufacturing practice, GST, glutathione S-transferase; OP, organophosphorus compound; OPAA, organophosphoric acid anhydrolase; OPAH, organophosphorus acid anhydride hydrolase; PEG, polyethylene glycol; PON1, paraoxonase 1; PTE, phosphotriesterase

1. THE BIOSCAVENGER CONCEPT

Enzyme systems located in skin, blood organs are involved in natural defences against endogenous and exogenous poisons. Detoxification processes occur through different types of reactions, including oxidation, hydrolysis, and conjugation. The role of liver, lung and kidney enzymes, cytochromes P450 (Brown *et al.*, 2008), oxidases, transferases (Miners *et al.*, 2006), amido-carboxylesterases (Redinbo and Potter, 2005; Potter and Wadkins, 2006; Satoh and Hosokawa, 2006) in the metabolism of drugs and xenobiotics is well known. The importance of plasma esterases in the inactivation of numerous toxicants has been recognized, too. Lastly, there is growing evidence that catalytic antibodies play also an efficient role in scavenging deleterious molecules and radicals (Belogurov *et al.*, 2009). These multiple enzymes constitute cellular and circulating barriers that protect physiological machineries

and systems against specific toxicants. Here we will examine endogenous and exogenous enzymes that react with poisonous carboxylic-, organophosphoryl- and carbamyl-esters. These enzymes act either as stoichiometric bioscavengers or catalytic bioscavengers. Catalytic bioscavengers are biocatalysts capable of degrading poisonous compounds with a turnover. Enzymes that are potential catalytic bioscavengers will be reviewed.

After a short survey of catalytic bioscavengers against cocaine, we will focus on biocatalysts to be used for prophylaxis and treatment of organophosphate poisoning. Indeed, OP poisoning is a major public-health problem. OP self-poisoning is responsible for 200,000 deaths a year in the world (Eddelston *et al.*, 2008). In addition, though 185 nations have joined the Chemical Weapons Convention (CWC), nerve agents and other organophosphorus compounds still represent military and terrorist threats. Significant progress has been made in the past twenty years in countermeasures of OP poisoning (Aas, 2003; Albuquerque *et al.*, 2006; Wetherell *et al.*, 2007; Eyer *et al.*, 2007; Thiermann *et al.*, 2007). However, classical pharmacological approaches have reached their optimum limit. Toxicity of OPs can be countered by reducing skin absorption and lowering OP concentration in the blood compartment, thus preventing the transfer of OP molecules towards physiological targets (Fig. 1). Neutralization of OPs has proved to be possible by using stoichiometric traps, first generation bioscavengers. The catalytic bioscavenger concept, second generation bioscavengers, is based on the idea of continuously trapping and degrading OPs in the blood stream before OP molecules reach their central and peripheral neuronal and neuromuscular targets.

2. DETOXIFICATION OF (-)COCAINE

Unlike the plasma of most mammalians, there is no carboxylesterase in human plasma (Li *et al.*, 2005). However, two enzymes are capable of degrading esters in the blood stream. Plasma paraoxonase (PON1; EC 3.1.8.1) displays an arylesterase activity, and butyrylcholinesterase (BChE; EC 3.1.1.8) — that has broad esterase specificity — plays a role in processing, catabolism and or detoxification of numerous poisonous esters: for instance, human BChE hydrolyzes ester-containing therapeutic and/or addictive drugs such as succinylcholine and its long-chain derivatives (Grigoryan *et al.*, 2008), aspirin, irinotecan, heroin (Lockridge, 1990; Li *et al.*, 2005). Plasma BChE also hydrolyzes prodrugs such as isosorbide diaspirinate, bambuterol (Li *et al.*, 2005), and ISDA, a new aspirin prodrug (Moriarty *et al.*, 2008).

Plasma BChE is the major detoxifying enzyme of cocaine in humans (Inaba *et al.*, 1978) and has been demonstrated to efficiently protect animals against cocaine toxicity (Hoffman *et al.*, 1996; Lynch *et al.*, 1997). However, BChE slowly hydrolyzes (-)cocaine with k_{cat}/K_m about 0.28 $\mu M^{-1}.min^{-1}$, so that under physiological conditions, a large part of the administered dose of cocaine reaches biological targets and triggers toxic effects. Mutagenesis efforts pursued over the last decade have dramatically enhanced the cocaine hydrolase activity of human BChE. A first mutation, A328Y, enhanced the catalytic efficiency 4-fold (Xie *et al.*, 1999). Molecular dynamics simulation and computer-based ligand docking led to the A328W/Y332A double mutant that displays a higher k_{cat}/K_m=8.5 $\mu M^{-1}.min^{-1}$ (Sun *et al.*, 2002). Further mutations using random mutagenesis raised k_{cat}/K_m. The simulation of activation transition state approach was also successfully applied to the design of new BChE mutants. Using the three-dimensional structure of human BChE (Nicolet *et al.*, 2003), molecular dynamic simulations of the deacylation transition state led to the design of highly active mutants against (-)cocaine. This was achieved by combining four mutations, A199S/S287G/A328W/Y332G, and yielded an enzyme with a catalytic efficiency 456-fold greater than that of wild-type BuChE (Pan *et al.*, 2005; Zheng and Zhan, 2008). The efficient quadruple mutant was fused to human albumin to improve its pharmacokinetics properties ($t_{1/2}$=8h in plasma) without altering its catalytic efficiency (Brimijoin *et al.*, 2008). At 10 mg/kg, the fusion enzyme stopped the symptoms of cocaine intoxication in rats at a lethal dose (100 mg/kg, i.p.). Interestingly, it also blocked cocaine-induced reinstatement of drug-seeking in rats that had previously self-administered cocaine. More recently, introduction of 5 mutations, A199S/F227A/S287G/A328W/Y332G, led to a more active enzyme (Zheng *et al.*, 2008). Therefore, the fusion enzyme and new mutants are promising for an efficient therapeutic approach to cocaine overdose rescue and the treatment of addiction. Immunopharmacotherapy for cocaine addition can be an alternative strategy. Monoclonal antibodies catalyzing the hydrolysis of (-)cocaine have been made (Landry *et al.*, 1993; Larsen *et al.*, 2004; McKenzie *et al.*, 2007). However, the kinetic parameters of catalytic antibodies have to be improved. Computational (transition state simulations, free energy barrier shift calculations) and mutagenesis approaches are expected to lead to more efficient biocatalysts (Pan *et al.*, 2008).

3. DETOXIFICATION OF ORGANOPHOSPHORUS COMPOUNDS

Organophosphates are widely used as pesticides. Some OPs are drugs, others are potent chemical warfare agents. OPs are irreversible inhibitors of cholinesterases (ChEs): acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (Fig. 2). AChE plays a major role in the cholinergic system terminating the action of acetylcholine. Thus, synaptic AChEs are the primary targets of OPs. Irreversible inhibition of AChE is the main cause of acute toxicity of OPs (Maxwell *et al.*, 2006). No clear physiological function has been ascribed to BChE. In the nervous system and at neuromuscular junctions, BChE may surrogate AChE under certain conditions (Lockridge *et al.*, 2009). However, BChE is of pharmacological and toxicological importance as shown in the previous section.

Endogenous enzymes are involved in natural defences against OP toxicity. The presence of OP detoxifying enzymes in skin contributes to reduce the amount of OP that penetrates into the body (Schallreuter *et al.*, 2007). Certain secondary targets of OPs found in various tissues are detoxifying enzymes, and they certainly play a role in the natural defences against OPs (Wang *et al.*, 1998; Nomura *et al.*, 2005, 2008). Natural blood bioscavengers significantly contribute to reduce the amount of OP molecules reaching physiological targets. BChE is the most important stoichio-

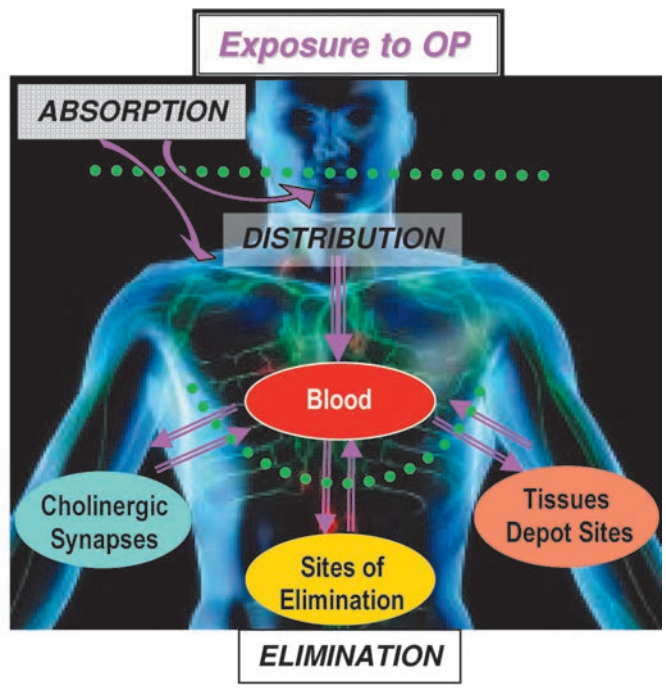

Figure 1: Biological fate of organophosphorus compounds in humans. Routes of penetration of OPs are absorption through the skin, eyes, and/or respiratory tract (nerve agents, pesticides), or ingestion (self-poisoning). OP molecules distribute from the blood compartment into tissues, including depot sites, biophase (physiological targets), and sites of elimination (liver and kidneys). ChEs of cholinergic synapses are the primary targets; their inhibition is responsible for the acute toxicity of OPs; reaction with secondary targets (carboxylesterases, serine-amidases, peptidases and other serine/tyrosine proteins) may be responsible for the non-cholinergic sub-lethal effects of OPs and chronic toxicity at low-dose exposure (Casida and Quistad, 2004; Costa, 2006).

metric OP scavenger in human plasma; its concentration is about 50 nM and its apparent second-order rate constant with OPs is $\approx 10^7$ M^{-1} min^{-1}. Human plasma PON1 displays activities categorized as "accidental" or "promiscuous" (Costa and Furlong, 2002; Mackness *et al.*, 2008). Albeit its primary function is likely to be a lipophilic lactonase (Khersonsky and Tawfik 2005) involved in protection against atherosclerosis (Shih *et al.*, 1998; Watson *et al.*, 1995), it hydrolyzes numerous OPs. It has been shown that animals in which the plasma concentration in PON1 is high are relatively resistant to OPs (Kaliste-Korhonen *et al.* 1996). Conversely, *knockout* mice for PON1 are very sensitive to OPs (Shih *et al.*, 1998). Albumin displays a low esterase activity and slowly reacts with carbamyl- and phosphoryl-esters. However, its concentration in the blood and lymph is high (≈0.6 mM), and it appears to play a significant role in the detoxification of carbaryl (Sogorb *et al.*, 2007). Plasma albumin could also play a role in detoxification of OPs (Tarhoni *et al.*, 2007; Li *et al.*, 2008), even at toxicologically relevant concentrations (Sogorb *et al.*, 2008).

OPs are also neutralized by tissues (liver) carboxylesterases (CaE; EC 3.1.1.1) and oxidized by oxidases such as cytochrome P450s, glutathione *S*-transferases, laccases, and peroxidases. Glutathione *S*-transferases (GST; EC 2.5.1.18) are 20-30kDa enzymes that catalyze glutathione conjugation (nucleophilic attack of the thiol group) to electrophilic substrates. These enzymes are involved in the cellular detoxification processes of endogenous compounds and of numerous xenobiotics, and their role in the resistance of insects to insecticides is well established. OP detoxification by GSTs results from a regioselective dealkylation of the alkyl or aryl side chain (Maturano *et al.*, 1997). There is evidence that GSTs contribute to OP detoxification in humans (Fujioka and Casida, 2007).

4. STOICHIOMETRIC BIOSCAVENGERS AGAINST OPS

Administration of human plasma has been used for treating OP poisoning. The effects of fresh frozen plasma on cholinesterase levels and outcomes in patients with OP-poisoning were evaluated (Güven *et al.* 2004). Results suggest that plasma therapy may be an effective alternative or adjunctive treatment method. Plasma BChE and possibly other abundant OP scavenging proteins in plasma (albumin and PON1) may have contributed to this result.

Research on stoichiometric bioscavengers mostly focused on enzymes that specifically react with OPs; i.e. cholinesterases (Wolfe *et al.*, 1987) and carboxylesterases (Redindo and Potter, 2005; Fleming *et al.*, 2007). Prophylactic injection of enzymes capable of inactivating OP quickly would allow first responders; i.e firemen, explosive ordnance disposal technicians, and medical personnels to work safely in a contaminated environment. Intravenous or intramuscular administration of bioscavengers to chemical casualties is expected to greatly improve the efficacy of implemented pharmacological countermeasures (Ashani *et al.*, 1998; Saxena *et al.*, 2006). However, enzymatic stoichiometric neutralization of OP needs the administration of a huge amount of a costly bioscavenger, e.g., about 3 mg/kg of highly purified plasma BChE for challenging several LD_{50} of OP (Ashani and Pistinner, 2004). Large-scale production of enzymes under GMP conditions at a reasonable cost has been the subject of intense research in North America.

Two industrial GMP processes exist for mass production of human BChE. The first one is purification of the natural enzyme from the Cohn Fraction IV of human plasma. One liter of plasma provides less than one milligram of GMP BChE. This process has been developped by Baxter Healthcare Corporation in the USA (www.baxter.com). Highly purified human plasma BChE was granted the status of Investigational New Drug by the Food and Drug Administration in 2006 for protection against nerve agents in the USA (Lenz *et al.*, 2007; Saxena *et al.*, 2007). Phase I of clinical trials on volunteers will be completed in Spring 2009, so that enzyme could be marketed soon. The second process has been developped by Nexia in Canada (www.nexiabiotech.com). It uses the recombinant human enzyme produced in the milk of trangenic goats. Several grams of enzyme can

be secreted in one liter of milk. This recombinant enzyme has been named Protexia™. Since 2005, the firm Pharmatheme in Maryland, USA (www.pharmathene.com), has been developping Protexia™, PEGylated derivatives of the recombinant enzyme (Huang *et al.*, 2007) and fusion proteins (Huang *et al.*, 2008).

Secondary targets of OPs and other enzymes interacting with OPs are potential stoichiometric bioscavengers. In particular, owing to the high number of amino acid residues in albumin that covalently bind OP molecules (5 tyrosines and 2 serines) (Ding *et al.*, 2008), the reactivity of tyrosine residues can be enhanced upon specific modification, e.g., nitration that causes a decrease in the pK_a of tyrosine by several orders of magnitude (Masson *et al.*, unpublished results). Modified human albumin could lead to a new generation of stoichiometric bioscavengers. Finally, low molecular stoichiometric scavengers could be an economic alternative to enzyme-based stoichiometric scavengers. Several serine- and tyrosine-containing hexapeptides from a random library of peptides have been selected, because they react with a fluorescent analogue of sarin (Landry and Deng, 2006; Deng *et al.*, 2008).

5. CATALYTIC BIOSCAVENGERS AGAINST OPS

As said, the detoxification of OPs involves the hydrolysis of the phosphoester bond by organophosphorus acid anhydride hydrolases (OPAH), also called phosphotriesterases (PTE), or oxidation to less toxic compounds by degrading their alkyl/aryl chain. Though works on catalytic antibodies (Vayron *et al.*, 2000; Jovic *et al.* 2005; Reshetnyak *et al.*, 2007) have made some progress, the re-design and engineering of enzymes capable of degrading OPs is the most promising research field. These enzymes could be used as catalytic bioscavengers for prophylaxis and treatment of OP poisoning, for topical protection (Fisher *et al.*, 2005), and for the decontamination of skin, mucosa and open wounds (Lejeune and Russell, 1999; Gill and Ballesteros, 2000). Immobilized OPAH in bioreactors can be used for the decontamination of water (Simo *et al.*, 2008), as well as genetically engineered bacteria producing OPAH can be introduced in water effluents of decontamination units for the purification of contaminated water before recycling or washing up in the environment (Chen and Mulchandani, 1998).

5.1. REQUIREMENT FOR AN ENZYME TO BE AN EFFICIENT CATALYTIC BIOSCAVENGER AGAINST OPS

There are several general requirements for the use of enzyme-degrading OPs as medical countermeasures against OP poisoning. Enzymes must have a wide spectra of activity, and ideally, enantioselectivity for toxic stereoisomers. Their mass production under GMP conditions must be realizable at a reasonable cost. Long-term storage without activity loss must be possible under field conditions. Conformational stability can be optimized by chemical modification or the addition of stabilizers.

Other requirements depend on the way of administration, the delivery system or galenic formulation of enzymes. For parenteral administration, it must be remembered that the toxicant concentration in blood, [OP], even in the most severe cases of poisoning, is always very low, well below the K_m of the enzyme for OP substrates. So, hydrolysis of OP in blood is pseudo first-order (Masson *et al.*, 1998): $v = k_{cat}/K_m \cdot [E] \cdot [OP]$. The product of the bimolecular rate constant (k_{cat}/K_m) and enzyme active site concentration ([E]) is the pseudo first-order rate constant. Thus, the higher the catalytic efficiency (k_{cat}/K_m), the lower the dose of enzyme to be administered for cleaning the blood of toxic molecules in a very short time (t). The catalytic efficiency of enzymes can be increased by several orders of magnitude by mutagenesis or chemical engineering (Griffiths and Tawfik, 2003; Hill *et al.*, 2003). The enzyme concentration in blood needed to drop [OP] to a non-toxic concentration in time, t, is $[E] = X/(k_{cat}/K_m)$. X is the factor by which [OP] is reduced ($X = Ln[OP]_0/[OP]_t$) (Masson *et al.*, 2008). Therefore, the second constraint is to maintain the bioscavenger concentration, [E], in the blood as high as possible for a long time. [E] is controlled by the enzyme pharmacokinetics and/or the frequency of injections. Bioavailability and the biological stability of injected stoichiometric or catalytic bioscavengers are important issues. Increasing the size of the enzyme by polymerization, conjugation to albumin, reducing the microheterogeneity of glycans, and chemical modifications of solvent-exposed surfaces improve the biological life of injected bioscavengers. Fast clearance of glycoproteins is often due to glycosylation defects. These can be corrected by chemical modifications. Pharmacokinetic studies of glycoproteins injected to animals showed that the enzyme clearance depends on sialylation of glycans. Rapid elimination of asialoglycoproteins from the blood stream is due to their capture by galactose receptors located on the surface of hepatocytes. Galactose is the residue that precedes sialic acid at the terminus of complex glycans. Pharmacokinetic studies with natural and recombinant ChEs confirmed the importance of sialic acid residues ending glycans (Kronman *et al.*, 1995; Saxena *et al.*, 1998; Cohen *et al.*, 2007; Kronman *et al.*, 2007). It was found that the half-life, $t_{1/2}$, is inversely proportional to the number of unoccupied attachment sites of sialic acid (Kronman *et al.*, 2000). To increase $t_{1/2}$ of administered recombinant ChEs, all galactosyl residues have to be sialylated. Full sialylation of recombinant enzymes can be achieved by the selection of an appropriate expression system (Chitlaru *et al.*, 1998) capable of synthesizing glycans similar to natural human glycoprotein glycans and adding inhibitors of sialidase in the cell culture medium. Co-expression of human AChE and sialyltransferase in HEK 293 cells was found to produce fully sialylated recombinant enzyme (Kronman *et al.*, 2000). Alternatively, *in vitro* polysialylation of purified enzymes is possible with a sialyltransferase or by using a chemical method (Gregoriadis *et al.*, 1999). PEGylation was also proved to be an effective chemical modification for increasing the circulatory half-life of administered recombinant ChEs (Cohen *et al.*, 2006, 2007; Huang *et al.*, 2007; Kronman *et al.* 2007; Mazor *et al.*, 2008; Chilukuri *et al.*, 2008). Recently, a 150 kDa recombinant fusion protein human albumin-human BChE showed substantially improved pharmacokinetics when administered to juvenile pigs, $t_{1/2} \approx 32$h against ≈ 3h for recombinant 70%-tetrameric BChE (Huang *et al.*, 2008).

Immunotolerance of injected enzymes is a major issue. For instance, bacterial enzymes are not suitable for use in

humans, but conjugation to dextran, PEG or inclusion in nanocontainers can reduce antigenicity and slow down clearance.

Enzymes in skin and eye lotions, immobilized in foams and on tissues for skin and eye decontamination (Gordon *et al.*, 2003; Simo *et al.*, 2008), or in topical skin protectants (Braue *et al.*, 2002), act under conditions where local [OP] can be very high. In this case, enzyme reaction order in [OP] tends to zero, so that the reaction rate is close to maximum velocity, k_{cat}.[E]. Thus, under these conditions, efficiency depends on the concentration and catalytic constant of the enzyme. Therefore, for external use, enzymes have to display high molecular catalytic activity and be highly concentrated. Co-immobilization of different enzymes could be an easy way to extend the spectra of agents to be degraded. This should allow simultaneous detoxification of different agents. Indeed, exposure to multiple agents has to be considered. In particular, in asymmetric conflicts, eschatological and criminal terrorism acts, the most extreme scenario has to be anticipated.

5.2. POTENTIAL ENZYMES

Cholinesterases - OPs may be regarded as hemi-substrates of ChEs (Fig. 2). When the enzyme reacts with carboxyl-esters, it is transiently acylated, the acyl group being rapidly displaced by a water molecule. On the contrary, with phosphyl-esters, the stereochemistry of the phosphyl-enzyme intermediate restricts the accessibility of water to the phosphorus atom. Thus, the spontaneous hydrolysis of the phosphylated enzyme is very slow or even impossible. Jarv postulated that the introduction of a second nucleophile pole in the active center could activate a water molecule. This water molecule could subsequently attack the phosphorus atom on the back face, leading to breakage of the P-serine bond (Jarv, 1989). The resolution of the three-dimensional structure of *Topedo californica* AChE (Sussman *et al.*, 1991) opened the way to the rational re-design of ChEs. Thus, the possibility to convert ChEs into OP hydrolases (OPHs) was hypothesized. Human BChE was chosen because its active center is larger (500 Å^3) than that of AChE (300 Å^3) and it is less stereospecific. Molecular modeling based on the structure of *Torpedo* AChE was used for making mutants of human BChE. The second nucleophile pole was created in the oxyanion hole of the active center where a glycine residue was replaced by a histidine. This first mutant, G117H, was capable of hydrolyzing paraoxon, sarin, echothiophate and VX (Millard *et al.*, 1995; Lockridge *et al.*, 1997). However, it was irreversibly inhibited by soman, because "aging" of the conjugate was faster than dephosphonylation. The mechanism of aging, i.e. dealkylation of an akyl chain on the phosphorus atom, is almost completely elucidated (cf. Fig. 2). This reaction involves a carbocationic transient that is stabilized by active site residues E197 and W82 and water molecules (Shafferman *et al.*, 1996; Viragh *et al.*, 1997; Masson *et al.*, 1999; Nachon et al., 2005; Li *et al.* 2007). Mutation of E197 into D, Q or G brought doun the rate of aging. As expected, the double mutant G117H/E197Q was capable of hydrolyzing soman (Millard *et al.*, 1999). However, the catalytic activity of the double mutant was too slow to be of pharmacological interest.

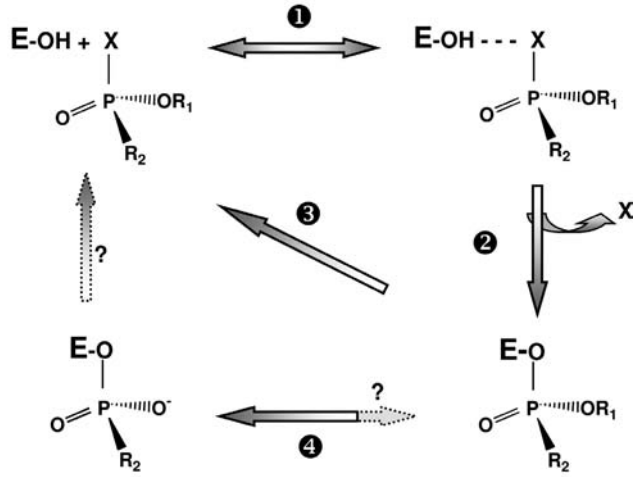

Figure 2: Inhibition of cholinesterases by OPs and reactivation of phosphylated enzymes
After formation of reversible complex between ChE and OP (step 1), the active serine (esteratic site, E-OH) is phosphylated and there is release of OP leaving group X (step 2). Phosphylated ChEs can be reactivated by nucleophilic agents, such as oximes (Pralidoxime, MMB4, Obidoxime, HI-6, etc) used as antidotes in emergency treatment of OP poisoning (Lundy *et al.*, 2006; Worek *et al.*, 2007) (reaction 3); water is a too weak nucleophile for fast spontaneous reactivation of phosphylated ChEs. Phosphyl-ChE conjugates may undergo a dealkylation ('aging') (Shafferman *et al.*, 1996; Viragh *et al.*, 1997; Masson *et al.*, 1999; Li *et al.*, 2007; Carletti *et al.*, 2008), resulting in irreversibly inactivated ("aged") enzyme (step 4). The dealkylation reaction can be very fast ($t_{1/2}$ = 3 min at 37°C for human AChE phosphonylated by soman). At the moment, drug-mediated reactivation of aged ChE is not possible.

The discovery of a fly (*Lucilia cuprina*) resistant to OPs because it carries a mutated carboxylesterase (CaE) at a position homologous to G117, i.e G137D, stimulated research on G117H-based mutants of BChE. Though the OPAH activity of the G137D mutant is low, it is balanced by the abundance of the enzyme in insect organs (Newcomb *et al.*, 1997). Mice *knockout* for AChE and carrying the G117H mutant of human BChE were found to be less sensitive to OP than wild-type mice (Wang *et al.*, 2004). Though these mice express the G117H mutant in all organs, unlike the resistance of *Lucilia cuprina*, their resistance to OP cannot be explained by the OP hydrolysis that is too slow, but rather by the hydrolysis of excess acetylcholine that accumulates in cholinergic synapses.

More than 60 double or triple mutants of human BChE with mutated G117 (Schopfer *et al.*, 2004) and mutants of human AChE and *Bungarus fasciatus* AChE were made using the same rationale (Poyot *et al.*, 2006). For a review of these works, see Masson *et al.*, (2008). None of the mutated ChEs was more active than the G117H mutant, and we provided evidence that mutations at position G117 cause dislo-

cation and loss of functionality of the oxyanion hole (Masson *et al.*, 2007).

However, computer-assisted design of new OPH mutants of ChEs is conceivable using a new approach called "intelligen" directed mutagenesis design based on the simulation of transition states. As mentioned, this approach was successfully applied for making BChE mutants that hydrolyze (-) cocaine at a high rate. Simulation of dephosphylation transition states is expected to indicate how to optimize interactions favouring productive crossing of the dephosphylation energetic barrier. Directed evolution of ChEs could be an alternative to computer-based methods. However, functional expression of ChEs is difficult in yeast (Durova *et al.*, unpublished results) and has failed in bacteria so far.

Certain ChE mutants sensitive to OPs not susceptible to aging are fully reactivatable by oximes. Thus, ChE mutants associated with oxime reactivators act as pseudo-catalysts in displacing the OP moiety bound to the enzyme. These enzyme-reactivator-coupled systems could lead to a new family of pseudo-catalytic bioscavengers (Taylor *et al.*, 2007; Kovarik *et al.*, 2007,2008; Mazor *et al.*, 2008).

Phosphotriesterases - Enzymes that catalyse the hydrolysis of phosphoester bonds in OPs are ubiquitous, e.g. a prolidase named organophosphorus acid anhydrolase (OPAA) was identified in a strain of *Alteromonas* (Cheng *et al.*, 1999), a diisopropylfluorophosphatase (DFPase) is abundant in the squid *Loligo vulgaris*, and paraoxonase-1 (PON1) is present in human plasma. Bacterial enzymes called phosphotriesterases (PTE; EC 3.1.8.1), or sometimes organophosphorus hydrolases (OPH), organo-phosphate-degrading enzymes (OpdA), show preference for OP compounds with P-O or P-S bonds. PTE are members of the amidohydrolases superfamily (Seibert and Raushel 2005).

Bacterial phosphotriesterases - Phosphotriesterases (PTE; EC 3.1.8.1) are encoded by the organophosphate degradation (*opd*) gene found in *Pseudomonas diminuta*, *Flavobacterium sp.*, and *Agrobacterium radiobacter*. Genes similar to *opd* were also found in Archaea (Merone *et al.*, 2005). *Pseudomonas diminuta* PTE is a 72 kDa dimeric bimetallic enzyme with Zn^{2+} involved in catalysis (Carletti *et al.*, in press). Substitution of Zn^{2+} ions in the active site with Mn, Co, Ni, or Cd ions results in the almost full retention of catalytic activity. Following the first determination of the three-dimensional structure of *P. diminuta* PTE (Benning *et al.*, 1994), a series of crystal structures, kinetic, and spectroscopic experiments were described. PTE catalysis proceeds via a SN_2 mechanism with the formation of a pentacoordinated transition state. The mechanism of the nucleophilic attack and enzyme regeneration are largely debated, and the functional roles of divalent metal cations and amino acids in the active centre are not yet fully understood (Aubert *et al.*, 2004; Samples *et al.*, 2007; Chen *et al.*, 2007; Wong and Gao 2007; Jackson *et al.*, 2008). Recently, the structure of *Sso*Pox (a hyperthermopholic PTE from the archeon *Sulfolobus solfataricus*) provided new information that led to a refined mechanism (Elias *et al.* 2008; Del Vecchio *et al.*, 2009). No natural substrate of PTE has yet been identified (Ghanem and Raushel, 2005) and PTE is thought to have evolved from lactonase (Afriat *et al.*, 2006), the PTE activity being considered as a promiscuous activity (Elias *et al.*, 2008). Whereas the catalytic efficiency of PTE for paraoxon, the best substrate identified so far, is approaching the diffusion-controlled limit, it is slow against OP nerve agents (Table 1). Meanwhile, directed evolution of PTE showed that only 3 amino acid changes dramatically enhanced the catalytic efficiency for an analogue of soman by ~3 orders of magnitude (Hill *et al.*, 2003).

Numerous studies have highlighted the potential of PTE for decontamination, skin protection, and biosensor detection of OP (Lejeune and Russell, 1999; Gill and Ballesteros, 2000; Létant *et al.*, 2005; Ghanem and Raushel, 2005; Karnati *et al.*, 2007). Administration of PTE before or after OP exposure was shown to improve pre-treatment and current treatment of OP poisoning (Doctor and Saxena, 2005). OpdA was shown to improve survival after poisoning by highly toxic OP pesticides (Bird *et al.*, 2008). To prevent abnormally fast pharmacokinetics and/or an immunological response of injected bacterial enzymes, PTE can be PEGylated (Jun *et al.*, 2007, 2008) or encapsulated. First attempts at using PTE encapsulated within sterically stabilized liposomes were promising, providing protection to rats from multiple LD_{50} of OP pesticides (Petrikovics *et al.*, 2004). Alternatively, blood detoxification could be achieved by extracorporeal circulation through a hollow fiber cartridge coated with immobilized PTE (Masson *et al.*, unpublished results).

PTEs possibly could also be used for skin protection as active components of topical skin protectants (TSPs) or covalently coupled to the cornified layer of epidermis (Parsa and Green, 2001). Thermostable PTEs from thermophilic bacteria (Merone *et al.*, 2005; Elias *et al.*, 2008; Del Vecchio *et al.*, 2009) or mutated/evolved highly stable enzymes from mesophilic bacteria are promising for topical protection and decontamination. PTE was also entrapped in additives for latex coating of biodefensive surfaces. Such PTE-based additives for paints and coatings were shown to retain catalytic parameters and the stability of the enzyme (McDaniel *et al.*, 2006). For the environment's decontamination and remediation, an alternative approach, phytodegradation by transgenic plants (e.g. tobacco) expressing a bacterial PTE, has been considered as a potentially low-cost, safe, and effective method (Wang *et al.*, 2008).

Human paraoxonase-1- PON1 is a 45 kDa glycosylated calcium-dependent enzyme expressed mainly in the liver and exclusively bound to high-density lipoproteins (HDL), in association with other apolipoproteins (Fig. 3). PON1 shows a genetic polymorphism; the most prominent determines the Q192R allozyme that has a substantial impact on PON1 activity with arylesters and OPs (Smolen *et al.*, 1991) (Table 1). Chemical modification and site-directed mutagenesis studies have identified essential amino acid residues for the activities of PON1 (Josse *et al.*, 1999; Yeung *et al.*, 2004; Khersonsky and Tawfik 2006; Amitai *et al.*, 2007; Tavori *et al.*, 2008; Hu *et al.*, 2009).

Attempts at solving the three-dimensional structure of human PON1 have failed so far. However, molecular modeling (Fokine *et al.*, 2003; Yeung *et al.*, 2004) and the crystal structure of a hybrid mammalian recombinant PON1 variant obtained by directed evolution (rPON1) (Harel *et al.*, 2004) showed that PON1 is a six-bladed β-propeller protein similar to *Loligo vulgaris* DFPase (Katsemi *et al.*,

Table 1. Catalytic efficiency (k_{cat}/K_m, M^{-1} min^{-1}) of different natural and engineered OP hydrolases towards different OPs

Source of enzyme	Paraoxon	DFP	Tabun	Sarin	Soman	GF	Echothiophate	VX
Human PON1 Q192	6.8×10^5 [a]	4×10^4 [b]		9.1×10^5 [c]	2.8×10^6 [c]			+ [d]
Human PON1 R192	2.4×10^6 [a]			7×10^4 [c]	2.1×10^6 [c]			+ [d]
Human rPON1 in 293T					6.2×10^5- 4.1×10^6 [e]			
Mammalian rPON1 G3C9	7.2×10^5 [f]							
Mammalian rPON1 V346A					8.7×10^4 [g]	3.6×10^5[g]		
Human BChE G117H	5.7×10^3 [h]	5.2×10^3 [h]		1.6×10^2 [i]	-		1×10^4 [h]	1.5×10^3 [i]
Blowfly CaE G117D	2×10^5 [j]							
B. fasciatus AChE HQT	64 [h]	7.6×10^2 [h]					24 [h]	
*Loligo vulg*aris DFPase		7.8×10^7 [k]		2.4×10^6 [k]	2.4×10^6[k]			0 [k]
P. diminuta OPAH	2×10^9 [l]	5.8×10^8 [m]		4.8×10^6 [n]	6×10^5 [n]	5×10^3 [o]		4×10^4 [p]
Alteromonas sp. JD6.5 OPAA		4.6×10^7[q]			14.6 [r]			
Alteromonas sp. JD6.5 cloned				5.8×10^6 [r]	1×10^7 [r]	6.2×10^7 [r]		
Alteromonas undina			21.8 [s]	30.4 [s]	1.6×10^2 [s]	1.3×10^2 [s]		
NG108-15 hybrid cells					2.5×10^3 [t]			

[a] Smollen *et al.*, 1991; [b] Masson *et al.*, 1998; [c] Davis *et al.*, 1996; [d] C.A. Broomfield, unpublished result; [e] Yeung *et al.*, 2008, with the four soman stereoisomers; [f] Harel *et al.*, 2004; [g] Amitai *et al.*, 2006; [h] Poyot *et al.*, 2006; [i] Lockridge *et al.*, 1997; [j] Newcomb *et al.*, 1997; [k] Hartlieb and Ruterjans 2001; [l] Kuo *et al.*, 1997; [m] Lai *et al.*, 1995; [n] Dumas *et al.*, 1990; [o] Hoskin *et al.*, 1995; [p] Rastogi *et al.*, 1997; [q] Cheng *et al.*, 1999; [r] Cheng *et al.*, 1994; [s] DeFranck *et al.*, 1993; [t] Ray *et al.*, 1988.

2005). The catalytic mechanism of DFPase was recently described (Blum *et al.*, 2006). Confirmation of a similar mechanism for human PON1 activity would considerably help to design more active PON1 mutants. The active site lid of PON1 is in close contact proximity to the area presumed to mediate binding of PON1 to HDL. It has been suggested that the hydrophobic N-terminus of PON1 mediates its anchoring to HDL, but the precise mode of binding of PON1 to HDL, as well as of other HDL-associated proteins, is still unknown.

As a naturally occurring plasma enzyme, human PON1 is the most promising catalytic bioscavenger candidate for pretreatment and therapy of OP poisoning (La Du, 1996; Rochu *et al.*, 2007a). Thus, PON1 is the focus of intensive research to improve its efficacy and functionalization. To provide a valuable medical countermeasure against intoxication by nerve agents, the catalytic efficiency of PON1 has to be enhanced by only 1 or 2 orders of magnitude. Mutants of rPON1 obtained by directed evolution and exhibiting enhanced OP-hydrolase activity (Amitai *et al.*, 2006) suggest this goal can be reasonably achieved soon. However, the instability of these mutants could impinge on their biotechnological development. Plasma PON1 works in a complex and dynamic milieu, HDL particles that include durably or transiently up to 90 associated proteins (Vaisar *et al.*, 2007). Thereby, PON1 requires association to apolipoprotein partners to retain its stable active conformation (James and Deakin, 2004; Gaidukov and Tawfik, 2005).

Human Phosphate Binding Protein (HPBP), an apolipoprotein that binds inorganic phosphate in blood was discovered recently. Its three-dimensional structure and complete amino acid sequence were solved (Morales *et al.*, 2006; Diemer *et al.*, 2008). The conditions to separate HPBP and PON1 *in vitro* indicate that HPBP is tightly associated with PON1 (Renault *et al.*, 2006). Moreover, stabilization of the active form(s) of human PON1 by HPBP shows that HPBP acts as a functional chaperone (Rochu *et al.*, 2007b,c; Cléry-Barraud *et al.*, 2009).

We are attempting at co-crystallization of the PON1-HPBP complex. For this, the first step was to construct a hybrid gene for co-expression of HPBP and PON1 in *E. coli*. For this purpose, a HPBP gene was synthesized from its amino acid sequence. Co-expression, aimed to favour correct folding of active PON1 and stabilization of the active functional conformation of the enzyme, is expected to provide a crystallizable PON1-HPBP complex. Finally, diffractable crystals of the complex are assumed to provide a three-dimensional structure of natural human PON1. This crucial phase will be the first step of the staircase, leading to the development of stable human PON1 mutants with enhanced catalytic efficiency against OPs. The fact that functional expression of human PON1 seems to be feasible (Stevens *et al.* 2008) would simplify the biotechnology of PON1 and open new perspectives.

Other enzymes - Other hydrolases are involved in biodegradation of OPs, such as organophosphorus acid anhydrolases (OPAA; EC 3.1.8.2) and organophosphate-degrading agents (OpdA) or prolidases (EC 3.4.13.9). Prolidases were first isolated from halophilic bacteria (*Alteromonas haloplanktis* and *A. sp.* JD6.5). Prolidase from *A. sp.* JD6.5 is an OPAA that displays the highest known activity against soman (k_{cat} = 3100 s^{-1}), but it is inactive against VX (Cheng *et al.* 1999). OpdA was incorporated as the active ingredient of Landguard™ OP-A, a formulation developped for the treatment of water run-off, equipment rinsing and soil decontamination (Dawson *et al.*, 2008). Whilst these applications are focussed on agricultural and remediation markets, modifications required for nerve agent detoxification will be relatively minor, as the enzyme can be applied in powder, liquid or matrix-bound forms.

A prolidase was also isolated from human liver. This prolidase displays a high catalytic activity against soman and exhibits sequence homology with the *Aleromonas haloplanktis* prolidase (Wang *et al.*, 1998). Human prolidase has been cloned and expressed in *Sacharromyces cerevisiae* and *Pichia pastoris*, and it appears to be one of the most interesting OP-degrading enzyme for protection against nerve agents (Wang *et al.*, 2005; Wang *et al.*, 2006). The newly discovered human cytosolic aminopeptidase (AMPP; EC 3.4.11.9) could also be a valuable catalytic bioscavenger against organophosphates and organophosphonates (Hsu *et al.*, 2008).

Laccases (EC 1.10.3.2) are fungal phenol oxidoreductases that have been used for detoxification of numerous xenobiotics (Richardt and Blum, 2008). The laccases from *Pleurotus ostreatus* and *Chaetomium thermophilium* were found to rapidly degrade VX and VR in the presence of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate (ABTS) as the mediator (Amitai *et al.*, 1998). We found that laccases from *Trametes versicolor* and *Coriolopsis polyzona* with ABTS display similar properties against V agents (Trovaslet *et al.*, unpublished results). The heme-containing chloroperoxidase (EC 1.11.1.X) from *Caldaromyces fumago*, with peroxide as co-substrate, is another efficient VX-degrading enzyme (Amitai *et al.*, 2003).

These enzymes are of interest for the destruction of chemical weapons stockpiles, soil remediation, the decontamination of materials, protective equipments, and water polluted by pesticides and nerve agents (Russel *et al.*, 2003). In particular, phosphorothiolates such as VX are relatively resistant to PTE. Thus, oxidative cleavage of the P-S bond could be achieved by oxidases like laccases. These enzymes could be used associated with other OP-degrading enzymes for skin decontamination and topical skin protection. Though no work has been performed on the combined action of oxidases and hydrolases, oxidation of P-bonded alkyl/aryl chains by oxidases is expected to alter enantioselectivity of PTE for parent OPs, and therefore to improve the efficiency of catalytic bioscavengers.

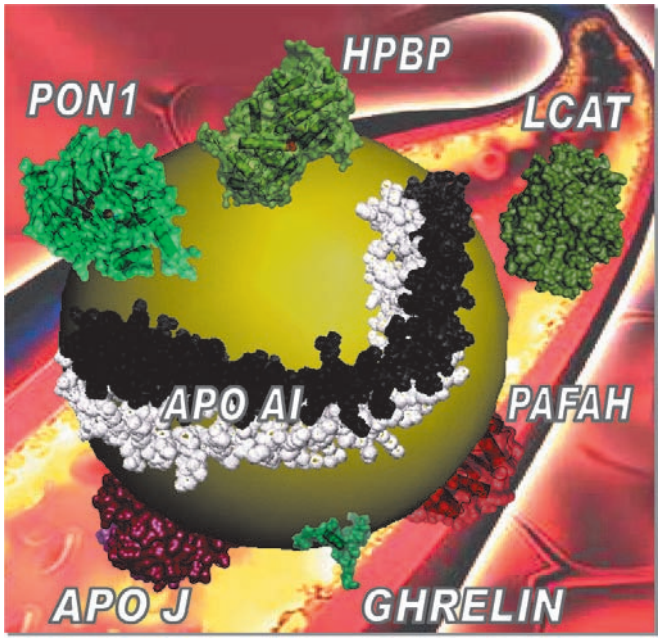

Figure 3: Scaled schematic representation for a PON1-containing HDL particle
HDL is ~ 10 nm-diameter sphere with a non-polar core of cholesteryl-esters and triglycerides encapsulated in a monolayer of amphipathic α-helical apolipoproteins and phospholipids. Among multiple HDL-associated proteins involved in lipid metabolism, complement regulation, acute-phase response and proteinase inhibition (Vaisar *et al.*, 2007), some of them, durably or transiently associated to PON1-containing HDL and described as having a propensity to contaminate purified PON1 fractions, are shown.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Enzymes that degrade OPs have been isolated from humans, different animal species, fungi and bacteria. Identification and isolation of new natural enzymes, in particular in insects resistant to OP pesticides and among secondary targets of OPs in humans is an active field of research. Also, potential extremozymes have been discovered in halophilic, thermophilic and piezophilic bacteria and other extreme environments (Merone *et al.*, 2005; Feerer *et al.*, 2007). All these enzymes can be purified from their natural sources. However, production of natural enzymes free of infectious agents under GMP is expensive. Thus, suitable biopharmaceuticals are mostly recombinant enzymes. These enzymes can be produced in prokaryotic expression systems (*E. coli*), eukaryotic expression systems (yeast, insect, mammalian cell cultures), transgenic animals (worm, rabbit, goat), transgenic plants (tomato, potato, tobacco), and also acellular biosynthetic systems. Current research's goal in protein engineering is to improve mass production of stable enzymes at low cost.

Improvement of *in vitro* and *in vivo* catalytic properties of cocaine hydrolases and OPAH-degrading nerve agents and pesticides is the main issue. Improvement of thermodynamic stability for long-term storage in solution or in lyophilized forms, and *in vivo* operational stability, improvement of immuno-tolerance and bioavailability are other major goals. Molecular modeling and transition state simulations, site-directed mutagenesis and directed-evolution approaches in combination with chemical modifications and medium manipulations have been successfully used to improve the properties of selected enzymes (Bershtein and Tawfik, 2008). Lastly, pharmacokinetic, toxicokinetic and immunological studies on animal models, and then on volunteers will validate enzymes of interest. Multiple enzymes will be associated in active topical skin protectants and decontamination tools. Soon, catalytic bioscavengers are going to take a place among medical countermeasures for prophylaxis and treatment of acute OP poisoning and treatment of cocaine overdose.

In the future, gene therapy could be considered to challenge OPs. This strategy will offer the possibility of transitory production of humanized OP-degrading enzymes in the body. Promising results have already been obtained with human AChE and BChE (Li *et al.*, 2006; Chilukuri *et al.* 2008) and human PON1 (Conwan *et al.*, 2001; Fu *et al.*, 2005; Bradshaw *et al.*, 2005; Miyoshi *et al.*, 2007; Guns *et al.*, 2007; Zhang *et al.* 2008). Again, the use of mutated PON1 genes encoding for an enzyme with high OPH activity against pesticides and nerve agents appears to be the most promising approach. Several studies, using different gene-delivery vectors in mice, showed that the level of PON1 in plasma was increased. High PON1 levels slowed and even prevented the entry of OP into the brain and reduced atherosclerosis signs (Conwan *et al.*, 2001; Fu *et al.*, 2005; Bradshaw *et al.*, 2005; Guns *et al.*, 2008). Local delivery of the PON1 gene using the Sendai virus vector inhibited neonatal hyperplasia after arterial balloon-injury in rabbits fed a high-fat diet (Miyoshi *et al.*, 2007). Transfer of the human PON1 Q gene in mice led to efficient expression of the enzyme that was capable of protecting the liver against oxidative stress (Zhang *et al.* 2008). Thus, enhanced expression of PON1 by gene therapy could be beneficial to the different functions of the enzyme. Meanwhile, the multiple and defectively identified PON1 activities make it apparent that strategy for repetitive administration of high concentration of PON1 in humans must be undertaken with caution. ●

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Life and Death Decisions in the CD95 System: Main Pro- and Anti-Apoptotic Modulators

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ABSTRACT Apoptosis is common to all multicellular organisms. Apoptosis can be triggered by the extrinsic (death receptor (DR)) or the intrinsic (mitochondrial) death pathways. CD95 (APO-1/Fas) is a prototypic member of the DR family. This review is focused on the mechanisms of CD95 (APO-1/Fas)-mediated apoptosis and the role in the apoptosis of the death effector domain (DED)-containing proteins: pro-apoptotic protein procaspase-8 and anti-apoptotic protein c-FLIP. Gaining insights into these processes will improve our understanding of the pathogenesis of diseases such as cancer, autoimmunity and AIDS, and will open new approaches to rational treatment strategies.

INTRODUCTION: CD95 AND CD95 SIGNALING

CD95 (also called APO-1; Fas; fas antigen; tumor necrosis factor receptor superfamily member 6, TNFRSF6 or apoptosis antigen 1, APT1) is a member of the death receptor (DR) family, a subfamily of the tumor necrosis factor receptor superfamily (1). All members of the DR family are characterized by a cytoplasmic region termed the Death Domain (DD) (2;3). DD are 80-100 amino acid long motifs involved in the transduction of the apoptotic signal. The DD belongs to the so-called 'death domain-fold superfamily'. This superfamily comprises the death domain (DD), the death effector domain (DED), and the caspase recruitment domain (CARD). Each of these motifs interacts with other proteins through homotypic interactions. All members of the DD-fold superfamily are characterized by similar structures that comprise six or seven antiparallel amphipatic α-helices.

Crosslinking of CD95 with its natural ligand, CD95L (CD178) (4), or with agonistic antibodies, such as anti-APO-1 (5), induces apoptosis in sensitive cells. The binding of CD95L or agonistic antibodies to CD95 leads to the formation of the receptor complex at the cellular membrane, which was named death-inducing signaling complex (DISC) (6). The DISC consists of oligomerized receptors, the DD-containing adaptor molecule FADD/MORT1 (Fas-Associated Death Domain), procaspase-8 (FLICE, MACHα, Mch5), procaspase-10, and the cellular FLICE-inhibitory proteins (c-FLIP) (Fig. 1) (7-9). The interactions between the molecules at the DISC are based on homotypic contacts. The DD of the receptor interacts with the DD of FADD, while the DED of FADD interacts with the N-terminal tandem DEDs of procaspases-8, -10 and c-FLIP. As a result of DISC formation procaspase-8 is activated at the DISC resulting in the formation of the active caspase-8. Caspase-8 cleaves and thereby activates downstream effector caspases-3, -6, and -7. This is followed by the cleavage of caspase substrates, which comprise a number of cellular proteins playing a central role for the normal functioning of the cell leading to demolition of the cell.

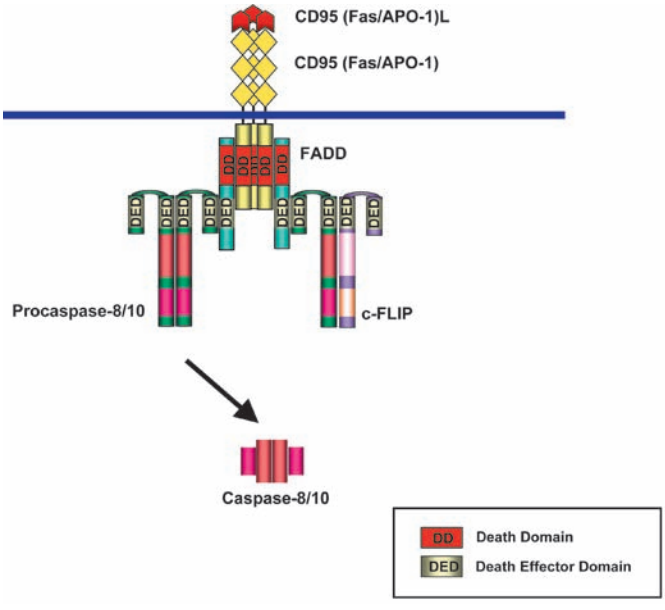

Figure 1. The CD95 death-inducing signaling complex (DISC). The DISC consists of CD95, (depicted in yellow), FAS-associated death domain, FADD, (depicted in light blue), procaspase-8/procaspase-10, (depicted in green), and cellular FLICE-inhibitory proteins, c-FLIP, (depicted in violet). DD are shown in red; DED are shown in light yellow. The interactions between the molecules at the DISC are based on homotypic contacts. The death domain (DD) of CD95 interacts with the DD of FADD while the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspase-8, procaspase-10 and c-FLIP.

DED proteins at the DISC play a central role in the regulation of DR-induced apoptosis. Recruitment of procaspase-8 to the DISC, followed by its activation at the DISC and formation of active caspase-8 heterotetramers, triggers the apoptotic pathway. Recruitment of c-FLIP proteins to the DISC has the opposite effect: c-FLIP proteins block procaspase-8 activation at the DISC and thereby apoptosis induction. Therefore, life/death decisions at the DISC are defined by the balance of two DED proteins: procaspase-8 and c-FLIP. In this review we describe in detail the DED-proteins procaspase-8 and c-FLIP and the mechanism of their pro- and anti-apoptotic action.

PROAPOPTOTIC DED PROTEINS OF THE DISC: PROCASPASE-8 AS A MEMBER OF THE CASPASE FAMILY

Procaspase-8 (FLICE, MACHα, Mch5) belongs to the family of caspases (7;10). Caspases, a family of cysteinyl aspartate specific proteases, are synthesized as zymogens with a prodomain of variable length followed by a large subunit (p20) and a small subunit (p10). Caspases are activated through proteolysis at specific aspartate (D) residues that are located between the prodomain, the p20, and p10 subunits (Fig. 2) (11). This results in the generation of mature active caspases that consist of heterotetramers $p20_2$-$p10_2$. Subsequently, active caspases specifically process various substrates that are involved in apoptosis and inflammation. Depending on their function and the structure of the prodomain, caspases are divided into initiator caspases and effector caspases and are typically divided into three major groups (Fig. 3) (11). The caspases with large prodomains are referred to as inflammatory caspases (group I) and initiator of apoptosis caspases (group II), while caspases with a short prodomain of 20-30 amino acids are named effector caspases (group III).

All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic processing and activation during apoptosis (12). The effector caspases are activated by initiator caspases. In turn, initiator caspase activation takes place in large protein complexes bringing together several caspase zymogens. All initiator caspases are characterized by the presence of a member of the 'death domain-fold superfamily' (DED or CARD), which enables their recruitment into their initiation complexes. Procaspases-8 and -10 possess two tandem DEDs in their prodomain (Fig. 3). The CARD is found in procaspases-1, -2, -4, -5, -9, -11, and −12 (Fig. 3). DEDs and CARDs are responsible for the recruitment of initiator caspases into death- or inflammation-inducing signaling complexes resulting in proteolytic autoactivation of caspases that subsequently initiates inflammation or apoptosis.

Procaspase-8 is activated at the DISC (13). Two isoforms of procaspase-8 (procaspase-8a and procaspase-8b) were reported to be bound to the DISC (14). Both isoforms possess two tandem DED domains, as well as the catalytic subunits p18 and p10 (Fig. 4). Procaspase-8a contains an additional 2 kDa (15 aa) fragment, which results from the translation of exon 9. This small fragment is located between the second DED and the large catalytic subunit resulting in the different length of procaspase-8a (p55) and, consequently, procaspase-8b (p53).

The activation of procaspase-8 is believed to follow an 'induced proximity' model in which high local concentrations and favourable mutual orientation of procaspase-8 molecules at the DISC lead to their autoproteolytic activation (15). There is strong evidence from several *in vitro* studies that autoproteolytic activation of procaspase-8 occurs after

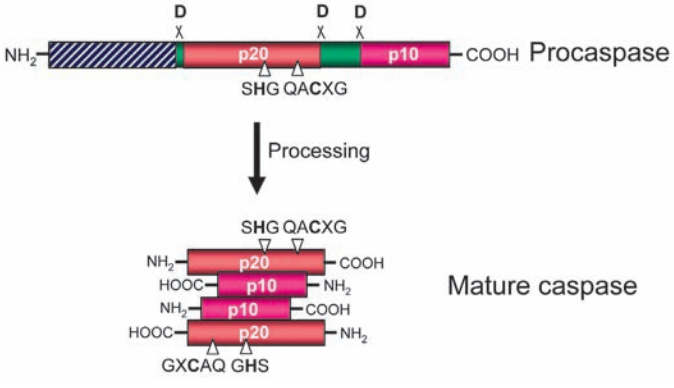

Figure 2. Scheme of procaspase activation. Cleavage of the procaspase at the specific Asp-X bonds leads to the formation of the mature caspase that comprises the heterotetramer $p20_2$-$p10_2$ and the release of the prodomain. The residues involved in the formation of the active center are shown.

oligomerization at the receptor complex (16). Furthermore, dimerization of two procaspase-8 molecules at the DISC has been shown to be necessary for procaspase-8 activation (17). Procaspase-8a/b at the DISC undergoes autocatalytic cleavage, for which a two-step mechanism has been described (Fig. 4) (14;18). The initial cleavage at Asp^{374} generates the two subunits p43/p41 and p12. In a second step, cleavage takes place at Asp^{216} and Asp^{384}, producing the active enzyme subunits p18, p10 and the prodomains p26/p24. As a result of procaspase-8 processing, the caspase-8 heterotetramer $(p18/p10)_2$ starts the apoptotic signaling cascade (19).

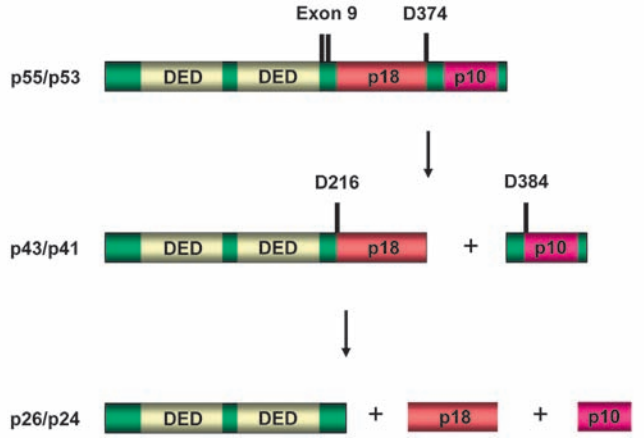

Figure 4. Scheme of procaspase-8 and the two-step mechanism of procaspase-8 activation. Procaspase-8a/b (p55/p53) is shown in green; DEDs are presented in light yellow. The N-terminal cleavage products: p43/p41 and prodomains p26/p24 as well as caspase-active domains: p18 and p10 are indicated. Two cleavage steps with the resulting products are presented.

ANTI-APOPTOTIC DED-PROTEIN OF THE DISC: CELLULAR FLICE-INHIBITORY PROTEINS (C-FLIP)

c-FLIP, also known as FLAME-1/I-FLICE/CASPER/CASH/MRIT/CLARP/Usurpin, is a well-described inhibitor of DR-mediated apoptosis. The current view on c-FLIP proteins is shown in figure 5. Five c-FLIP proteins have been characterized so far: three c-FLIP isoforms and two cleavage products (9;20-24). The three c-FLIP isoforms comprise: Long (L), Short (S), and Raji (R), *e.g.* $c\text{-}FLIP_L$, $c\text{-}FLIP_S$, and $c\text{-}FLIP_R$, respectively (Fig. 5). All three isoforms possess two DED domains and thereby bind to the DISC. In this way, the short FLIP isoforms, $c\text{-}FLIP_S$, and $c\text{-}FLIP_R$ block procaspase-8 activation and apoptosis. The role of the long c-FLIP isoform, $c\text{-}FLIP_L$, at the DISC is controversial. It has been shown that depending upon its concentration at the DISC it can act either as an anti-apoptotic molecule, functioning in a way analogous to $c\text{-}FLIP_S$, or as a pro-apoptotic molecule, facilitating the activation of procaspase-8 at the DISC (25;26). This pro-apoptotic role is in agreement with the phenotype of c-FLIP-deficient mice, which are characterized by heart failure and death at embryonic day 11 (27).

In addition, two cleavage products of c-FLIP have been reported until now: p43-FLIP and p22-FLIP (9;24). P43-FLIP was shown to be generated from $c\text{-}FLIP_L$ at the CD95 DISC as a result of procaspase-8 cleavage at D376. P22-FLIP was shown to be the N-terminal cleavage product of c-FLIP resulting from procaspase-8 cleavage at D196. In contrast to p43-FLIP, p22-FLIP is formed in the cytosol independently of DR-stimulation. In addition, p22-FLIP turned out to be a prominent inducer of NF-κB activity by binding to the IKK complex.

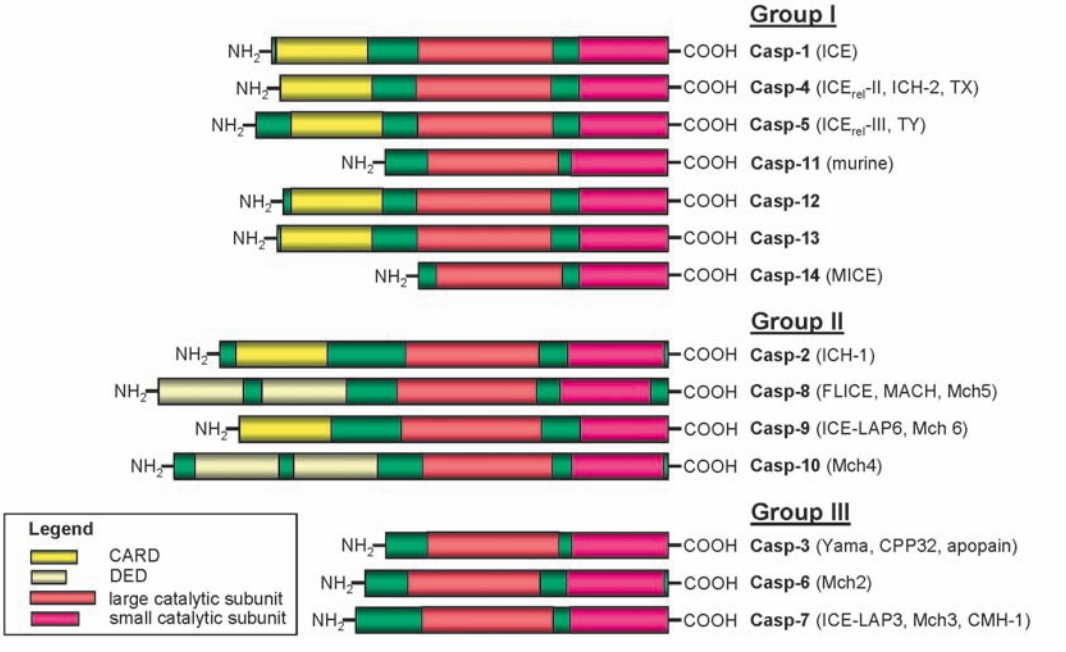

Figure 3. The caspase family. Three major groups of caspases are presented. Group I: inflammatory caspases. Group II: apoptosis initiator caspases. Group III: apoptosis effector caspases. The caspase activation recruitment domain (CARD), the death effector domain (DED), the large (p20), and the small (p10) catalytic subunits are indicated.

REGULATION OF LIFE AND DEATH BY DED PROTEINS

DED proteins procaspase-8 and c-FLIP play a central role in the regulation of DR-induced apoptosis and might also induce the NF-κB pathway (6). Regulation of DR-induced apoptosis by procaspase-8 and c-FLIP occurs at the DISC. (Fig. 6. left side). Procaspase-8 is activated at the DISC inducing the apoptotic process, while this activation can be inhibited by all reported c-FLIP proteins. The only exception is the c-FLIP$_L$ isoform, which might induce procaspase-8 activation when expressed at low concentrations and block procaspase-8 activation when expressed at high concentrations. Therefore, procaspase-8 at the DISC has a pro-apoptotic role and c-FLIP proteins, except for the c-FLIP$_L$ isoform, possess an anti-apoptotic function.

Interestingly, in the cytosol, interactions between procaspase-8 and c-FLIP have been reported to induce the NF-κB pathway rather than apoptotic pathways. Recently, a new NF-κB-activating pathway initiated by procaspase-8 has been described. It has been shown that, independently of DR stimulation, non-apoptotic procaspase-8 activity generates the p22-FLIP cleavage product that leads to the induction of NF-κB (Fig. 6, right side). The role of procaspase-8 in this pathway is different from its pro-apoptotic activity at the DISC. Procaspase-8 does not undergo processing leading to apoptosis induction with active heterotetramer formation but rather utilizes its so-called proform activity processing c-FLIP to the p22-FLIP cleavage product. It is likely that

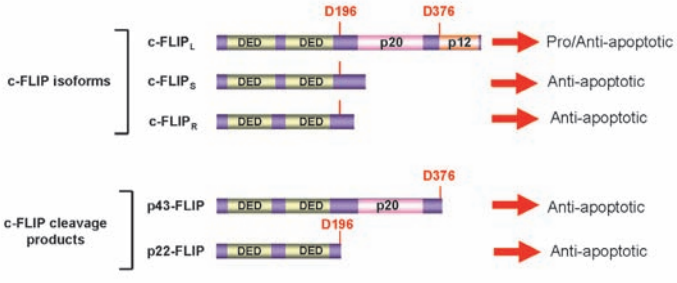

Figure 5. The scheme of c-FLIP proteins. c-FLIP isoforms and c-FLIP cleavage products are shown. DED (Death effector domains) and caspase-like domains (p20 an p12) are indicated. D376 and D196, leading to the generation of p43-FLIP and p22-FLIP, respectively, are presented in red.

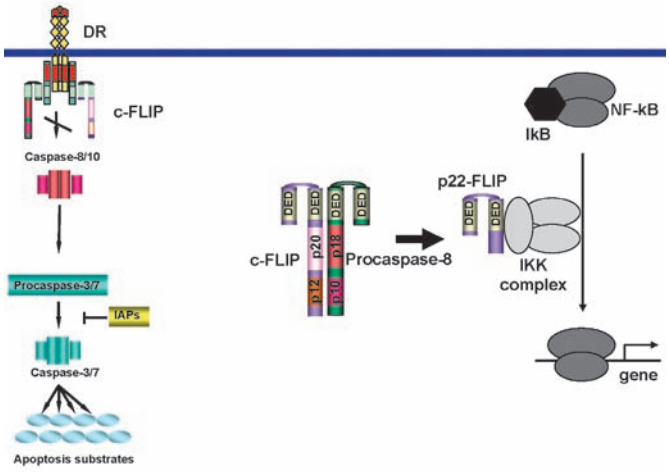

Figure 6. DED proteins: procaspase-8 and c-FLIP at the DISC and in the cytosol. c-FLIP proteins block procaspase-8 activation at the DISC (left side). Procaspase-8 and c-FLIP form dimers in the cytosol leading to generation of p22-FLIP. P22-FLIP binds to IKK complex *via* IKKγ, which leads to the induction of NF-κB (right side).

procaspase-8 constitutively forms heterodimers with c-FLIP cleaving c-FLIP to p22-FLIP. Thus, the ratio between procaspase-8 and c-FLIP in cells would be the crucial factor defining the amount of generated p22-FLIP and, correspondingly, the potential to induce NF-κB.

The balance between DED-containing proteins may provide sensitive signaling check points that cells use for signaling cross-talk and switching between apoptosis-resistant and sensitive phenotypes and, thus, between life and death. Furthermore, the balance between DED-proteins also depends on the subcellular localization. The regulation by c-FLIP and procaspase-8 of life/death decisions at the DISC is different from the cytosolic events. At the DISC, c-FLIP mostly acts as a devoted procaspase-8 inhibitor, while in the cytosol it uses procaspase-8 activity to initiate cleavage to p22-FLIP and the subsequent NF-κB induction (Fig. 6). Consequently, procaspase-8 initiates apoptosis at the DISC and NF-κB in the cytosol. The crosstalk between the DED proteins in the cytosol and at the DISC will be a topic of future studies. ●

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Analysis of Myelin Basic Protein Fragmentation by Proteasome

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ABSTRACT The proteasome is a high molecular protein complex whose purpose is specific protein degradation in eukaryotic cells. One of the proteasome functions is to produce peptides, which will then be presented on the outer cell membrane using main histocompatibility complex (MHC) molecules of the first or second class. There are definite reasons to believe that proteasome directly takes part in the specific degradation of myelin basic protein (MBP), which make up to 30% of all proteins in the myelin sheath of neuronal axons. The details of the proteasomal degradation of MBP are still unclear. In this work, the features of specific MBP degradation by proteasome were studied.

It was demonstrated that MBP (non-ubiquitinated) is a good substrate for 20S and for the 26S proteasome. This is the first work on detecting the sites of MBP proteolysis by proteasome from brains of SJL/J/J and Balb/C mice's lines. Substantial differences in the degradation pattern of this neuroantigen were found, which could indicate the better presentation MBP parts on MHC molecules in the case of mice predisposed to the development of experimental autoimmune encephalomyelitis.

INTRODUCTION

Multiple sclerosis (MS) – a chronic neurodegenerative disease of autoimmune nature – is an outstanding medical–social problem, because it affects mainly the young and middle-aged. The problem of MS treatment still has no satisfactory solution, and to this day there are several medicines (therapies) able to suppress MS to some extent, but not to fully cure it. Neuronal degradation occurs in the brain of MS patients due to the destruction of the neuron's myelin sheath. One biochemical characteristic which differentiates myelin from other biological membranes is the high lipid/protein ratio. Proteins comprise 25–30% of the mass of the myelin sheath dry matter. About 30% of all myelin proteins are three isoforms of the myelin basic protein (MBP). MBP is one of the main autoantigen in MS. Earlier, we and other authors showed that catalytic antibodies [2–5] and some proteases [6–9] may be involved in MBP degradation. It is known that every eukaryotic cell contains a special compartment for targeted protein degradation (proteasome), which is a high molecular protease complex. One of the proteasome's functions is to produce peptides, which will then be presented on the cell membrane using main histocompatibility complex (MHC) molecules of the first or second class [10]. There are definite reasons to assume that proteasome directly takes part in specific MBP degradation. The details of this process are still unclear. In this work, the specific features of MBP degradation by proteasome were studied.

It is well-known that the 20S proteasome (a multicatalytic proteinase complex) is an oligomeric high-molecular-weight (700 kDa) proteinase that can be isolated separately. This complex is the catalytic core of the larger 26S proteasome, which also contains one or two regulatory 19S subunits. It was shown that both 20S and 26S proteasomes are able to degrade proteins, including the MBP [11, 12]. The question of the site-specificity of MBP degradation by the proteasome remained open. It is also known that, during many inflammatory pathological processes, the standard protease complex (constitutive proteasome) transforms into a form of immunoproteasome, which has an alternative specificity and catalytic efficiency with respect to intracellular proteins processing. It is very likely that this 'switching' is closely re-

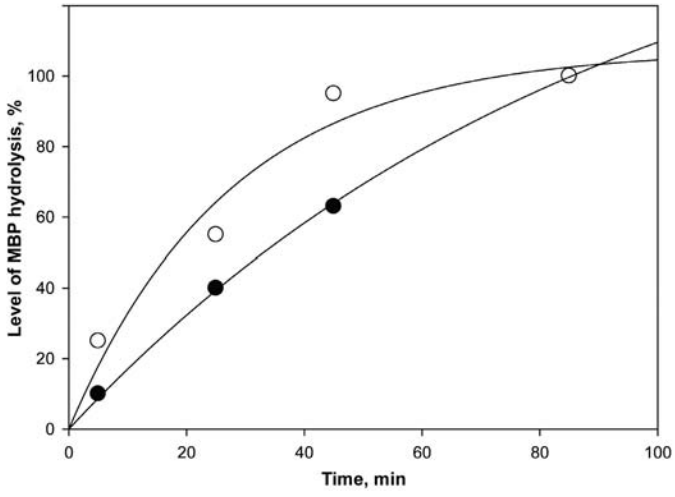

Fig. 1. The time dependence of the level of MBP hydrolysis by proteasome. Labels: ○ 20S proteasome, ● 26S proteasome, isolated from outbreed mice liver.

lated to different antigen presentation in healthy and in the pathological states. The pattern of MBP degradation by proteasome has not been studied before.

RESULTS AND DISCUSSION

Proteasome was isolated and purified using the technique described in [13] with slight modifications. At the first stage, the degradation of MBP (from bovine brains, isoform with MW 18,5 kDa) was performed by a full 26S complex and catalytic 20S subparticle isolated from mice liver. It is shown in Fig. 1 that the incubation of MBP with 20S and 26S proteasomes leads to progressive MBP degradation. The 20S proteasome completely hydrolyzed MBP in 45 min, while the 26S proteasome requires 85 min to degrade the same amount of MBP. The variation in reaction rates could be ascribed to different proteasome concentrations: in the case of 20S proteasome, the enzyme/substrate ratio was 2.7 : 1 (⌈g/⌈g of protein) or 1 : 14.5 (mol/mol); in the case of the 26S proteasome, the enzyme/substrate ratio was lower, namely 1 : 1 (⌈g/⌈g of protein) or 1 : 110 (mol/mol). The proteasome amount was estimated by the Lowry method, using bovine serum albumin as a standard.

The MBP hydrolyzates, processed using 20S and 26S protease complexes from the liver of outbreed mice, were fractionated by reverse-phase HPLC on C4 column (Waters, Delta- Pak, 300 Å). Although the general patterns of elution profiles of hydrolyzates were similar, several differences in elution profiles were observed. In particular, it should be noted that some peaks that concurred for 20S and 26S proteasomes differed in their amount of matter; moreover, in the 26S hydrolyzate, new fractions appeared. Thus, the 26S degradation pattern of MBP somewhat altered compared to the 20S pattern. These differences can be explained by the unequal accessibility to proteolysis of MBP sites located on the surface and in the depth of the protein globule, as well as by their pronounced secondary structure. In the case of the 26S proteasome, the accessibility of different sites of MBP was not of great importance, because the 19S subparticle contains subunits responsible for the denaturation of protein molecules that are to be degraded.

The pool of proteasomes is heterogenous and consists of macromolecular complexes of several types, with catalytic subunits referred to the so-called constitutive type (β1, β2, and β5) or immune type (β1i, β2i, and β5i) (Fig. 2). Six catalytic subunits of proteasome expressed three types of activities, namely chymotrypsin-like (cleavage of the peptide bond on the carboxyl site of hydrophobic and aromatic amino acids Leu, Tyr, Phe), trypsin-like (hydrolysis after positively charged Lys and Arg), and caspase-like (hydrolysis after negatively charged Asp and Glu) [14].

The constitutive-to-immunoproteasome ratios have clearly defined tissue-specificity and, to a considerable degree, depend on the immune state of the organism. For example, more than 90% of the total amount of proteasomes in the brain are constitutive, but in the spleen about 90–95% of proteasome are immunoproteasomes. Besides, in any tissue under interferon gamma exposure, the immune catalytic subunits are produced extensively, being integrated into the newly assembled multicatalytic complexes [15].

It was shown earlier that replacing constitutive catalytic subunits with immune leads to a change in hydrolysis specificity and to an increase in its velocity. Immunoproteasome complexes almost lose their ability to hydrolyze peptide bonds after aspartic and glutamic acids residues (caspase-like activity);however, a lot more often the hydrolysis takes place after hydrophobic and, especially, branched hydrophobic amino acid residues. Therefore, during immunoproteasome processing, an increased amount of peptides bearing hydrophobic amino acids on the C-end is produced. Because hydrophobic amino acids on the C-end of peptides are important anchor fragments for binding with MHC I class mol-

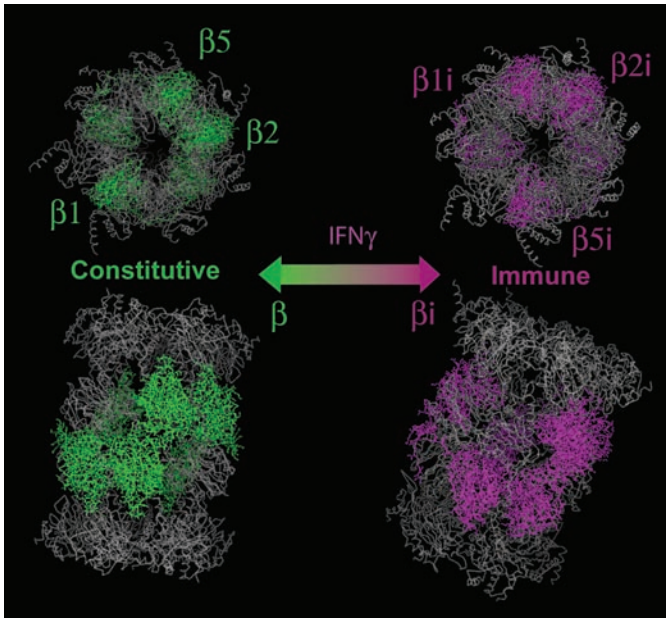

Fig. 2. The proteasome–immunoproteasome equilibrium. Immune catalytic subunits produced in cells exposed to interferon gamma.

Fig. 3. Amino acid sequence of MBP. Arrows mark the proteolytic peptides determined by chromato-mass spectrometry in hydrolyzates of MBP by proteasome from the brains of SJL/J/J mice (upper picture) and of Balb/C mice (lower picture). The arrow's thickness indicates the frequency of occurrence of the corresponding peptide. Color rectangles show the immunodominant regions of MBP.

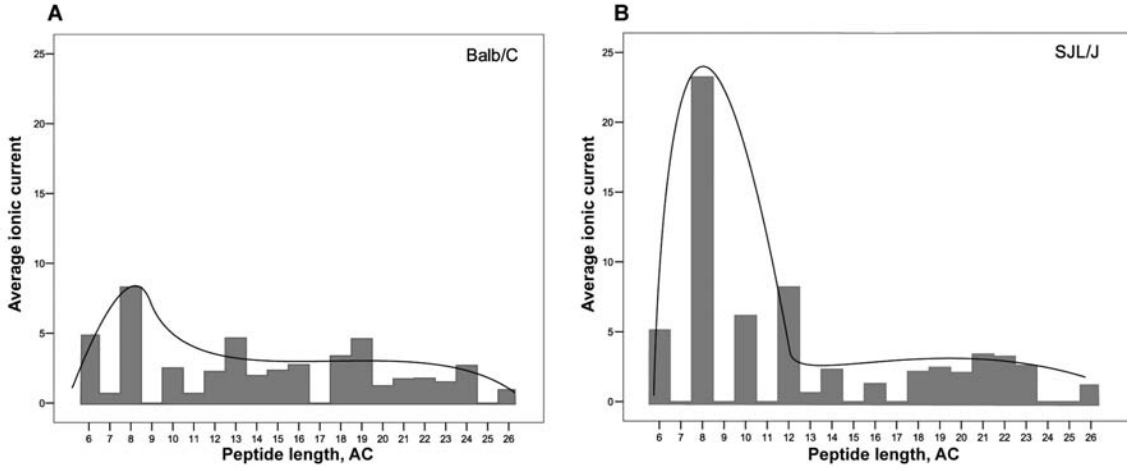

Fig. 4. The length distribution of peptides found in MBP hydrolyzates obtained using proteasome pools from the brains of (a) Balb/C mice and (b) SJL/J/J mice. Bars on the diagram correspond to the experimental value of the ionic current for the peptides of relevant lengths as determined by LC-MS.

ecules, the change in hydrolysis specificity leads to an increase in the production of peptides able to form complexes with MHC molecules. Then, the fragments bounded to MHC are presented on the outer cellular membrane to immune cells. Thus, cells bearing immunoproteasome should more effectively present their own antigens.

It is also known that ⟨-subunits of the proteasome catalytic 20S subparticle function as a gate forming an axial channel that regulates the influx and efflux of proteins and their degradation products by the opening and closing of the entrance to the so-called catalytic chamber. Therefore, the closing of this channel may enable complete substrates degradation by preventing the efflux of partially hydrolyzed peptides [16]. It was also shown [17] that the opening of the channel strongly affects the proteolysis kinetics and the distribution of hydrolytic fragments obtained *in vitro* with respect to the peptide length. If the channel is open, the hydrolysis rate increases; however, in this case, the mean length of produced fragments also increases by 40%. Thus, the higher hydrolysis rate of immunoproteasome should result in longer degradation products (peptides), which will better bind to MHC molecules and, thus, will more effectively be presented on the cell's surface.

It is well established that SJL/J mice are genetically predisposed to the development of experimental autoimmune encephalomyelitis. This pathology is the clinically relevant animal model of multiple sclerosis (MS). Using immunoblotting we studied the composition of the proteasome pool in the brains of SJL/J mice, and it was revealed that the immunoproteasome amount is increased in comparison to Balb/C mice brains (data not shown). Therefore, at the next stage of the study, the proteasomes from the brains of SJL/J and Balb/C mice were isolated, and the proteolysis of MBP by those proteasome samples was studied.

The MBP hydrolyzates obtained using proteasomes isolated from mice of the two strains were studied by LC-MS. Figure 3 shows the complete amino acid sequence of MBP, and the arrows indicate the major fragments produced by proteasome pools from the above-mentioned sources. The thickness of the arrows indicates the relative amount of corresponding peptide in hydrolyzate.

In the MBP sequence, several regions could be attributed to immunodominant protein regions: 12-31, 82-98, 110-128 and 144-169; fragment 85-98 is the so-called encephalitogenic epitope, which can activate the immune response.

It should be noted that MBP degradation by proteasome from outbreed mice liver, both by the full 26S complex and the catalytic core, does not lead to the generation of immunogenic epitopes, all of which were exposed to subsequent fragmentation inside the proteasome catalytic chamber.

In MBP hydrolyzate by proteasome isolated from the brains of SJL/J and Balb/C mice, almost the only hydrolytic site where proteasome exhibits its caspase-like activity was the bond between amino acid residues Asp81-Glu82, close to the beginning of the encephalitogenic peptide.

The MBP hydrolysis pattern by proteasome isolated from the brains of SJL/J and Balb/C mice differed to some extent. In the brains of the autoimmune mice, the generated epitopes could be considerably better colocalized with immunodominant regions of the protein. Under the action of the SJL/J mice proteasome pool on the myelin basic protein, up to a quarter of all obtained hydrolytic fragments were made up of encephalitogenic peptides. The Balb/C mice strain proteasome produces half the amount of that peptide. Moreover, fragments obtained using a Balb/C mice-brain proteasome poorly correlate to the recognition regions of MHC class II molecules.

Figure 4 shows the length distribution of peptides in corresponding hydrolyzates. On the vertical axis, the experimental values of the ionic current obtained using LC-MS for a peptide of a given length is shown. It can be seen that the maximum of the distribution is on peptides having 8 amino acids in length, both in case of proteasome isolated from the brains of SJL/J or Balb/C mice. However the relative values of the average ionic current for these two samples differed drastically, which can be an argument for substantially more fragments of the given length in the case of autoimmune mice. In the hydrolyzates studied, peptides with an even number of amino acid residues predominate, and no peptides shorter than 4 residues were detected. These data are in good agreement with the literature [18] and with the opinion that one of the main roles for proteasome in cells is the generation of peptides for subsequent presentation on MHC class I molecules, which can bind peptides of 8–10 amino acids in length. Longer peptides found in hydrolyzates could be further processed to shorter fragments inside the cell and presented on the MHC I class molecules, and they could also participate in presentation on MHC II class molecules [10].

CONCLUSIONS

In conclusion, in our work it was shown that both 20S and 26S proteasomes are able to hydrolyze the myelin basic protein, and the proteasome/MBP molar ratio was found to be 1 : 14.5 and 1 : 110, respectively; the complete hydrolysis time was 45 and 85 min, respectively. After separating hydrolyzates by HPLC, the molecular weights of the fragments were determined by MALDI mass spectrometry. After analyzing the amino acid sequence of MBP, the proteolytic sites were identified.

It was demonstrated that the nonubiquitinated myelin basic protein is a good substrate for both 20S and 26S proteasomes. This was the first work to identify the sites of MBP proteolysis using a proteasome isolated from the brains of SJL/J and Balb/C mice and to show significant differences in the degradation pattern of this autoantigen. These findings could argue for a better presentation of MBP fragments on the MHC molecules in the case of mice genetically predisposed to the development of experimental autoimmune encephalomyelitis.

Acknowledgements

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UDK 577.214.3

The Interaction between the RNA-Dependent RNA-Polymerase of the Hepatitis Virus and RNA Matrices

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Hepatitis C is one of the most dangerous and widespread viral diseases. Currently, the World Health Organization estimates that about 170 million people are infected with the hepatitis C virus (HCV), the causative agent of infection, in almost every country in the world. The RNA-dependent RNA-polymerase (R-RNAP, virus nonstructural protein) is a key fragment that carries out HCV genome replication. R-RNAP is about 65 kDA in molecular weight and localized on the endoplasmic reticulum membrane of infected hepatic cells by the C-tail α-spiral transmembrane domain (21 a.r.). One characteristic feature of R-RNAP is its ability to catalyze RNA synthesis by both primer-dependent and primer-independent (*de novo*) mechanisms [1]. In the first case, in the *in vitro* experiments, the primer-matrix poly(rA)-oligo(rU) duplex is used as the RNA matrix; in the second case, the HCV genome fragments are used. It is suggested that oligomer from several identical R-RNAP molecules takes part in the replication. Moreover, oligomer was discovered to be composed of H502 and E18 amino-acid residues located in the interaction area of protein globules [2].

Earlier, we obtained the *E.coli* strain (the HCV R-RNAP producer) which allows a highly purified recombinant protein with a cell culture yield of up to 6 mg/l to be created and we developed a procedure for enzyme purification to the homogeneous condition (the data of electrophoresis in the polyacrylamide gel) [3]. The purification procedure included methods similar to those described in the corresponding literature [4]; the kinetic parameters of the primer-dependent reaction determined for the polymerase sample were consistent with the literature data as well [5]. However, anomalously high incorporation of the radioactive label was noted for the primer-independent replication of the heterogeneous RNA (for instance, (-)IRES matrix, which represents a 3'-nontranslated region of the HCV virus genome chain). The enzyme was also incapable of single-step oligonucleotide elongation in the primer-dependent system and, thus, could not be used to the full extent in investigations of specific inhibitors. Those facts testified to the possible impurities of the RNA cell in the protein sample, which formed a stable complex with the enzyme molecule and could act as an "endogenic" matrix. Hence, the need to modify the enzyme purification method and to more thoroughly determine the parameters of its interaction with the different types of matrices obtained.

HCV R-RNAPs were expressed in the *Escherichia coli* cells using the pET21-2c-5BΔ55 plasmid, as was described earlier [3]. Initially, protein containing six histidine residues on the C-tail of the polypeptide chain was recovered by cell disintegration with supersonic rays, and the chromatography of lyzate was cleared by imidazol gradient centrifugation using the Ni-NTA-agarose column. Enzyme activity was determined by the measurement of labeled [γ^{32}P]-UTP incorporation into the high-molecular products using the poly(rA)-oligo(rU) primer-matrix complex or (-)IRES matrix. The produced HCV R-RNAP sample was characterized by high polymerase activity in the primer-dependent system. At the same time, the high incorporation of radioactive

label was typical of the *de novo* RNA replication in both the presence and absence of a matrix.

Modifying the standard recovery method involved the lysis of bacterial cells with lysozyme (1 mg/ml, Sigma, United States) with the following cryolysis (2–3 freezing/thawing cycles) in liquid nitrogen; then NH_4Cl was added to lysate up to a concentration of 1M. After the complete dissolution of NH_4Cl, a 10% solution of polyethyleneimine was added (Serva, Germany) up to the final 1% concentration. The solution was mixed for 30–40 minutes at 40°C and then centrifuged at 3–5,000 r/min for 3 minutes. These procedures ended with the selection of supernatant. Ammonium sulfate was added to the supernatant up to a saturation of 80%, and the mixture was incubated at 40°C for the night. Proteins were subject to centrifugal sedimentation at 10 000 r/min and 40°C for 20 minutes. The sediment was dissolved in a minimum volume of the buffer, which contained 20 mM Tris-HCl pH 7.5, 350 mM NaCl, 5% glycerin, 1 mM β-mercaptoethanol, 1 mM of phenilmethylsulfonyl fluoride (PMSF), and 1 mcl/ml of Pi protease inhibitor mixture (Sigma, United States). Dialysis was carried out in two stages (1 h for each stage) against 50 ml of the same buffer. Dialysate was applied twice to the Ni-NTA-agarose column equilibrated with the same buffer. The column was washed with 50 mM imidazol. Protein was eluated with 200 mM imidazol and assembled from fractions. Eluate was dialyzed in two stages against 200 and 100 ml of the buffer. The first buffer contained 20 mM Tris-HCl pH 7.5, 350 mM NaCl, 10% glycerin, 0.5 mM EDTA, and 1 mM β-mercaptoethanol. The second buffer content was the same, except for 50% glycerin. The protein concentration was measured by the Bradford method [6].

Total and specific enzyme activity was determined by the previously described methods [3].

The phosphorylation of RNA and R-RNAP samples was carried out with T4-polynucleotide kinase (Fermentas, Lithuania) and [γ^{32}P]-ATP according to the manufacturer's instructions. Phosphorylated RNA was purified on Micro-Spin G-50 microcolumns (GE Healthcare, United States).

The kinetic parameters of enzyme and RNA interaction were determined with the help of dot–blot hybridization. A 48-well dotter with nylon and nitrocellulose membranes were used (Bio-Rad, United States). A nitrocellulose membrane was put on the nylon membrane in the dotter. The membranes were preliminarily moistened in the buffer containing 50 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, and 10 mM β-mercaptoethanol. The enzyme was diluted twice with an interval from the highest concentration value at 500 mM NaCl. The RNA assembly was carried out at 4°C for 30 minutes in the membrane moistening buffer; then it was applied to the membranes. The RNA concentration was determined experimentally and reduced to 2 nM. Dried membranes were subject to exposure for 40–60 minutes and visualized on a Storage Phosphor Screen and Phosphor Imager (Packard, United States). Mathematical treatment of the results obtained was carried out with the Total Lab and Origin 7.0 programs.

The necessity of modifying the R-RNAP purification method was related to the possible presence of short impurity RNA, which could be the bacterial ribosome or transfer RNA, in the purified recombinant enzyme sample. Those impurity RNAs could competitively block the binding sites of nucleic acids in the enzyme molecule and thus impede the *in vitro* interaction with the RNA-matrices added in the course of the reaction. As is known, the ultrasonic treatment of bacterial cells causes the mechanical destruction of the cell's nucleic acids, which are fractionized into fine fragments of different lengths. In the case under consideration, some of them were likely bound to the recombinant R-RNAP. Moreover, this process was characterized by a high affinity that inhibited their separation at the following purification stages. Indeed, at least three discreet low-molecular RNAs 12–60 nt in length were established in the course of treating the [γ^{32}P]-ATP enzyme samples in the presence of polynucleotide kinase, which was responsible for the specific phosphorylation of the free polynucleotide 5'-tail (Figs. 1A and 1B). Moreover, we managed to show the protein-RNA complex formation in the course of the experimental covalent linking the complex components with formaldehyde (Fig. 1C). Attempts to get rid of those impurities using different methods (for instance,

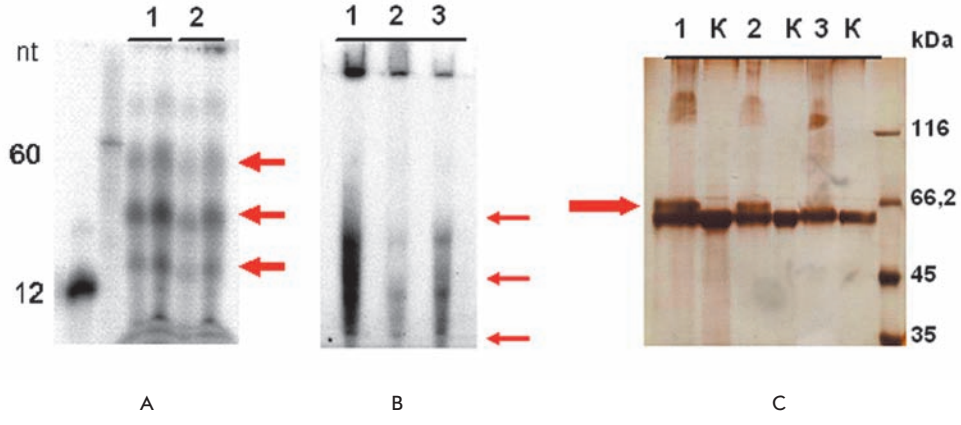

Fig. 1. Low-molecular RNA in the R-RNAP samples recovered by the standard method; the of [γ32 P]-ATP samples (track 1, wild-type type; track 2, double mutant) (A); the wild-type R-RNAP sample recovered by the modified method contains far fewer impurity RNAs; the phosphorylation of [γ32 P]-ATP samples (track 1, wild type of the standard recovery; track 2, wild-type of the modified recovery; track 3, double mutant of the standard recovery; K, negative control without linking) (B); formation of the R-RNAP complex, standard recovery, with impurity RNA, linking with 1% formaldehyde (track 1, wild-type; track 2, mutant with single substitution; track 3, double mutant) (C); (A, B) autoradiography, (C) argentation.

treatment with high NaCl concentrations (up to 1.5 M) and additional chromatography with heparin-agarose) were not successful (the data are not provided).

Finally, the previously offered method of R-RNAP recovery was modified in such a way that the active enzyme sample did not contain impurity RNA. The modification consisted in the replacement of the bacterial cell destruction method, while all the following purification stages remained unchanged. The considered method excluded cell ultrasonic treatment, which caused the formation of short RNA, while the high NaCl concentration resulted in the dissociation of the protein-nucleic complexes and inhibited the coprecipitation of the intentional protein with nucleic acids. This method has been successfully used before to purify recombinant T7 RNA-polymerase [7].

Because the problem of enzyme oligomerization upon RNA binding remained unsolved, we used, along with the wild-type enzyme, the created double mutant (DM) of R-RNAP (H502L, E18A), which, according to the literature data, was capable of oligomerization [2], as well as two mutant forms of R-RNAP with single substitutions near the active site (R222A and C223A), which presumably could influence the binding of the enzyme with RNA. The wild-type R-RNAPs recovered by a new method were distinguished by their high values of specific activity (9.2 nmole/min · 1 μg of enzyme). According to the literature data [2], the double amino-acid replacement (H502L, E18A) in HCV R-RNAP causes the inactivity of double mutant. According to our data, the specific activity of a double mutant is somewhat higher than that of the wild-type enzyme (20.4 nmole/min · 1 μg of enzyme).

However, it should be noted that the use of the new method led to a decrease in the intentional protein yield. If the R-RNAP yield attained 3-5 mg/l of the cell culture when using the standard recovery method, then the recovery by a new method reduced the R-RNAP yield to 1 mg/l. Moreover, as it turned out, the R-RNAP samples depleted in impurity polynucleotides are less stable. In contrast to the standard samples, which are stable at a storage temperature of −20°C, new samples remained active for no longer than one month at −85°C. This phenomenon is explained by the possible participation of impurity polynucleotides in the stabilization of enzyme and protection from inactivation.

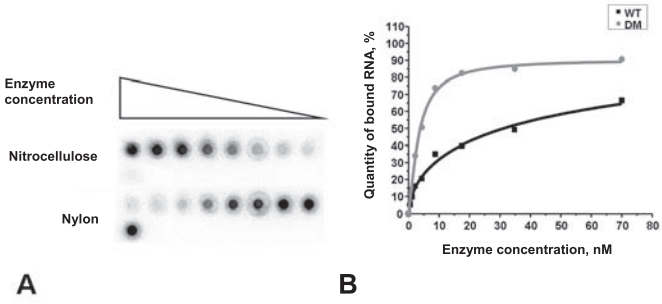

Fig. 2. Parameters of R-RNAP and RNA binding. (A) dot hybridization on nylon and nitrocellulose membranes; (B) dependence of RNA binding on enzyme concentration. Designations: WT, wild type and DM, double mutant.

The R-RNAP samples devoid of impurity polynucleotides had higher *in vitro* affinity to different RNAs. Three types of RNAs were used in the experiments: short (rA20) and long (poly-rA) homopolymers, as well as the long heteropolymer HCV RNA (-IRES). Figure 2 and Table 2 demonstrate the results obtained.

The parameters of R-RNAP and RNA interaction were calculated in accordance with the Hill equation [8], because it was suggested that long RNAs were characterized by cooperative binding [9]. As follows from Table 2, in case of (rA20), the dissociation constants are significantly different for WT and DM R-RNAP; i.e., R-RNAP oligomerization inhibits the binding of short RNA. At the same time, upon the binding of a long homopolymer matrix, both enzymes (WT and DM) behave alike and the interaction is not cooperative. On the basis of these data, it is possible to suggest that, firstly, the matrix binding site does not exceed 20 nt and, secondly, the oligomer structure changes upon the binding of a long polynucleotide with wild-type protein. This situation was not typical of mutants with single substitutions, and both proteins showed similar results. ●

Table 2. Parameters of RNA binding with enzyme recovered by two methods. Designations: WT, wild type; DM, double mutant; n, Hill coefficient; K_D, complex dissociation constant.

RNA type	Parameters	Standard method		Modified method	
		WT	DM	WT	DM
rA20	K_D (nM)	57.86 ± 1.82	-	17.34 ± 6.62	2.73 ± 0.31
	Hill coef.	0.59 ± 0.14	-	1.08 ± 0.21	1.38 ± 0.19
poly-rA	K_D (nM)	-	-	0.81 ± 0.12	1.03 ± 0.13
	Hill coef.	-	-	0.97 ± 0.21	0.83 ± 0.12
(-)IRES	K_D (nM)	>3,000	>3,000	7.80 ± 1.21	14.69 ± 2.94
	Hill coef.	-	-	1.24 ± 0.16	1.20 ± 0.18

Hence, we modified the method of HCV R-RNAP recovery, which allows the production of recombinant protein devoid of impurity cell nucleotides. The R-RNAPs sample obtained is characterized by a high affinity to different RNA, which makes it possible to specify the parameters of this interaction.

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UDK 577.2

Studying of Membrane Localization of Recombinant Potassium Channels in *E.coli*

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ABSTRACT The effective expression of recombinant membrane proteins in E.coli depends upon the targeting and insertion of proteins into the cellular membrane, as well as on those proteins adopting the correct spatial structure. A significant technological problem involves the design of approaches for detecting the location of target proteins within a host cell. Using a hybrid potassium channel KcsA-Kv1.3 as a model, we developed a technological scheme which is suitable for the study of membrane localization in *E.coli* cells of recombinant proteins containing voltage-gated eukaryotic potassium channels as the functional active site. The scheme involves both biochemical and fluorescent methods for detecting target proteins in the cytoplasmic membrane of *E.coli*, as well as the study of the ligand-binding activity of membrane-embedded proteins.
Key words: membrane proteins; KcsA; cell fractionation; fluorescent methods

Studies on trans-membrane proteins and membrane-bound proteins are one of the current trends in biology. Membrane proteins participate in most cellular processes – signal reception and intercellular communications, molecular and ionic transport— and they play a role in the pathogenesis of many diseases and, as such, are the targets for most pharmaceutical preparations [1].

Because of the low level of biosynthesis of many membrane proteins in biological tissues, the main source of these proteins for structural-functional studies is from recombinant molecules produced in various systems for heterologous expression [2]. Bacterial cells (in particular, *Escherichia coli*) represent the most widely used, and most productive, system for the biosynthesis of recombinant membrane proteins [3]. At the same time, the heterologous expression in *E.coli* of membrane proteins is associated with numerous problems involving the general toxicity of these proteins to the host cells. Besides, recombinant proteins are often produced in aggregated form (with inclusion bodies) necessitating careful preparation to refold such proteins. It would seem more practical to work out an approach for the functional expression of membrane proteins in a bacterial membrane [4].

The development of such an approach can be facilitated with the help of simple and effective tests to ensure the correct folding of the target protein within the cellular membrane. These tests, for example, can be based on measuring the functional activity of the protein, or its ability to bind ligands. Furthermore, simple biochemichal assays for determining the location of the target proteins within the cell enable one to more accurately control the insertion of the target protein into the membrane. Such approaches will increase efficiency in the functional expression of target membrane proteins.

To develop an approach for the controlled functional expression of recombinant membrane proteins in *E.coli*, we used a hybrid potassium channel KcsA-Kv1.3, which was successfully expressed in bacterial cells [5, 6]. This, and also

hybrids KcsA-Kv1.X, which are similar, have been obtained by insertion of the ligand-binding site of eukaryotic Kv1 channels into a homologous site on the bacterial channel KcsA.

Eukaryotic voltage-gated potassium channels like Kv1 are known for their important role in the propagation of nerve impulses, in the regulation of muscle contractions, and in the proliferation of cells [7]. Now, channel Kv1.3 is also being considered as a therapeutic target in the treatment of various autoimmune disorders [8], and the testing of its ligand-binding activity provides the basis for the development of new medications [9].

Production of hybrid proteins KcsA-Kv1.X seemed quite possible due to a high structural and functional homology with potassium channels. These channels are tetramers composed of four α-subunits, each one containing six (voltage-gated eukaryotic channels) or two (bacterial channels) transmembrane helices. In the case of eukaryotic channels, C-terminal helices S5 and S6, which are connected by a loop, form the pore domain, which catalyzes the transport of potassium ions [10]. The bacterial potassium channel, KcsA [11], which has a more simple structure involving an α-subunit, shares a high degree of homology with the pore domains from various bacterial and eukaryotic voltage-gated channels. The most homologous of these is an amino acid sequence comprising a pore loop, which connects transmembrane helices M1 and M2 (Fig. 1a).

The S5-P linker sequence of eukaryotic Kv1 channels participates in the formation of a ligand-binding site for peptide toxins – the natural blockers of voltage-gated channels [12]. The possibility of replacing the S5-P KcsA linker with the corresponding linker from Kv1.3 resulted in the formation of hybrid protein KcsA-Kv1.3 (Fig. 1b), which represents a receptor with a very high affinity for peptide toxins [5]. This hybrid acquired ligand-binding specificity for toxins, which is inherent to eukaryotic cells as a model system. Channel Kv1.3 is thus a suitable bioengineered protein to be used in the search for new modulators of the activity of the Kv1.3 channel.

KcsA-Kv1.3 was chosen for the present study primarily because of its rather high level of biosynthesis in *E.coli* (about 2.5 mg/l [5]), which permits the detection of this protein in a cell lysate by means of a denaturing electrophoresis in PAGE. Secondly, the tendency of hybrid proteins to binding labeled ligands can be used for developing alternative methods of membrane protein localization.

The aim of the present work study is to develop a technological approach for achieving the functional expression of recombinant membrane proteins under the control of fluorescent methods.

MATERIALS AND METHODS

MATERIALS

Reagents were from Merck and Sigma; detergents Mega 9 and N-lauroylsarcozine, sodium salt, were from Amresco; Triton X-100 was from Merck; kits for purification of plasmid DNA, isolation of PCR fragments and extraction of DNA from agarose gel were from Qiagen; restriction endonucleases and DNA-modifying enzymes were from Fermentas.

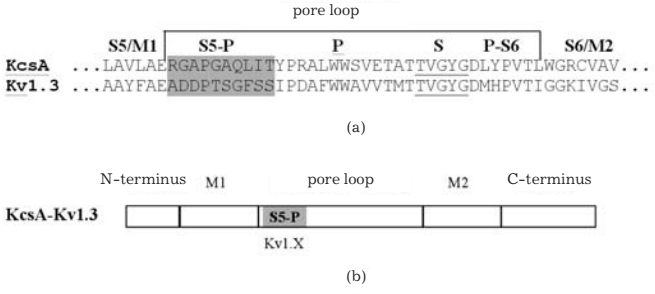

Fig. 1. Homology of amino acid sequences between the pore loop of eukaryotic voltage-gated channel Kv1.3 and the bacterial channel KcsA (a) and the schematic representation of the hybrid protein molecule KcsA-Kv1.3 (b). S5/M1 and S6/M2 – transmembrane helices; S5-P and P-S6 –linkers; P – pore helix; S – a sequence for selectivity filter (underlined).

METHODS

General protocols for recombinant DNAs were followed in accordance to [13].

The KcsA expression was conducted in *E.coli* strain BL21(DE3) (Novagen). Overnight culture of *E.coli* previously transformed with the target plasmid was inoculated in 30 ml of M9 medium enriched with kanamycin (40 μg/ml) to an initial density of 0.25 at OD_{560}. The culture was induced at an OD_{560} of about 1.0 with 50 μM of IPTG, and it was grown with shaking at 37˚C for 18 h. The level of hybrid protein expression was analyzed through 13.5% SDS-PAGE [14].

Immunoblotting. Proteins from SDS-PAGE were transferred onto a nitrocellulose BA-85 filter (Schleisher & Schuell) by means of electrophoresis on a Mini-Protean 3 Cell (Bio-Rad) in 25 mM $NaHCO_3$, pH 9.2, 10% methanol for 1.5 h with a current of 0.8 mA/cm at a temperature of 20 ˚C.

Immunochemical staining was carried out using monoclonal mouse IgG1 antibodies (Penta-His, Qiagen) diluted to 1:1000, and secondary anti-mouse antibodies conjugated with HRP (Novagen) diluted to 1:3000, according to the manufacturer's instructions. TMD (tetramethylbenzydin) was used as a chromogene in a 0.03% solution of hydrogen peroxide.

Cell fractioning. Probes for fractioning were prepared by pelleting cells from 30 ml of culture (4000 × g, 10 min, 10^0C). Cells were lysed in 4 ml of buffer A (100mM NaCl, 5mM KCl, 50mM Трис pH 8.0) containing 0.5 mg/ml of lysozyme, 1 mM EDTA and 1mM PMSF at 4 ˚C for 20 min, sounded for 5 min on a Digital Sonifier model 250 (Branson) with 10s pulses at an output of 200 W.

The suspension was centrifuged for 10 min at 10,000 g and a temperature of 5 ˚C, a pellet was removed (pellet I), and the supernatant was further centrifuged for 1 h at 80,000 g at 5 ˚C on a TLA 100 (Beckman) producing pellet II. Each pellet was extracted with buffer B (0.1 M Na_3PO_4, pH 7.0, 5mM KCl) containing 40 mM Mega 9 (1% of N-lauroylsarcozine or 1% Triton X-100) with gentle shaking for 3 – 4 h, then the suspensions were re-centrifugated, and the final pellets were dissolved in an electrophoresis buffer.

Carbonate wash. Cells were lysed in buffer C (50 mM Tris pH 8, 5 mM EDTA) containing 1mg/ml of lysozyme. The suspension was frozen and thawed 3 consecutive times and then sounded for 1 min with 10 s pulses. Then, an equal volume of 0.2 M Na_2CO_3, pH 12, was added to it, and the mixture was incubated on ice for 5 min. Integral membrane proteins were pelleted by centrifugation for 1 h at 80,000 g at 4 ˚C. The obtained pellet was dissolved in an electrophoresis buffer.

Protein purification by means of metal-affinity chromatography. Protein extracts of pellets I or II were applied onto a 1ml-column of Ni-NTA agarose (Qiagen) equilibrated in buffer B with 40 mM Mega 9**.** The resin was washed with a solution of 20 mM imidazole, and the target protein was eluted with 0.4 M imidazole in the same buffer.

Preparation of spheroplasts and binding procedure. Host strain BL21(DE3), carrying pETKcsA-Kv1.3 plasmid, was cultivated, following induction, with IPTG at 37 ˚C for 18 h in a minimal M9 medium. The cells were harvested by centrifugation (5000 ×*g*, 10 min, 4 °C), incubated in buffer A (10 mM Tris-HCl, pH 8.0, 0.5 M sucrose, 0.3 mM EDTA) containing lysozyme (20 μg/ml) for 20 min and then stabilized by the addition of $MgCl_2$ to a final concentration of 10 mM.

Obtained in this way, the spheroplast suspension with OD_{560} of about 0.5 was diluted 100-200 times, transferred into the 12-well flexiPERM silicon chamber (Perbio, Aalst, Belgium) attached to a thin (0.17 mm) glass slide and incubated with Rh-AgTx2 at room temperature on a shaker for 1.5 h. The concentration of Rh-AgTx2 (AgTx2 labeled with 5(6)-carboxytetramethylrhodamine-N-succinilmidyl ester) was 10 nM. (Rh-AgTx2 was kindly provided by Yu.V. Korolkova, IBCH RAS). The cells were then analyzed using the inverted laser scanning confocal microscope (LSM510-META, Zeiss, Germany).

The confocal fluorescent images of spheroplasts stained with Rh-AgTx2 were measured with the C-Apochromat water immersion objective (63×, NA = 1.2, Zeiss) at approximately 0.25 μm lateral and 0.5 μm axial resolution. The fluorescence of Rh-AgTx2 was excited with a He,Ne laser (543.5 nm, 12 μW on the sample), and emission was registered on a 585-nm long pass filter.

RESULTS AND DISCUSSION

STUDYING THE CELLULAR LOCALIZATION OF KcsA-Kv1.3 BY MEANS OF FRACTIONING AND DETERGENT EXTRACTION

A gene for the hybrid KcsA-Kv1.3 protein was constructed according to the specifications in [5] and cloned into plasmid pET28a (Novagen). Gene expression was then carried out in *E.coli* BL21(DE3). The level of expression was determined by analyzing the total protein content with SDS-PAGE, and the presence of the target protein was confirmed by means of immunoblotting with anti-His antibodies to the C-terminal hexahistidine tag.

It is known that recombinant KcsA and also a hybrid protein KcsA-Kv1.3 accumulate in the cytoplasmic (inner) membrane of *E.coli* [5, 15, 16]. So, for this reason, isolation of hybrid proteins [5] was carried out from the membrane fraction of cells obtained by high-speed centrifugation of cellular lysate (110 000 g, 45 min). We decided to use this procedure for working out the fractioning procedure aimed at the analysis of the cellular localization of the target membrane proteins – the hybrid potassium channels.

The procedure of fractioning the cells grown after induction was based on a sounding of cells, pelleting of cellular debris and insoluble components with low-speed centrifugation (10 000g, for 10 min) (pellet I), and subsequent pelleting of membrane vesicules by high-speed centrifugation (pellet II). As shown in Fig. 2a, hybrid protein KcsA-Kv1.3 is contained within an insoluble cell fraction, and a considerable amount of it is detected in pellet II (Fig. 2a, lane 3), suggesting that KcsA-Kv1.3 is inserted into the cellular membrane. Noteworthy, recombinant KcsA-Kv1.3 protein is found in the pellet I (Fig. 2a, lane 2). This low-speed pellet is usually considered as a fraction of inclusion bodies – insoluble protein aggregates of the target protein formed by the nascent chains of recombinant proteins in denatured form [17]. In the case of soluble protein expression, the isolation of active proteins from inclusion bodies by means of a re-naturing procedure is carried out. In the case of membrane protein biosynthesis, the composition of pellet I, as well as the mode of its formation, might be different. So, in work [18] it is shown that the distribution of proteins within the cytoplasmic membrane of *E.coli* is uneven: with the help of differential centrifugation, "light" membrane fractions without protein, "heavy" membrane fractions overloaded with protein, and intermediate fractions were identified. Taking into account that KcsA-Kv1.3 protein is overexpressed in BL21(DE3) cells, it is conceivable that pellet I contains membrane vesicules saturated with the target protein molecules and, thus, has a higher density than pellet II. This mechanism of pellet I formation is mentioned in review [2]. It cannot be excluded that pellet I contains denatured molecules of the target protein.

Pellets I and II were studied by means of detergent extraction. For this, nonionic detergent Mega 9 was used, which is usually the detergent of choice for the extraction of KcsA and KcsA hybrid proteins from the membrane fraction [19]. As shown in Figs. 2b and 2c, incubation of each pellet with Mega 9 resulted in the dissolution of the target protein. Then, pellets were subjected to extraction with the mild ionic detergent lauroylsarcosine. Solutions of 1 – 2% lauroylsarcosine are usually used for the selective dissolution of *E.coli* cytoplasmic membrane [20, 21] and, in some cases, for dissolution of the low-speed pellet to recover a target membrane protein [22, 23]. The target protein was quantitavely extracted from pellets I and II using lauroylsarcosine (results of the dissolution of pellet I are shown in Fig. 2c). Pellets I and II were totally dissolved in 1% solution of Triton X-100 according to [24] (Figs. 2d and 2f). Triton X-100 (in 0.5 – 1 % solutions) is frequently used for dissolving the cellular membrane fraction, as well as for washing the inclusion bodies of any contaminating proteins [17].

To confirm that the target protein was indeed localized in the cellular membrane, we determined the total membrane protein fraction using the method of "carbonate wash" [25]. This method is based on the selective solubility of both cytoplasmic protein aggregates and membrane-bound proteins in an alkaline solution at pH=12, and the subsequent pelleting of integral membrane proteins by high-speed centrifugation. As shown in Fig. 2g, the whole amount of the hybrid KcsA-Kv1.3 is found in the pellet fraction.

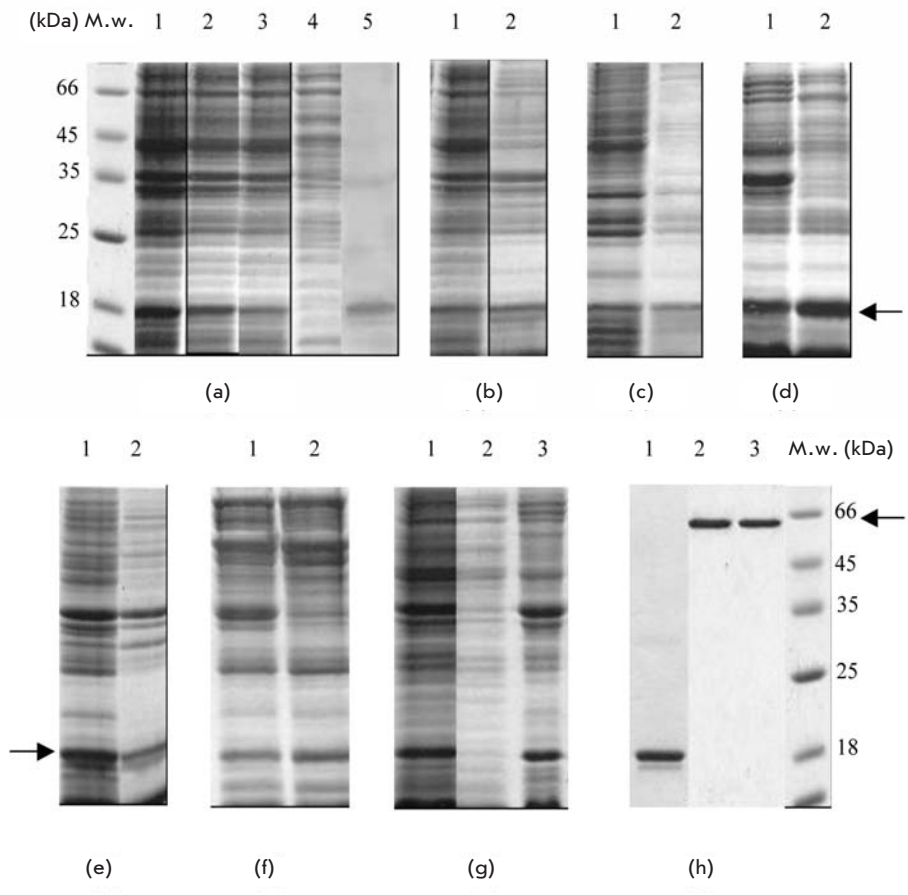

Fig.2. Fractioning of BL21(DE3) cells producing KcsA-Kv1.3 (a). 1 –cellular lysate; 2 – pellet I; 3 – pellet II; 4 – supernatant following high-speed centrifugation; 5 – immunoblotting of cellular lysate with anti-Hisx6 antibodies.
Extraction of pellet I with detergent solutions: 40 mM Mega 9 (b), 1% lauroylsarcosine (c); 1% Triton X-100 (d). Extraction of pellet II with detergent solutions: 40 mM Mega 9 (e), 1% Triton X-100 (f).
1 – suspension of pellet in a detergent solution; 2 – extract.
Fractioning of cells by the "carbonate wash" method (g). 1 –cellular lysate; 2 – supernatant following high-speed centrifugation; 3 – a pellet.
Analysis of KcsA-Kv1.3 tetramer formation (h). 1 – KcsA-Kv1.3 preparation preheated for 10 min at 96 °C; 2 - KcsA-Kv1.3 preparation isolated from the pellet I by extraction with 40 mM Mega 9 preheated for 10 min at 37 °C; 3 – KcsA-Kv1.3 isolated from pellet II prepared under the same conditions. M.w. – molecular weight markers. The position of a target protein (monomer or tetramer) is identified by an arrow.

Next, we purified the KcsA-Kv1.3 from the protein extracts on a column of Ni-NTA Sepharose, and then the isolated target protein was analyzed in SDS-PAGE using two protocols – one with preliminary heating at 96 °C for 10 min in a buffer containing 1% SDS, and the other without heating. This test, which is used for determining the native tetramer formation of KcsA and its mutations, is based on the unique thermal stability of the KcsA tetramer in solutions of different detergents [16]. For example, in the 0.1% SDS solution the melting temperature of KcsA is 68 °C. It is clear from Fig. 2h that hybrid protein KcsA-Kv1.3, which is recovered either from pellet I or from pellet II, forms a tetramer. Taking into consideration that the presence of a lipid environment is one of the prerequisites for KcsA tetramer formation [36], and on the basis of the data showing a high degree of solubility of KcsA-Kv1.3 from both pellets in detergents (Mega 9, lauroylsarcosine and Tritone X-100), one can assume that KcsA-Kv1.3 in pellet I is properly folded and integrated into membrane vesicles. Additional confirmation of the presence of a membranous environment in both pellets comes from the location of the whole amount of KcsA-Kv1.3 within the insoluble fraction following the "carbonate wash." The obtained results allow one to correctly evaluate the data from the fractioning of *E.coli* cells producing membrane proteins, indicating that the location of a target protein within a low-speed pellet (pellet I) does not exclude the assumption, by this protein, of native fold in a membranous or membrane-like surrounding.

DETECTING THE MEMBRANE LOCALIZATION OF KCSA-KV1.3 BY MEANS OF FLUORESCENT MICROSCOPY

The presence of a ligand-binding site in the hybrid KcsA-Kv1.3 molecule creates an opportunity to detect the insertion and folding of this recombinant protein into the cytoplasmic membrane of *E.coli*. For this, we used a procedure for binding KcsA-Kv1.3 to a fluorescently labeled peptide toxin – agitoxin2, on the entire surface of *E.coli* cells [26]. Agitoxin (AgTx2), a 38-a.a. peptide from the venom of a scorpion, *Leiurus quinquestriatus*, is an effective blocker of the Kv1.3 channel [27]. Binding to the channel from the outside of the pore, it inhibits the transport of potassium ions through the channel. Agitoxin2 does not exhibit affinity for KcsA, but it effectively interacts with the purified KcsA-Kv1.3 (IC50=6.4 nM) [5]. Furthermore, AgTx2 is sensitive to the structural integrity of KcsA, and it is usually used for probing the quaternary (tetramer) structure of this channel [28].

For fluorescent detection of binding agitoxin to KcsA-Kv1.3, BL21(DE3) cells with KcsA-Kv1.3 were first converted into spheroplasts by lyzosyme treatment in order to disrupt the cell wall and thus allow a labeled toxin to reach the cytoplasmic membrane. Then, spheroplasts were incubated with agitoxin that had been labeled with a rhodamine dye and subjected to analysis with a scanning confocal microscope. Figure 3 shows that a fluorescent signal is detected at the surface of the cellular bacterial membrane. Control BL21(DE3) lacking a plasmid and BL21(DE3) with the KcsA protein did not demonstrate any binding. These results sug-

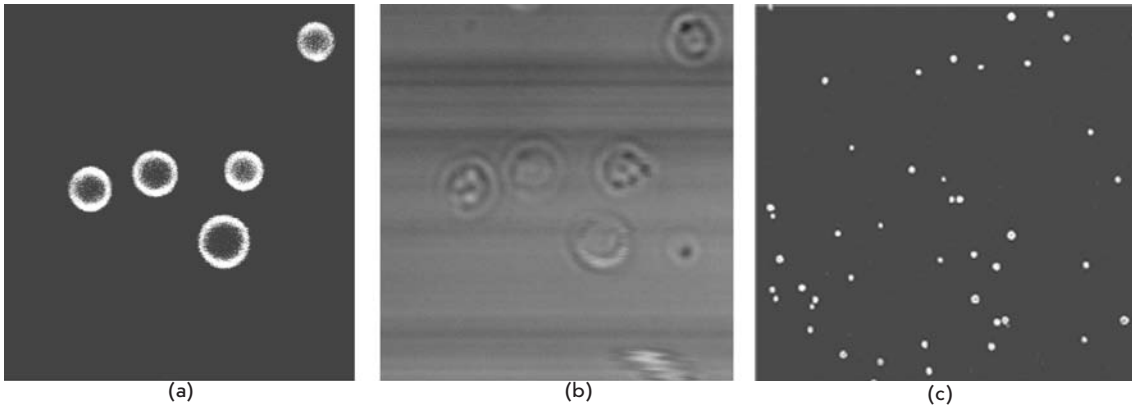

Fig. 3. Binding of Rh-AgTx2 to the membrane of spheroplasts. (a, c) Typical confocal fluorescent images of Rh-AgTx2 bound to *E.coli* BL21(DE3) producing KcsA-Kv1.3. (b) Transparent light image of spheroplasts. Length of the bar corresponds to 5 μm.

gest that hybrid KcsA-Kv1.3 is located in the inner membrane in a functional active form and is able to specifically bind a ligand; in this case, a peptide toxin.

Our experiments show that the set of methods used (biochemical and fluorescent methods of analysis) allow one to adequately estimate the location of recombinant proteins — hybrid potassium channels, within the cytoplasmic membrane of *E.coli*.

CONCLUSIONS

1. Using the KcsA-Kv1.3 hybrid protein as a model, we developed an approach for the study of the membrane localization, within *E.coli* cells, of recombinant channels containing functionally active sites composed of eukaryotic, voltage-gated potassium channels. The technical scheme is based on commonly used biochemical techniques, as well as on fluorescent methods of analysis. It includes:

- fractioning of E.coli cells, producing recombinant membrane proteins by means of the differential centrifugation of the cellular lysate;
- analysis of the pellet fractions with the help of extraction using different detergents;
- determination of membrane proteins within the cellular lysate through a "carbonate wash" method;
- determination of KcsA-Kv1.3 tetramer formation by means of SDS-PAGE electrophoresis;
- detection of the insertion and proper folding of a target protein within the cellular membrane by means of fluorescent methods of analysis using whole *E.coli* cells.

2. The present study demonstrates that a low-speed pellet, produced during cell fractioning, contains the target protein, which forms a proper tetramer structure in a membranous surrounding. ●

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UDK 577.2

Cell-free Production of the Extracellular Domain of the Nicotinic Acetylcholine Receptor

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The nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel which is incorporated into the postsynaptic membrane of neurons [1]. NAChR is composed of five homologous subunits, whose transmembrane domains form an ion pore and the N-terminal domains contain the binding sites for the ligand (Fig.1). One of the most common subtypes of nAChR receptor in mammalian nervous systems is a homopentameric, α7 nAChR (α7nAChR), and several neurodegenerative disorders are associated with its dysfunction [2]. An effective system for the production of the individual subunits and other domains of nAChR is a prerequisite for studies focused on the receptor itself, and for the design of biomedical drugs to be used in the treatment of the disease. The tendency of these proteins to form insoluble aggregates in solution makes the development of such systems difficult [3]. Recently, a research group from the USA published the x-ray structure of acetylcholine binding protein (AchBP) from *Lymnaea stagnalis* [4]. AChBP is a water-soluble protein composed of five identical subunits. This protein shares 25% sequence homology with the extracellular domain of α7nAChrR (α7ED), and it is capable of interacting with some of the nAChR ligands (for example, acetylcholine, α-conotixins, and α-neurotoxins) [4]. It has been shown that replacing the fragment of α7ED which is located between Cys128 and Cys142 (the so-called Cys-loop) with a homologous loop from AChBP increases the solubility of the domain [5].

During the past 10 years, cell-free systems, especially continuous-z exchange cell-free systems, have been successfully used in the production of recombinant proteins [7, 8]. These systems have some advantages over host-based gene expression systems: (i) the direct addition of special agents or co-factors into the reaction mixture can prevent the aggregation of the target protein, and (ii) the method permits the synthesis of selectively labeled proteins which can then be used in structural studies.

The main objective of the present study was the development of an effective cell-free system for the production of

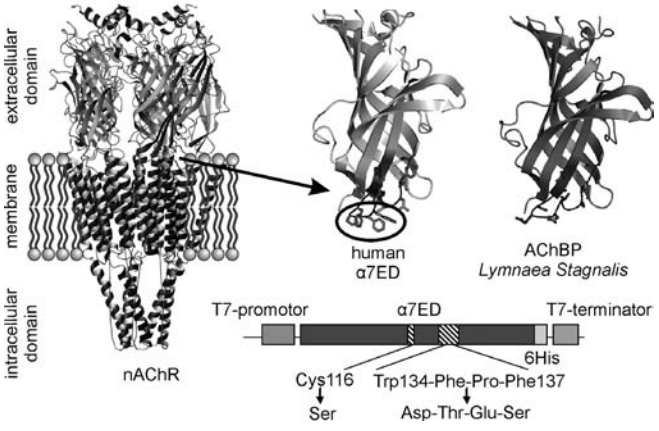

Fig.1. Model of the spatial organization of nAChR [1]. Comparison of a model of α7ED [6] with the spatial structure of AChBP subunit [4]. Scheme of mutations.

human α7ED. For this purpose, from a full-length gene of α7nAChR (a generous gift from Prof. J. Lindstrom) two mutant α7ED genes were constructed, with substitutions that were intended to increase solubility. The first gene (*α7ED/C116S/Cys-loop*) coded for α7ED with two substitutions: (i) unpaired Cys116 was replaced by Ser [3], and (ii) Cys-loop of α7ED (Cys128-Cys142) was replaced by Cys-loop from AChBP [5]. An analysis of the model of α7ED [6] revealed that only a small fragment (Trp134-Phe-Pro-Phe137) of the Cys-loop is hydrophobic in nature and that it probably interacts with the membrane portion of the receptor (Fig.1). At the same time, the homologous region of AChBP contains only hydrophilic residues (Fig.1). Thus, the second mutant gene (*α7ED/C116S/DTES*) encodes α7ED with the replacement of this hydrophobic site with Asp-Thr-Glu-Ser from AChBP and the substitution of Cys116Ser.

Mutant genes, with an additional sequence coding for a His 6-tag on the C-terminus, were cloned into the *pET22b(+)* vector. In both cases, the majority of the synthesized protein was in the form of insoluble aggregates, and only a small percentage remained soluble (Fig.2, lanes 2-4). The most likely cause for the observed aggregation is the incorrect formation of disulfide bonds and/or the tendency of α7ED toward spontaneous pentamerization when in solution [9]. The addition of reduced (GSH) and oxidized (GSSG) glutathiones, in concentrations of 0.1 mM and 0.5 mM, respectively, to the translation solution increased the yield of soluble proteins by up to 30% (Fig.2, lanes 5-7). The presence in the translation solution of a low-molecular-weight nAChR agonist (carbamylcholine, CCh) yielded similar results (Fig.2, lanes 8-10). The use of a soft, non-ionic detergent (Brij-35) at a concentration of 0.5% to prevent spontaneous pentamerization, together with GSH and GSSG, caused a significant decrease in the fraction of insoluble protein (Fig.2, lanes 11-13). It should be noted that both chimeras of α7ED displayed identical properties during the process of synthesis and during subsequent manipulations, so the experiment proceeded using only α7ED/C116S/DTES.

Purification of the recombinant proteins using metal-affinity chromatography caused the proteins to precipitate. Thus, the conditions for the refolding of α7ED/C116S/DTES from a precipitate of the translation mixture were determined. Several different approaches (for example, 8M urea, 6M guanidine hydrochloride and 1% sodium laurylsarcosine, LS) for initial precipitate dissolution were tried. The highest efficiency was achieved using a mixture of 3M urea and 1% LS in the presence of DTT. The refolding of α7ED/C116S/DTES was carried out on metal-affinity resin by washing with GSSG/GSH in a descending concentration gradient of both LS and Urea. However, the protein that was obtained turned out to be highly unstable in solution. The alternative protocol involved the replacement of 1% LS with 0.1% β-dodecylmaltoside (DDM) or 0.1% dodecylphosphocholine (DPC).

As a result, highly stable (more than 1 month at +4^{0}C) protein preparations, with a final yield of 1 mg per ml of the translation mixture, were obtained. The samples of α7ED/C116S/DTES in DDM and DPC solution were analyzed using size-exclusion chromatography (Fig.3). In the case of DDM, recombinant α7ED was found in the form of large soluble aggregates (Fig.3). Use of DPC yielded a much more homogenous (>90%) sample with an average particle diameter of 7mm, which corresponds to a size of α7ED monomer (5 nm) associated with DPC micelle (4 nm). An analysis of the secondary structure of α7ED/C116S/DTES in DPC solution using CD spectroscopy revealed a prevalence of the β-structure, which was in keeping with our expectations (Fig.3). The ability of α7ED/C116S/DTES in DPC solution to interact with nAChR antagonists was studied using NMR spectroscopy with an ^{15}N-labeled long-chain neurotoxin

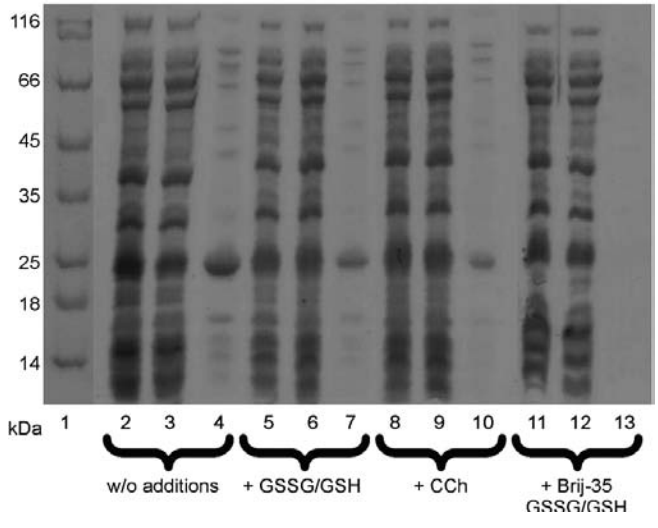

Fig. 2. Electrophoresis analysis of the cell-free synthesis of α7ED/C116S/DTES. 1- markers of molecular weights; 2, 5, 8, 11 – total protein fraction of translation mixture; 3, 6, 9, 12 – soluble protein fraction of translation mixture; 4, 7, 10, 13 - insoluble protein fraction of translation mixture.

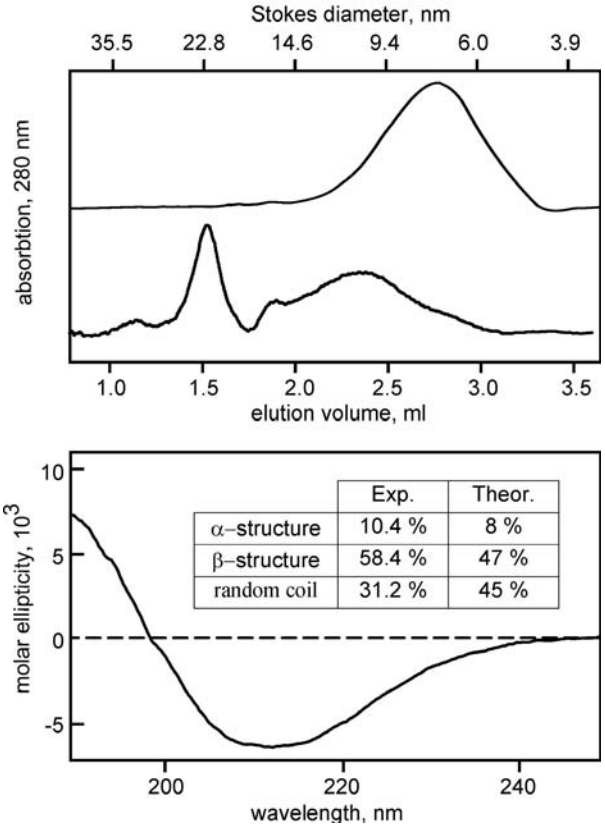

Fig.3. Analysis of α7ED/C116S/DTES in DDM and DPC solution using size-exclusion chromatography on Superdex-200 (GE Healthcare). CD spectrum of α7ED/C116S/DTES in DPC solution.

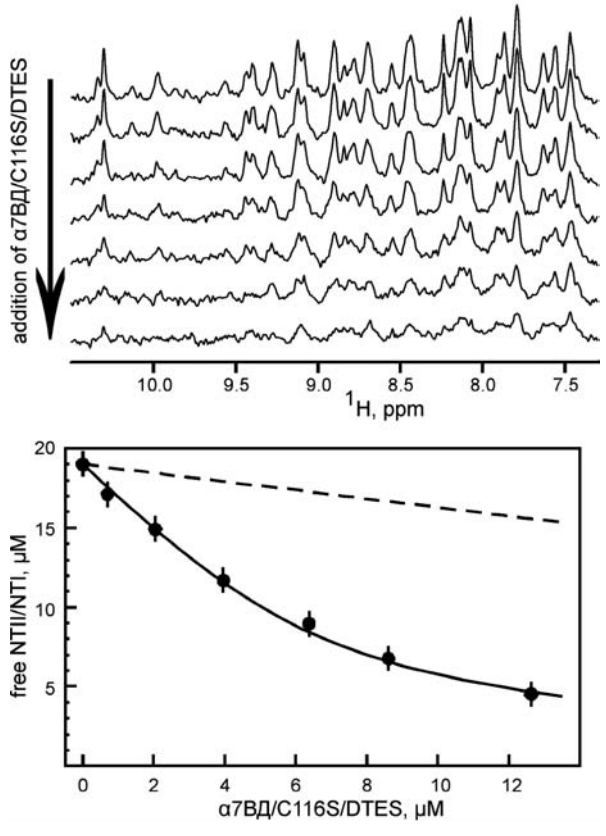

Fig.4. 1D ^{15}N-HSQC NMR spectrum of NTII/I toxin in the presence of DPC micelles and following an increase in α7ВД/C116S/DTES concentrations. The obtained isotherm binding and dilution curves (dashed line) are shown.

NTII/I obtained as in [10]. The NTII/I sample in DPC was titrated by α7ED/C116S/DTES, and an attenuation of the toxin signals in 1D ^{15}N-HSQC spectra was observed (Fig. 4). An analysis of the attenuation curve revealed that one molecule of the domain cooperatively binds two molecules of the toxin (Hill coefficient approximately 1.8) and an apparent dissociation constant of approximately 2 mkM.

In summary, we developed a new cell-free system for the production of the active extracellular domain of α7nAChR. The addition of DPC to the protein sample stabilizes the domain in solution, preserves the secondary structure, and doesn't prevent the binding of antagonists. The development of this system creates new possibilities for future structural-functional studies of nAChR/ligand interactions.

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Complete Sequencing of the Mitochondrial Genome of *Opisthorchis felineus*, Causative Agent of Opisthorchiasis

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ABSTRACT *Opisthorchis felineus*, a hepatic trematode, is the causative agent of opisthorchiasis, a dangerous disease in both human beings and animals. Opisthorchiasis is widespread in Russia, especially Western Siberia. The purpose of the present study was to determine the complete mitochondrial DNA sequence of this flatworm. Two parallel methods were employed: (1) capillary electrophoresis to sequence the mitochondrial genome fragments obtained through specific PCR amplification, and (2) high throughput sequencing of the DNA sample. Both methods made possible the determination of the complete nucleotide sequence of the *O. felineus* mitochondrial genome. The genome consists of a ring molecule 14,277 nt in length that contains 35 genes coding 2 rRNA, 22 tRNA, and 12 proteins: 3 subunits of cytochrome-C-oxidase, 7 subunits of NADH-dehydrogenase, B apocytochrome, and subunit 6 of ATP-synthetase. Like many other flatworms, *O. felineus* is characterized by the absence of the ATP-synthetase subunit 8 gene. Nineteen out of the 22 tRNAs have a typical "clover leaf" structure. The tRNA(AGC) and tRNA-Cys genes lack DHU-loops, while the tRNA-Ser(UCA) has 2 alternative structures: one with a DHU-loop, and one without it. Analyzing the results obtained from the high throughput sequencing revealed 45 single-nucleotide polymorphisms within the mitochondrial genome. The results obtained in this study may be used in the development of molecular diagnostic methods for opisthorchiasis. This study shows that high throughput sequencing is a fast and effective method for decoding the mitochondrial genome of animals.

INTRODUCTION

The flatworm *Opisthorchis felineus* (class: *Trematoda*, family: *Opisthorchiidae*) is a parasitic liver fluke in both human beings and animals. An estimated 2 million people worldwide are infected with opisthorchiasis, most of them in Russia and countries of the former Soviet block, such as Ukraine, Belarus and Kazakhstan [1, 2]. Within some of the northern settlements in these regions, up to 90% of the population is infected with opisthorchiasis [1].

Although *O. felineus* has been studied for over a century, the lack of knowledge about its specific indentifying characteristics has meant that many questions about its prevalence and about how it evolves remain to be answered. Previous molecular analyses of these flukes have not provided molecular markers specific enough to be effective for the purposes of present-day studies [3,4,5], but the complete decoding of this trematode's mitochondrial genome may enable specific and effective molecular mark-

ers to be created, which would have far-ranging applications in research.

The mitochondrial DNA (mtDNA) of most species of animals has some unique features, such as its maternal pattern of inheritance, the absence of recombination and its higher replication rate, which distinguish it from nuclear DNA[6], and which make it a potentially unequalled tool for identification in phylogenetic and phylogeographic studies.

The number of sequenced genomes continues to increase, and now they are widely used for selecting genetic markers characterized by a high evolution rate, and for creating high-resolution phylogenetic trees in which both the sequences proper and the individual gene sequences can be used as markers.

Two of the methods available for the complete sequencing and structural analysis of the mitochondrial genome of *O. felineus* are the subject of the present review.

MATERIALS AND METHODS

BIOMATERIAL SOURCE AND DNA RECOVERY

The *O. felineus* samples were recovered from an infected cat from the Ust-Tula settlement (Novosibirsk Region, Russia). The morphological features allowed specialists from the Parasitology and Ichthyology Laboratory (Institute of Systematic and Ecology of Animals, Russian Academy of Sciences) to determine the species. Both sequencing procedures involved the recovery of DNA from the pooled samples using the phenol-chloroform method [7].

Table 1. Gene pattern of the *O. felineus* mitochondrial genome.

Gene	Length, pn	Start-codon	Stop-codon	Gene	Length, pn	Start-codon	Stop-codon
cox3	642	ATG	TAG	nd3	354	GTG	TAG
tRNA-His	67			tRNA-Ser(AGN)	61		
cob	1110	ATG	TAG	tRNA-Trp	68		
nd4L	261	ATG	TAG	cox1	1560	GTG	TAG
nd4	1275	ATG	TAG	tRNA-Thr	63		
tRNA-Gln	63			16S rRNA	994		
tRNA-Phe	66			tRNA-Cys	60		
tRNA-Met	68			12S rRNA	780		
atp6	513	ATG	TAG	cox2	639	ATG	TAG
nd2	867	ATG	TAG	nd6	459	ATG	TAG
tRNA-Val	65			tRNA-Tyr	62		
tRNA-Ala	62			tRNA-Leu(CUN)	64		
tRNA-Asp	67			tRNA-Ser(UCN)	72		
nd1	900	GTG	TAG	tRNA-Leu(UUR)	65		
tRNA-Asn	71			tRNA-Arg	68		
tRNA-Pro	64			nd5	1602	ATG	TAG
tRNA-Ile	62			tRNA-Glu	72		
tRNA-Lys	65			tRNA-Gly	67		

DECODING OF THE O. FELINEUS MTDNA SEQUENCE USING CAPILLARY ELECTROPHORESIS, AFTER P. SENGER

The conserved sequences characteristic of the trematode genomes were identified by comparing the mitochondrial genomes of the *Fasciola hepatica* (AF216697), *Paragonimus westermani* (AF216698) and *Schistosoma mansoni* (AF216698) trematodes using the MEME/MAST programs (http://meme.sdsc.edu/). Universal primers were selected on the basis of those sequences, as well as on the basis of such published sequences as *Clonorchis sinensis* (DQ116944, AY264851) and *O. viverrini* (DQ882172, DQ119551). These primers helped to create a set of amplicons, approximately 1,000 pn long, whose sequences were then used to synthesize new primers. Then, the remaining overlapping fragments of the mitogenome were amplified. Most amplicons were directly sequenced; some amplicons were cloned, and then at least three clones were subjected to sequencing. The mtDNA sequencing was performed using the Applied Biosystems ABI PRISM 3100 Avant Genetic Analyzer in the DNA Sequencing Institute, Siberian Branch of the Russian Academy of Sciences. The complete sequence of *O. felineus* mtDNA can be found in the GenBank (NC_011127).

DECODING OF THE O. FELINEUS MTDNA SEQUENCE USING THE HIGH THROUGHPUT SEQUENCING METHOD

In order to determine the *O. felineus* mtDNA sequence using the high throughput sequencing method, we employed the techniques developed by the 454 Life Science Company with the GS FLX genome analyzer. Having obtained the library of random DNA fragments, we carried out the clonal amplification of the DNA molecules related to the microparticles in the water-in-oil emulsions, as well as the sequencing with the GS FLX genome analyzer using a reagents kit and following the protocols established by the Roche Laboratory. One run of the device (12 hours) allowed us to determine 100 mln. nt; the average length of "reading" was about 220 nt.

The set of overlapping sequences obtained using the GS FLX genome analyzer was then assembled into contiguous clones using the GS *de novo* Assembler program pack (Roche Diagnostics, Roche Applied Science). Finally, the complete nucleotide sequence of the contiguous clone was determined to be mitochondrial genome, 14, 277 nt in length. The average mtDNA reading frequency was 30.

ANALYSIS OF BIOINFORMATION

The analysis both of the sequences and of the assembled genome was performed with the Vector NTI 7 program (Informax Inc.). Similar sequences were searched for in the GenBank´s biological sequences databases (http://www.ncbi.nlm.nih.gov/blast). The flatworm´s mitochondrial genetic code was used to translate protein-coding sequences [8]. Most tRNA were detected by the tRNAscan-SE program, [9] while secondary structures of other flatworms were found manually.

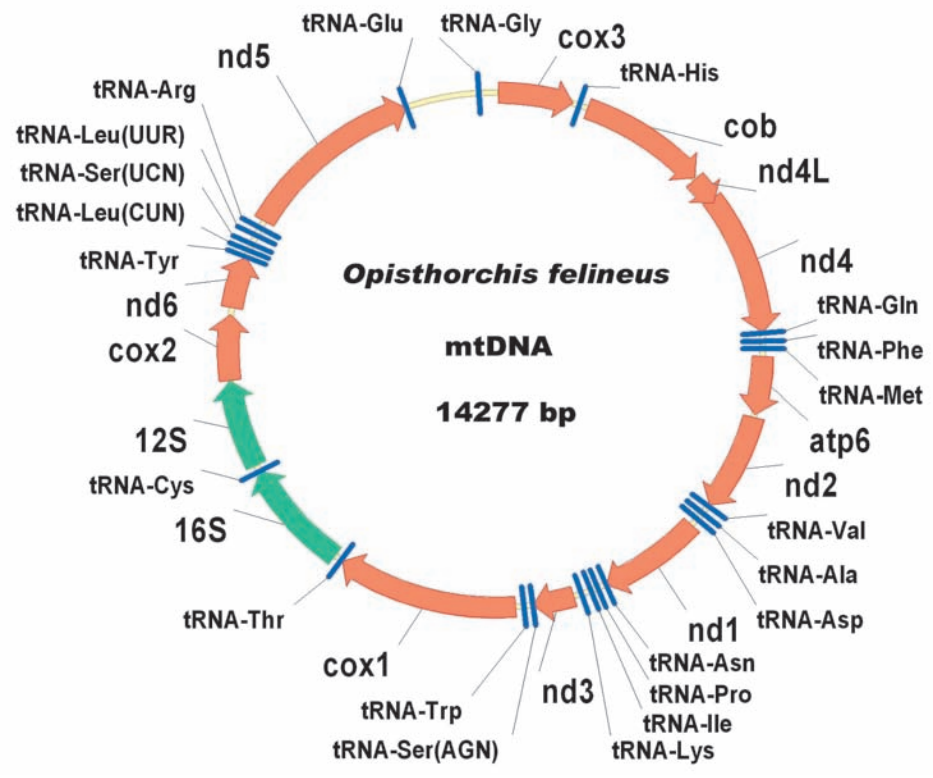

Fig. 1. Genetic map of the *O. felineus* mitochondrial genome.

In order to identify potential single nucleotide polymorphisms (SNP), some sequences determined during the course of sequencing were aligned relative to the "consensus" sequence of *O. felineus* mtDNA using the GS reference mapper program (Roche). SNPs were detected during the course of at least three individual readings at those points where their sequences did not coincide with the "consensus" sequence. All points where the complete mtDNA sequences determined by capillary electrophoresis and high throughput sequencing methods were not consistent were referred to as SNP as well.

RESULTS AND DISCUSSION

METHODS OF MTDNA SEQUENCING

Due to their relatively short lengths, animal mitochondrial genomes were among the first objects of genomic investigation [10], and to date, hundreds of mtDNA sequences are known.

The standard method for decoding the mitochondrial genome involves the recovery of mitochondria from the cells and the creation of a mitochondrial DNA sample maximally purified of genome DNA. The following Sanger sequencing suggests the genome decomposition into randomly chosen fragments, cloning using the plasmid vector (library of random fragments), and sequencing of the clones produced using capillary electrophoresis. Since the fragments are overlapping, the sequences produced may be combined into a complete mtDNA sequence. In the present study, for the specific recovery of mitochondrial sequences, we used data on the mtDNA structure of closely related helminthes, which allowed us to identify the conserved sites of the genome, and to amplify the *O. felineus* mtDNA fragments occurring between them using the PCR method. The sequences of the fragments obtained were determined by capillary electrophoresis and were combined into a complete mtDNA sequence 14,277 nt in length.

A new method, which makes it possible to detect the genome sequences *de novo*, is the high throughput sequencing method [11], developed by the 454 Life Science Company using the GS FLX genome analyzer. This method involves the fragmentation of up to 300-800 nt of DNA, the amplification of the individual DNA fragments related to microparticles in microdrops formed in the water-in-oil emulsions, the injection of nanoparticles containing immobilized amplified fragments into the microcells on the glass sheet, parallel high throughput sequencing, and the registration of the results obtained from each of the few hundred thousand cells on the glass sheet. The average reading length is approximately 200 nt, and one run of the device can analyze a sequence up to 100 mln nt in length. The large volume of sequences detected using this method allowed us to reject the specific recovery of the mitochondrial genome fragments and to use the sample of "total" *O. felineus* genome DNA for the sequencing. In spite of the fact that the share of the mtDNA sequences was less that 1% of the whole sequencing volume, it was enough for the reading of mtDNA with 30-fold overlapping that provided a complete "assembly" of the mitochondrial genome sequence following only one run of the GS FLX genome analyzer.

MAJOR CHARACTERISTICS OF THE O. FELINEUS MITOCHONDRIAL GENOME

The *O. felineus* mitochondrial genome is a ring molecule, 14,277 nt in length. It is the shortest among the currently known mitochondrial genomes of trematodes [12]. Analysis

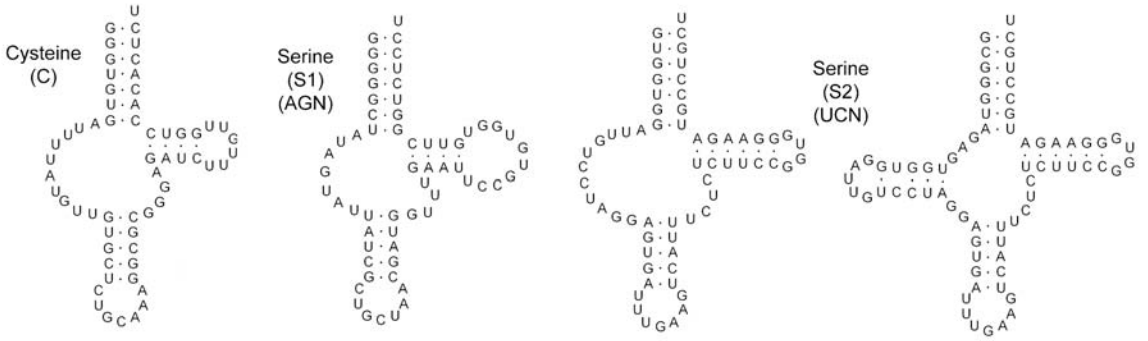

Fig. 2. Secondary structures of tRNA: tRNA-Ser(AGN), tRNA-Cys, and two possible alternative structures of tRNA-Ser(UCN).

Table 2. Frequency of occurrence of different codons in the *O. felineus* mitochondrial genome.

UUU	Phe	334	9.9 %	UCU	Ser	107	3.2 %	UAU	Tyr	146	4.3 %	UGU	Cys	89	2.6 %
UUC	Phe	40	1.2 %	UCC	Ser	22	0.7 %	UAC	Tyr	17	0.5 %	UGC	Cys	15	0.4 %
UUA	Leu	125	3.7 %	UCA	Ser	26	0.8 %	UAA	-	0	0 %	UGA	Trp	33	1.0 %
UUG	Leu	236	7.0 %	UCG	Ser	36	1.1 %	UAG	stop	12	0.4 %	UGG	Trp	80	2.4 %
CUU	Leu	107	3.2 %	CCU	Pro	43	1.3 %	CAU	His	49	1.5 %	CGU	Arg	47	1.4 %
CUC	Leu	14	0.4 %	CCC	Pro	19	0.6 %	CAC	His	7	0.2 %	CGC	Arg	4	0.1 %
CUA	Leu	26	0.8 %	CCA	Pro	7	0.2 %	CAA	Gln	12	0.4 %	CGA	Arg	3	0.1 %
CUG	Leu	41	1.2 %	CCG	Pro	21	0.6 %	CAG	Gln	21	0.6 %	CGG	Arg	23	0.7 %
AUU	Ile	103	3.1 %	ACU	Thr	60	1.8 %	AAU	Asn	44	1.3 %	AGU	Ser	72	2.1 %
AUC	Ile	18	0.5 %	ACC	Thr	11	0.3 %	AAC	Asn	7	0.2 %	AGC	Ser	15	0.4 %
AUA	Met	55	1.6 %	ACA	Thr	8	0.2 %	AAA	Asn	17	0.5 %	AGA	Ser	15	0.4 %
AUG	Met	101	3.0 %	ACG	Thr	16	0.5 %	AAG	Lys	44	1.3 %	AGG	Ser	47	1.4 %
GUU	Val	215	6.4 %	GCU	Ala	21	0.6 %	GAU	Asp	69	2.0 %	GGU	Gly	133	3.9 %
GUC	Val	15	0.4 %	GCC	Ala	76	2.3 %	GAC	Asp	3	0.1 %	GGC	Gly	35	1.0 %
GUA	Val	39	1.2 %	GCA	Ala	5	0.2 %	GAA	Glu	6	0.2 %	GGA	Gly	28	0.8 %
GUG	Val	138	4.1 %	GCG	Ala	36	1.1 %	GAG	Glu	71	2.1 %	GGG	Gly	105	3.1 %

The corresponding amino-acid and the frequency of occurrence in the mtDNA genes are indicated for each codon. Differences from the standard genetic code are underlined.

of the genome sequence confirmed the presence of typical mitochondrial genes: 12 protein-coding genes (ATP-synthetase subunit 8 is absent), 22 tRNA-, and 2 rRNA-coding genes (Table 1).

As with other flatworms, all genes are transcribed from one chain (Fig. 1). The gene sequence of the *O. felineus* mitochondrial genome is similar to that of *F. hepatica* [13]; 40 pn of *nd4L* and *nd4* genes are overlapped for different reading frames.

All well-known flatworm mitochondrial genomes, except for the *P. westermani* genome, are A/T-rich. The *O. felineus* mitochondrial genome contains 60% A+T; moreover, the coding strand is rich in thimine (43%) compared to adenine (17%), guanine (28%), and cytosine (12%). The nucleotide composition is variable in different parts of the *O. felineus* genome, especially in the third position of codons of protein-coding genes, where the cytosine content is only 8%. Codons ending in T and G are more frequent than those ending in A and C. The most frequently appearing codons are TTT, GTT, and TTG. The percentage of TTT codons represents almost 10% of the total number, while all codons composed of A and C account for only 2% (Table 2). As with other trematode mitochondrial genomes, the start-codons are ATG and GTG, while the stop-codon is TAG. The TGA codon codes for tryptophan, while TAA is not used at all. Truncated stop-codons were not found in the *O. felineus* mitochondrial genome (Table 1).

The length of tRNA genes in the *O. felineus* mitochondrial genome ranges from 59 to 72 nucleotides. Most tRNA genes are combined in clusters composed of up to five genes. Nineteen out of 22 tRNA genes are characterized by the typical "clover leaf" structure. As in all trematodes, tRNA-Ser(AGN) is lacking in the DHU-loop. The tRNA-Cys, as in some schistosomes, does not have the DHU-loop [14]. The tRNA-Ser(UCN) gene can have two alternative structures: one with the DHU-loop and one without it (Fig. 2).

In addition to short intervals between consecutive genes, flatworm genomes often have long non-coding regions, which are believed to be sequences necessary for the initiation of the mtDNA replication and transcription. As in the *F. hepatica* genome, the *O. felineus* non-coding region located between the *tRNA-Glu* and *cox3* genes is divided into 2 parts

Table 3. Single nucleotide polymorphisms detected in the *O. felineus* mitogenome.

№	Number of occurrences in mtDNA and allele determined by Sanger sequencing		Allele frequencies in sequences determined duringt the course of high throughput sequencing	Gene	Replaceable codon	Amino-acid replacement
1	361	T	C-3 / T-27	cox3	tgg/cgg	W/R
2	1068	C	T-3 / C-25	cytB	tac/tat	-
3	1195	C	T-8 / C-17	cytB	cta/tta	-
4	1300	C	T-4 / C-23	cytB	ctg/ttg	-
5	1524	A	A-1 / G-28	cytB	caa/cag	-
6	1599	T	C-5 / T-28	cytB	ctg/ccg	L/P
7	1860	C	C-1 / T-24	cytB	ggc/ggt	-
8	1899	C	T-4 / C-24	nd4L	cct/tct	P/S
9	2025	T	C-4 / T-28	nd4L	tta/cta	-
10	2034	C	T-4 / C-28	nd4L	cgg/tgg	R/W
11	2039	C	T-4 / C-28	nd4L	ggc/ggt	-
12	3104	T	C-5 / T-19	nd4	ttg/ctg	-
13	3228	C	T-3 / C-25	nd4	gcg/gtg	A/V
14	3245	T	C-3 / T-27	nd4	tta/cta	-
15	3260	C	T-3 / C-26	nd4	ctg/ttg	-
16	3674	A	G-6 / A-28	atp6	aat/agt	N/S
17	3827	G	G-0 / A-39	atp6	ggt/gat	G/D
18	3915	A	A-0 / G-34	atp6	cta/ctg	-
19	3921	T	C-6 / T-28	atp6	tat/tac	-
20	3935	T	T-0 / C-35	atp6	gtg/gcg	V/A
21	4507	A	A-1 / G-20	nd2	ata/gta	I/V
22	4548	C	T-11 / C-14	nd2	agc/agt	-
23	4707	T	T-0 / C-33	nd2	tct/tcc	-
24	4710	T	T-0 / G-33	nd2	cct/ccg	-
25	5390	T	T-0 / A-36	nd1	aat/aaa	N/K
26	5684	C	C-2 / T-21	nd1	gcc/gct	-
27	6158	C	T-5 / C-32	-		
28	6175	C	T-5 / C-33	tRNA-Asn		
29	6314	T	C-3 / T-24	tRNA-Ile		
30	6650	A	A-2 / G-30	nad3	gta/gtg	-
31	6810	C	T-4 / C-30	-		
32	7865	A	A-0 / G-31	cox1	tca/tcg	-
33	8669	A	G-4 / A-27	16S rRNA		
34	10692	T	C-3 / T-17	cox2	ata/aca	I/T
35	11152	A	G-8 / A-23	nd6	cca/ccg	-
36	11880	C	T-4 / C-36	tRNA-Arg		
37	12186	G	G-1 / C-25	nd5	gtt/ctt	V/L
38	12326	T	A-3 / T-24	nd5	cgt/cga	-
39	12533	G	A-5 / G-22	nd5	gtg/gta	-
40	13403	G	A-4 / G-23	nd5	tcg/tca	-
41	13589	G	G-0 / A-11	-		
42	13993	C	C-0 / T-30	-		
43	14001	G	G-0 / A-18	-		
44	14007	C	C-0 / T-8	-		
45	14212	C	C-0 / T-9	-		

by the tRNA-Gly gene. In contrast to the non-coding regions of the mitochondrial genomes of other flatworms, the *O. felineus* non-coding regions contain neither long tandem repeats, nor sequences able to form long hairpin structures.

An open reading frame, 402 pn in length, was detected in the *O. felineus* non-coding region. A search for similar sequences within the database of biological sequences using both the nucleotide and the amino-acid sequence did not yield any results. Quite long open reading frames different from well-know proteins were also found in the non-coding regions of the mtDNA of other flatworm species: *F. hepatica*, cestodes *Hymenolepis diminuta* [15], and monoge-

neas *Microcotyle sebastis* [16]. These reading frames likely code for functional proteins; however, this hypothesis needs to be investigated further in future studies.

The mtDNA non-coding region may be used to develop a molecular method for the specific identification of *O. felineus*. The homology levels between the *O. felineus* mtDNA sequences and that of two related trematodes, C. sinensis (FJ381664) and F. hepatica (AF216697), from which the mitochondrial genome sequences are well-known, amount to 78% and 64%, respectively. However, these three sequences of non-coding regions located between the *tRNA-Glu* and *cox3* genes do not have significant homology either between themselves or with other sequences contained in the GenBank.

SINGLE NUCLEOTIDE POLYMORPHISMS IN THE O. FELINEUS MTDNA

In the course of the mtDNA sequencing using the high throughput sequencing method, each nucleotide in the genome was "read" an average of 30 times in the process of sequencing of the clonal-amplified individual fragments of the *O. felineus mtDNA* molecules. Comparison of the sequences obtained during the course of individual reading with the consensus sequence permitted the identification of single nucleotide polymorphisms (SNP), which are present in different mtDNA molecules in one organism. Since both Sanger sequencing and high throughput sequencing were performed with DNA recovered from several *O. felineus* species, comparing the corresponding mtDNA sequences makes it possible to estimate the frequency of hyplotypes occurrence in each SNP.

Data from 45 detected SNPs is presented in Table 3. Most SNPs in both animal and human mtDNA [17] involve T:C and A:G substitutions (corresponding to T:C on the lower strand), which do not cause an amino-acid substitution in the protein products of the corresponding genes. It should be noted that some SNPs looked specific for the mtDNA sequence decoded by one of two technologies and were not found (or were only rarely found) in other sequences. The difference in allele frequency is likely to be the result of errors specific to the PCR-based methods for the amplification and sequencing of the heterogeneous amplicon mixture, while the ratio of SNP alleles obtained in the process of sequencing individual fragments must be extremely precise.

In the future, the data on specific SNPs and their frequency of occurrence in mtDNA may be used as molecular markers in studies of the natural populations of *O. felineus*, as well as in the analysis of the pathogenic pathways of this trematode in human populations.

CONCLUSION

This review contains the results of the complete sequencing of the *O. felineus* flatworm mtDNA obtained using two methods. The first method involved the amplification and sequencing of the mtDNA using capillary electrophoresis. Parallel high throughput sequencing of the animal genome DNA sample is performed without any preliminary enrichment with the mtDNA sequences. This enables the complete *de-novo* sequencing of the mitochondrial genome. The high throughput sequencing method using the GS FLX genome analyzer may be used for the rapid decoding of animal mitochondrial genomes and for the identification of polymorphisms. The newly generated data on the nucleotide sequence of the *O. felineus* mitochondrial genome may be utilized in the development of specific molecular diagnostic methods for opisthorchiasis.

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The Organization in Micro-Loops of an Extended Fragment of Chicken Chromosome 14, Including the Alpha Globin Gene Cluster in the Erythroid Cells

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ABSTRACT It has been shown that the activation of tissue-specific gene transcription during the course of cell differentiation is associated with a spatial reorganization of the genomic domains harboring those specific genes. This reorganization consists of the relocation to the nuclear matrix of the whole genomic domain containing one or more of the genes being transcribed. However, it remains unclear whether, during this process, extended areas of the genome also become attached to the nuclear matrix. We studied the genome´s pattern of interaction with the nuclear matrix in both erythroid and non-erythroid cells of chickens, using a 220Kb region of chromosome #14, which contains the alpha-globin gene cluster and some surrounding house-keeping genes. The results show that in erythroid cells, the fragment of the genome containing the alpha-globin gene domain became spatially arranged into micro-loops which could not be detected by mapping experiments.

In eukaryotic cells, chromosomal DNA is organized into a series of loops which are attached to the nuclear matrix [1,2]. The attachment sites for DNA on the nuclear matrix are all different, yet it is possible to distinguish between permanent (stable, structural) DNA attachment sites, which exist in cells of different lineages, and facultative (i.e. temporary, functional, tissue-specific) attachment sites, which are found only in cells in a particular lineage or during a particular stage of differentiation [2,5]. In the present study, we characterized the spatial organization of a large (220 Kb) segment of the chicken chromosome 14, which includes a cluster of erythroid-cell specific alpha-globin genes, as well as a number of open reading frames (Fig. 1) [6]. For the purpose of distinguishing between the various types of interactions between DNA and the nuclear matrix, the spatial organization of the above-mentioned region was studied in both erythroid- and non-erythroid cells. Virus-transformed chicken erythroblasts, from the HD3 cell line (A6 clone of the LSCC cell line), and cells from the lymphoid cell line DT40 (CRL-2111, ATCC) were used as cellular models.

In previous experiments with lymphoid cells, using *in-situ* hybridization with nuclear halos of BAC-probes representing the analyzed area of the genome, we have shown that this area is spatially arranged in small loops [8]. However, in erythroid cells, the spatial arrangement of the same area of the genome is quite different; here the entire genomic segment being studied collapsed onto the nuclear matrix and could be visualized as a dot following *in-situ* hybridization of the corresponding DNA BAC clone with the nuclear halos[8].

It has been previously reported that active, tissue-specific genes associate with the nuclear matrix [9-11]. In this respect it seemed only logical to assume that the collapse of the DNA loop which was observed in chicken erythroblasts was a consequence of the activation of tissue-specific genes

during the course of erythroid differentiation. In order to obtain a map of the DNA interaction with the nuclear matrix for the area of the genome currently being studied, we adopted the following approach: short test fragments were distributed over the whole length of the genome being studied, with a distance of 5 Kb separating consecutive test fragments, and then the relative representation of each of these test fragments in the nuclear matrix DNA (compared to the total DNA) was determined using a semi-quantitative PCR analysis (Fig. 1). The primer sequences used for these PCR reactions are listed in Table 1.

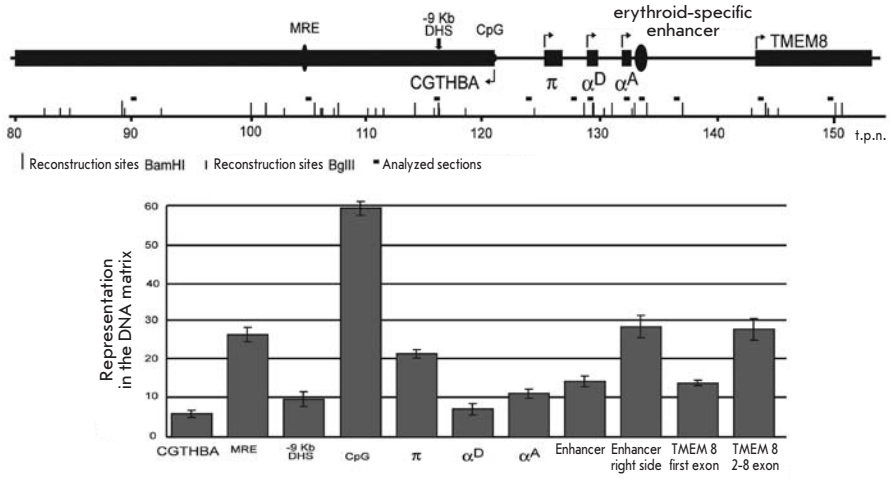
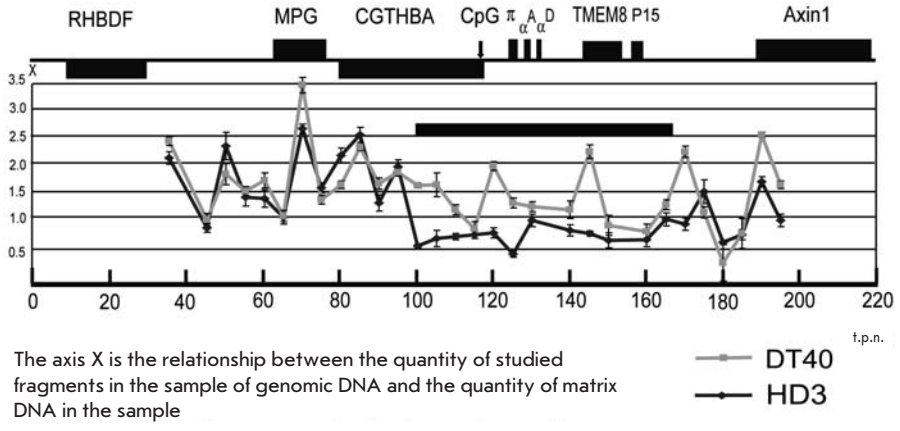


Figure 1. Mapping attachment sites of DNA to the nuclear matrix for a 220 Kb segment of chicken chromosome 14, which includes the α-globin gene cluster.
At the top of the figure, a scheme of the genome area under study is shown. Within the scheme: RHBDF - a gene encoding the epidermal growth factor receptor; MPG - a gene encoding the N-methylpurine-DNA glycosylase; CGTHBA - a housekeeping gene of unknown function; CpG – CpG island containing an origin of DNA replication; ϖ - embryonic α-type globin gene; α^A and α^D "adult" α - globin genes; TMEM8 – an ORF encoding an unknown transmembrane protein; P15 – a gene encoding mitochondrial ribosomal protein L28; Axin1 – a gene encoding axin 1. Kb – thousand pairs of nucleotides.
In the bottom part of the figure, the results of mapping sites of DNA attachment to the nuclear matrix are presented in the form of a graph (see the text for details). The black rectangle represents the extended area, which appears entirely attached to the nuclear matrix in HD3 cells.



The axis X is the relationship between the quantity of studied fragments in the sample of genomic DNA and the quantity of matrix DNA in the sample

Figure 2. The analysis of relative representation within the nuclear matrix DNA of DNA test-fragments located within genes and various regulatory elements. The positions of test fragments are shown by black horizontal lines under the map of the studied genome area. The sites of DNA cleavage by Bam HI and Bgl I restriction enzymes are shown by long and short vertical lines, respectively, above the scale. Columns in the diagram at the bottom of the figure indicate the relative representation of test fragments within the nuclear matrix DNA.

Table1. List of the primers used in semi-quantitative PCR for mapping sites of DNA attachment to the nuclear matrix

name	Sequence
01 dir	5' CTCATTTGCCAGCGAGATAT 3'
01 rev	5' GCCTCGATGGTGCAGTAAGC 3'
02 dir	5' CCCTTGTAGGCTGCAACCCG 3'
02 rev	5' ACAGTCCCTTTTCCATCACC 3'
03 dir	5' CATCTGTGCATCCGTTCTAC 3'
03 rev	5' TCTTCTAAAGTGCCACCATC 3'
04 dir	5' CCCTATTTTCAGGGTTATTA 3'
04 rev	5' AAATGTAAAGCGATTGGTAG 3'
05 dir	5' GCGTGGAGTAGTTCAGGTAA 3'
05 rev	5' GACACTGAGTCCCACCAAAG 3'
06 dir	5' CACTTGTGAATGCAGGTTAA 3'
06 rev	5' TCTTCTAAGATTGCCGTGTT 3'
07 dir	5' GGCTGCATAGCATTACTTTC 3'
07 rev	5' GTAGGACTTAACACCAACGT 3'
08 dir	5' TCCATGTAAGGAGCAGATTT 3'
08 rev	5' GTGTTTTGGGAGCAGTGAGT 3'
09 dir	5' TCATTCTGCCTGACCACTTT 3'
09 rev	5' CCTCCTCAGAGCCAACCAAA 3'
10 dir	5' GGAAACTCAGGGCTCACAAT 3'
10 rev	5' GCAAGGCCAAGAAGCAATAT 3'
11 dir	5' AAGCACTCAGAAGCACAACA 3'
11 rev	5' GCCAGACTTACGAAATCAAA 3'
12 dir	5' CACAGGACTATCCAGGTATG 3'
12 rev	5' AGGGCTGTCAGTCTTCAGTA 3'
13 dir	5' GCTGCTCTACCTTGTTCTCA 3'
13 rev	5' GCCTTGTTCTCTTCCCTACC 3'
14 dir	5' CTGCCTCATGTTTGTTAAGA 3'
14 rev	5' CAAAGTCCCAAAAGCTATAA 3'
15 dir	5' ATTACCAAGCCTACTTCATT 3'
15 rev	5' GTTGAGACTTTGATCTGTGG 3'
16 dir	5' CAGAGCTCAATTCCATAGG 3'
16 rev	5' TTATCTGGGGTACCTGCAT 3'
17 dir	5' TGTTCCCTGGTACTCGTCAG 3'
17 rev	5' TCACCGCATATCGACTCCGT 3'
18 dir	5' GCAGACTCTTAGATTGGCAT 3'
18 rev	5' CTCAGTCAGAACAGAGGAAA 3'
19 dir	5' GTGAAAAAAATCCACTGTAAA 3'
19 rev	5' ATCTAAAGCCAATGAAGAAAA 3'
20 dir	5' TATCCCTCCCTGCCTTACCC 3'
20 rev	5' AGGCAGCCACTACCTTCTTG 3'
21 dir	5' GCCCTTCGTGTCCTTGATTT 3'
21 rev	5' ATTCCAGCAGCCTTTCTTCC 3'
22 dir	5' ACCTCATCACCCTTCCACAT 3'
22 rev	5' TGCCACAAACCATCGTCTCA 3'
23 dir	5' TCAGGAGCACCACCTTTAGA 3'
23 rev	5' TTGTGGCAGCACTTCGGTAA3'
24 dir	5' GGAGTGCTACTTCCTTTGAT 3'
24 rev	5' TGGTAATGTTCCTACTGGGT 3'
25 dir	5' AAGCGTGGTGCATGTGGAAA 3'
25 rev	5' TGAGTCTGCTGATGGGTCTG 3'
26 dir	5' TCCCTAACAATGTGAGTTCC 3'
26 rev	5' CTGCTGCATACAGTCTTGGA 3'
27 dir	5' GTGCTTATCTGAGCCCTTCC 3'
27 rev	5' CAGTATCACCCAGCTCCACA 3'
28 dir	5' TTCAAATCACTTACGCTACA 3'
28 rev	5' CTGGTTATCTGCCTACTCTG 3'
29 dir	5' TGGATGCTGACAGTGCTTGA 3'
29 rev	5' TGCGGTGAAAGAGTTGGAGT 3'
30 dir	5' CAGTCTATCTCCCGTTGCTA 3'
30 rev	5' AAACCTTACGGCTGGTCTCA 3'

The nuclear matrix DNA was recovered as described [11-12], employing a relatively mild nuclease treatment, so that the average size of the nuclear matrix DNA fragments recovered was ~ 5 Kb. The results of this analysis are presented in Fig. 1, as a ratio of the concentration of each test fragment in the total DNA versus its concentration in the nuclear matrix DNA. On the graph shown in Fig. 1, the regions bound to the nuclear matrix would be expected to lie close to the x-axis, while regions arranged in loops should be represented by peaks. The regions containing the CpG island upstream from the α-globin gene domain were previously shown to be permanently bound to the nuclear matrix [5] and thus served as a kind of internal positive control. An analysis of the results shown in Fig. 1 suggests that in the DT40 lymphoid cell line the attachment sites on the matrix DNA are distributed regularly along the entire length of the genome being studied, at an average distance of approximately 20 to 30 Kb. These results are in keeping with previous data obtained using a different experimental approach [8]. In erythroid cells, the larger part of the genome area under study (fragment corresponding to the coordinates 95-165 Kb in Fig. 1) appeared attached to the nuclear matrix. Within this fragment, there was no alternation of nuclear matrix-bound regions and unbound regions, while in the segments flanking this region, this alternating arrangement can be clearly seen. The long DNA fragment attached to the nuclear matrix harbors the cluster of α-globin genes, as well as genes P15, TMEM8, and a part of gene CGTHBA. The spatial arrangement of this segment of the genome, whose coordinates in Fig. 1 are 35 – 95 Kb, is similar in both HD3 and DT40 cell lines. This area of the genome also contains housekeeping genes that are transcribed at a low rate in both erythroid and lymphoid cells [13]. All of the results obtained correlate well with previous data showing the spatial arrangement of the alpha-globin gene cluster and the flanking areas of the genome obtained using galo-FISH [8]. It remains unclear, however, whether the area of the genome represented by coordinates 95 – 165 Kb (Fig. 1) is entirely attached to the nuclear matrix. An alternative model suggests that this area of the genome is spatially arranged into small loops (micro-loops) which cannot be recognized in our experiments due to the relatively large (5 Kb) distances between the test fragments, and the large size of the matrix DNA fragments. In order to test this possibility, we conducted a more detailed analysis of the association of several functionally important regions within the area under study and the nuclear matrix. In these experiments, the HD3 erythroid cells were used as the cellular model. The nuclear matrix DNA was obtained after the distal portions of the DNA loops were cleaved using the restriction enzymes Bam HI and Bam HII. The relative representation of DNA fragments containing globin genes and different regulatory elements within this preparation of matrix DNA was determined by real-time PCR analysis.

The nucleotide sequences of the TaqMan probes and the PCR primers used are presented in Table II. The results (diagram in Fig. 2) demonstrate that the relative representation in the nuclear matrix DNA of different DNA fragments from the apparently attached area was not the same. DNA fragments containing the CpG island upstream from

Table2. Primers and TaqMan probes for mapping sites of DNA attachment to the nuclear matrix using real-time PCR

name	sequence
TMEM8 2-8 exon probe	5' FAM CACTGTAACT(TBHQ1) TTGTGTTTTGTGCCTGTAGC 3'
TMEM8 2-8 exon dir	5' AGGCTCCAGCAGTGAGATCC 3'
TMEM8 2-8 exon rev	5' GACCTGGGCATACAAGATAAGC 3'
TMEM 1- exon probe	5' FAM CTACAACAGCCTCACT(BHQ1) GTGAAGCTCTCTC 3'
TMEM 1- exon dir	5' AGGAGCTATCAAATGCAGTGTCT 3'
TMEM 1 exon rev	5' AGGTACAGAAAGGTCCAGAAACA 3'
DHS -9 probe	5' FAM ATTTGATCCTAGATT(BHQ1) GCCAGTGAATTGAA 3'
DHS -9 dir	5' GCGATATTGAATGTTCTCTAGGA 3'
DHS -9 rev	5' GCTTTGTACTGGATGACTGCC 3'
MRE probe	5' FAM AAGTGTTGACT(BHQ1) CATGGTTTGCTAGTTTGC 3'
MRE dir	5' GCTGCCTCATGTTTGTTAAGATA 3'
MRE rev	5' GTGACTCAGCAAGAACAGCAGA 3'
CGTHBA probe	5' FAM TGAACACAGCAGAACT(BHQ1) GGAAGGCAA 3'
CGTHBA dir	5' CACCAGCATGACTAGGTCTTTG 3'
CGTHBA rev	5' ATCAGGACACATGGTTGGACA 3'
CpG probe	5' FAM CCACAAAT(BHQ1) CAAAGCGATGCGGTAT 3'
CpG dir	5' TTCACAGCACAAGGGATAACT 3'
CpG rev	5' GATCTGAGCTGCATCACTAAATG 3'
alphaD probe	5' FAM AACGCCGT(BHQ1) GAAGAACGTGGACAAC 3'
alphaD dir	5' TGTTCACCACCTATCCCCA 3'
alphaD rev	5' GTTGCTCAGCTCAGCCATG 3'
alphaA probe	5' FAM AGGTAGGTGT(BHQ1) CCTTCTCTGTCCTCCG 3'
alphaA dir	5' AGGGCATCTTCACCAAAATC 3'
alphaA rev	5' GTGGAGCACAGTGAGTCAGG 3'
enh probe	5' FAM AAGTGCTGATGGTTCCT(BHQ1) GTTGGAGTGT 3'
enh dir	5' GCAGACAGGCTGGAGAAGAC 3'
enh rev	5' GGTCATAGCCCAAAGAGCAG 3'
enh right probe	5' FAM TTCAGAGAGTAAGTTCCT(BHQ1) ATGCGTTGCCT 3'
enh right dir	5' TTAGGCTGTGCTCCTCCAAC 3'
enh right rev	5' AACAGGTCGATAAACAGATGCT 3'
Pi probe	5' FAM ACGCAT(BHQ1) GATCCGCACTTGAAATACA 3'
Pi dir	5' GCTCACAGCAGTTTGAAGACCT 3'
Pi rev	5' CAAAAAGCCTGGAGGAGAAC 3'

the α-globin gene domain, the central part of the TMEM8 ORF gene, the embryonic alpha-globin gene π, the distant upstream regulatory element of the α-globin gene domain (MRE), and a region located centrally from the erythroid-specific enhancer were much more abundant within the nuclear matrix DNA than were fragments containing the erythroid-specific enhancer itself, and the globin genes α^D and α^A. Some other regions studied in the above-mentioned experiments were also underrepresented within the nuclear matrix DNA. These include the first exon of open reading frame TMEM8, the erythroid-specific regulatory element located in a hypersensitive DNAse I 9 Kb upstream from the π gene, and part of a housekeeping gene CGTHBA. Based on the above findings, it is possible to conclude that the area of the genome with coordinates 95-165 Kb (Fig. 1) is not entirely and uniformly attached to the nuclear matrix along its length (Fig. 1). Different levels of representation, within the nuclear matrix DNA, of several individual fragments from this area suggest rather that the area is spatially arranged in micro-loops. Due to their small size, these loops could not be identified following hybridization of BAC-probes with nuclear halos.

The spatial organization of the chicken α-globin gene domain has been recently studied using the Chromosome Conformation Capture (3C) experimental approach [14, 15]. It has been found that in HD3 cycling cells (in which globin genes are transcribed at a very low rate) a potentiated chromatin hub is assembled which includes MRE, the CpG island upstream from the α-globin gene domain, and the α^D gene promoter. Here we showed that two of the elements of this chromatin hub are associated with the nuclear matrix, which can promote their mutual interaction. ●

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The Production and Characteristics of a Mouse's Embryonic Stem Cell Lineage, Transfected by the Glia Neurotrophic Factor and Gene Fused with the Green Fluorescent Protein Gene

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ABSTRACT The influence that the expression of the human (glial-derived neurotrophic factor (GDNF)) neurotrophic factor has on the morphology and proliferative activity of embryonic stem cells (SC) of a mouse with R1 lineage, as well as their ability to form embroid bodies (EB), has been studied. Before that, using a PCR (polymerase chain reaction) coupled with reverse transcription, it was shown that, in this very lineage of the embryonic SC, the expression of the receptors' genes is being fulfilled for the neurotropfic RET and GFRα1 glia factor. The mouse's embryonic SC lineage has been obtained, transfected by the human GDNF gene, and has been fused with the "green" fluorescent protein (GFP) gene. The presence of the expression of the human GDNF gene in the cells was shown by northern hybridization and the synthesis of its albuminous product by immunocitochemical coloration with the use of specific antibodies. The reliable slowing-down of the embriod-body formation by the embryonic SC transfected by the GDNF gene has been shown. No significant influence of the expression of the GDNF gene on the morphology and the proliferative activity of the transfected embryonic SCs has been found when compared with the control ones.

Key words: embryonic stem cells, glial-derived neurotrophic factor, transfection, proliferation, immunocitochemistry, emroid bodies.

INTRODUCTION

Glial-derived neurotrophic factor (GDNF) belongs to the GDNF-similar ligand (GFL) family, which includes neurturin (NRTN), persipin (PSPN), and artemin (ARTN). All of these are necessary for the survival of the dopaminergic neurons of the mesencephalon, as well as for peripheral sensory and sympathetic neurons (with the exception of PSPN) [12, 14]. In addition, beyond the nervous system, GDNF fulfills the following important functions: it regulates the differentiation of spermatogonia and is needed for the embryonic development of kidneys [14].

All representatives of the GFL family influence the cells through bonding with the heteroreceptor complex, which contains the RET receptor and GFRα co-receptor [14]. The specificity of the RET receptor activation, in relation to different factors of the GFL family, depends on the GFRα type

contained in the cells. GDNF, NRTN, ARTN, and PSPN have strong affinities to GFRα1, GFRα2, GFRα3, and GFRα4 co-receptors, respectively. GFL binding with the "alien" GFRα is possible, but it happens with less efficiency. It has been shown that GDNF increases the survival rate of the dopaminergic neurons of rats' embryonic mesencephalon in a specific way, making the dopamine metabolism stronger; it also increases the differentiation level of tyrosine hydroxilase-positive cells (TH+), raising the axons' growth and increasing the dimensions of the cell's body. Analogous effects have also been observed in rats' mesencephalon dopaminergic neurons [12]. Similar results have been obtained on fruit-fly transgenic lines. It appeared that a distinct synthesis of tyrosine hydroxilase has been found in the fruit fly's lines bearing the GDNF gene and in differentiating nerve cells, and the fruit fly's cells bearing the GDNF gene were actively producing acetylcholinesterase [2, 3]

Embryonic SCs are able to self-renew and differentiate to the derivatives of all three primary embryonic leaves both *in vitro* and *in vivo*. Most importantly, in the course of the embryonic SCs, the differentiation of the order of expression and the tissue-specific genes correspond to the order of these processes during the development of the organism *in vivo* [7].

The ability of the SCs to differentiate in definite directions *in vitro* makes it possible to study the cell and the molecular mechanisms of early development on this type of model, as well as to receive different types of donor cells for transplantation. Prioritized embryonic SC differentiation in a definite direction is achieved only with specific cultivating conditions [9] after exogenous growth factors are applied and after differentiation [7] by way of their genetic modification [1, 10, 11, 13]. The first research works on embryonic SC-directed differentiation were conducted mostly on mouse embryonic SCs, whose differentiation with the help of the above-mentioned methods could be directed to the hemopoetic cells [16], cardiomyocytes [11], insulin-secreting cells [15], and neurons and glia cells [4, 6]. The use of the combined methods of genetic manipulation with mouse embryonic-SC cultivaton on the feeder layer of stromal cells has shown a significant increase in the number of dopaminergic neurons (approximately twice as many) in the pilot test as in the control one. The results of tests on the transplantation of such cells have shown the efficient integration of the TH+ cells into the mouse's striatum [10]. The induction of dopaminergic differentiation was shown on human embryonic SCs with condition of their cultivation on the stromal P06 cells [18]. Preserving the vitality of the dopaminergic neurons during their transplantation remains essential. Buytaert–Hoefen *et al.* [5] showed that, in order to improve the vitality of the dopaminergic neurons, the presence of a factor such as the GDNF factor or astrocytes from the striatum is necessary. Japanese researchers [17], using the primate embryonic-SC cocultivating technique on Sertoli cells secreting GDNF, found not only an increase in the number of induced dopaminergic neurons over the control-test number, but they also successfully transplanted these cells to the mouse's striatum, which has a pathology similar to Parkinson's disease in humans. Embryonic SCs obtained as a result of definite experimental manipulations, including genetic ones, and possessing a specific type of directed differentiation may be used in the future in cellular therapy of several critical human illnesses.

In connection with this, examining embryonic SCs with increased GDNF expression genes is essential. In order to achieve that, in this article embryonic-SC polyclonal cultures of mice were obtained and transfected with the human GDNF gene fused with the gene that codes the "green" fluorescent protein (GFP); they were then described.

EXPLORATORY PROCEDURE

CULTIVATING MOUSE EMBRYONIC SCS Cultivating the embryonic SCs was done at 37^{0}C and 5% CO_2 in α-MEM (Sigma, United States) medium containing 15% cow fetal serum (CFS) (Gibco, United States), 0.1 mM 2- mercaptoethanol, 2 mM L-glutamine, nonessential amino acids (Gibco, United States), nucleosides, vitamins, and gentamicin antibiotic (20 microgram/ml). As a nourishing (feeder) layer for the embryonic Scs, primary fibroblasts received from embryonic mice in the 11th–12th day of development were used, the proliferation of which was blocked by mitomycin C (3 microgram/ml). The medium for growing the primary fibroblast culture was DMEM (Sigma, United States) containing 10% CFS, 2 mM L-glutamine, and gentamicin antibiotic (20 microgram/ml). When cultivating embryonic SCs without the feeder layer, leukemia inhibiting factor (LIF) (Sigma, United States) was added in a final concentration of 10 ng/ml, which blocked the spontaneous differentiation of these cells. The cells were reseeded and medium-changed every 3 days.

ESTIMATING THE EXPRESSION LEVEL OF THE RET AND GFRα1 RECEPTORS WITH THE HELP OF POLYMERASE CHAIN REACTION COUPLED WITH THE REVERSED TRANSCRIPTION (RT-PCR) The total DNA from the non-differentiated embryonic Scs, which were cultivated without the feeder layer in the presence of LIF, were extracted following the manufacturer's recommendations by the method of phenol-chloroform extraction with the use of the YellowSolve (Clonogen, United States) kit. The reversed transcription procedure was held with the use of the Silex Company (Russia) kit according to the protocol and recommendations of the manufacturer. kDNA synthesis was done on 2 micrograms of the total RNA for 1 h at 37^{0}C in 25 microliters of reaction mixture containing 0.05 micrograms of accidental hexaprimers and 100 units of MMLV (moloney murine leukemia virus) reverse transcriptase. After the cessation of the reaction (10 min incubation at 70^{0}C), the samples of kDNA were kept at −20^{0}C.

Polymerase chain reaction (PCR) was conducted in a 25 microliter reaction mixture composed of a Taq-buffer, 1.5 mM of the dNTP mixture, 1.25 units of "colored" Taq polymerase (Sintol Russia), 0.5 micro grams of kDNA sample, and 10 Pmol of each primer.

The primers' sequences used in the tests are given below. [8]

For the RET gene: 5'-CCTCCGTGACAGCCGCAAGA-3' (forward), 5'-GGGAATCCGGCCCTTGCTTT-3' (reverse); size of the product 297 bps

For the GFRα1 gene: 5'-TCATTGGCAGAAACATCG-

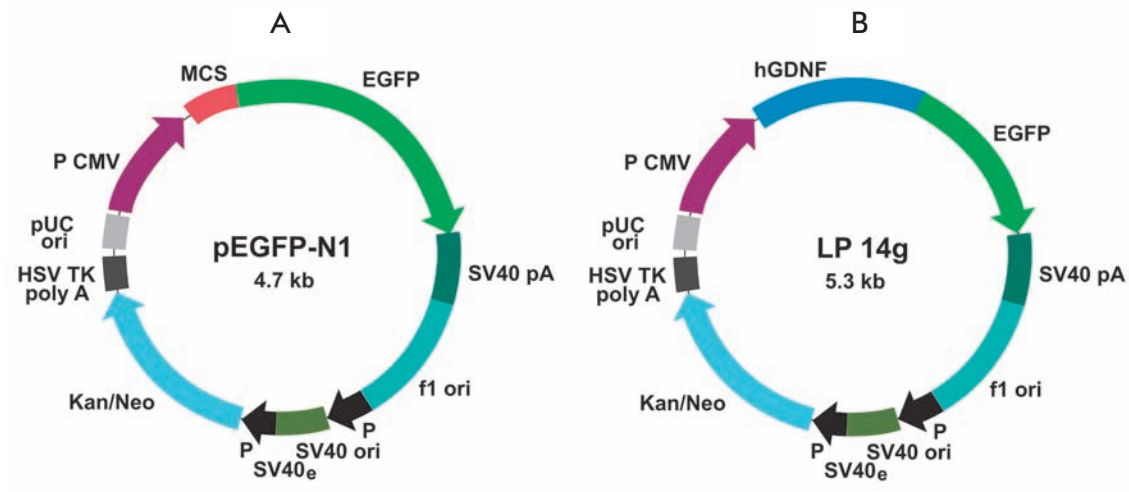

Fig. 1. *pEGFP-N1* and *LP14g* plasmid construction schematics for embryonic SCs transfection. Kan/Neo is a gene providing resistance to antibiotics, EGFP is the "green protein" gene, MCS is the sequence containing restriction sites, and hGDNF is the human GDNF gene insertion.

TAG-3' (forward), 5'-GCTCAGCTTGCTTTACAGTCC-3'(reverse); size of the product 285 bps

Conditions of the PCR were the same for both fragments: 5 min at 52°C, 5 min at 95°C, then 35 cycles, including at 95°C for 30 sec; at 68°C for 30 sec; at 72°C for 30 sec, and at 72°C for 10 min. The products of the PCR were separated by electrophoresis in 1.5% agarose gel with visualization with the help of lower bromide etidium.

NORTHERN HYBRIDIZATION mRNA was extracted from the embryonic SCs using TRI REAGENT (Sigma, United States) according to the protocol of the manufacturer and separated by electrophoresis in 1.5% agarose gel. Then, mRNA was transferred on the nylon membrane. The probe for GDNF gene was marked by the α-^{32}P ATP with the help of the Random Prime Labeling Kit (Promega, United States) in accordance to the instructions of the manufacturer. Prehybridization was carried out in the hybridization buffer (X5 SSPE, 0.1% SDS, X5 Denhardt solution) for 1 h at 65°C. The labeled probe was denaturized for 5 min at 100°C and added to the hybridization buffer.

Hybridization was conducted for 16–20 hours at 65°C on the water bath. Then, the filter was washed 3 times (for 15 min each time) in the X0.l SSC, 0.1% SDS solution and wrapped in a waterproof plastic film and put into the photo film holder with the photo film. Then it was exposed at –70°C for 1 to 5 days.

RECOMBINANT PLASMIDS Following genetic engineering, constructions were used for the cells transfection. *pEGFP-N1* control plasmid (Fig.1A) contained the GFP gene under the (CMV) cytomegalovirus promoter and a gene resistant to neomycin *neo* antibiotic. The size of the plasmid is 4.7 thousand bts. *LP14g* plasmid (Fig.1B) carrying the human GDNF gene fused with the GFP gene was constructed on the *pEGFP-N1* basis. The plasmid's size is 5.3 thousand bps, and the plasmid's structures are shown in Fig. 1.

EMBRYONIC SCS TRANSFECTION AND SELECTION Insertion of the plasmid DNA into embryonic SCs was carried out with the help of electroporation using a SUM4 device (V.A.Engelgardt Molecular Biology Institute, Russian Academy of Sciences) at the following characteristics selected through experiments: impulse duration was 1.5 microseconds, and 400 V voltage. For the transfection of one million cells, 6–8 micrograms of plasmid DNA were used. Trnsfected cells were seeded, 300,000 in each Petri cup (35 mm diameter, gelatin covered (0.01%) in 2 ml of the standard for the embryonic-SC medium with the addition of LIF (10ng/ml)). Selection began on the second day after seeding by adding the antibiotics G418 (200 microgram/ml) to the medium. The selective medium was changed every 3–4 days. Polyclonal cultures of each transfection version were gathered on the 10th day from the cups in the form of a summary pool of G418-resistent clones. Transfected cells were analyzed visually under an Axioscope (Karl Zeiss, Germany) microscope. The average transfection efficiency was about 10^{-4}.

DETERMINING THE PROLIFERATIVE ACTIVITY OF THE EMBRYONIC SCS An estimation of the proliferative activity of the control and transfected lines was conducted on the third day after seeding by directly calculating the cells under an Olympus CKX41 (Olympus, Japan) microscope in the Goryayev chamber.

EMBROID BODY PRODUCTION For the induction of differentiation with the production of embroid bodies (EB), the embryonic SCs were isolated from the fibroblasts of the feeder layer. In order to do this, the cells were processed in tripsin, centrifugated, and then the obtained suspension was incubated in Petri cups (d = 60mm) (Nunc, Denmark) for 15–30 min. During that time, most of the mass of fibroblasts sticks to the cup's bottom while the embryonic SCs remain in the suspension. To form EB, the embryonic SCs suspension was transferred either to the Petri cup (d = 60mm) (Nunc, Denmark) in an amount of 500,000 or to the 96-well immunology plate (1,000 cells on each well) and then placed into a CO_2-incubator (5% CO_2). Calculating the number of the produced EB was done on the 3th–4th cultivation day.

To form single EBs, the "hanging drop" method was applied [7]. Embryonic SCs cultivated on the gelatin substrate in the medium with LIF (Sigma, United States) added were

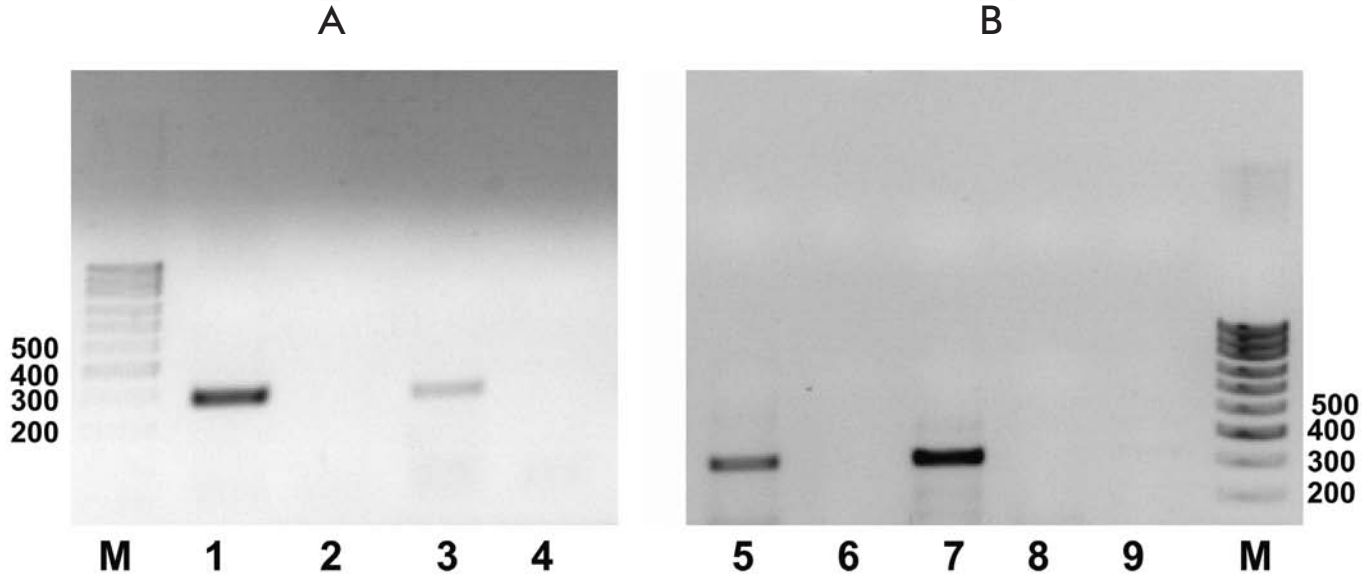

Fig. 2. Receptors GFRα1 and RET expression analysis in the R1 line mouse embryonic SCs.
(A) is the GFRα1 receptor's expression. M is the fragments lengths marker, 1 is the mouse's hippocampus (positive control), 2 is the mouse's hippocampus control on the genomic DNA impurity, 3 is the line R1 embryonic SCs, and 4 is the line R1 embryonic SCs control on the genomic DNA impurity. The length of the product is 285 bps
(B) is the RET receptor expression, 5 is the hippocampus (positive control), 6 is the mouse's hippocampus control on the genomic DNA impurity, 7 is the R1 line embryonic SC R1- line, 8 is the R1 line embryonic SC control on the genomic DNA impurity, and 9 is the water. The length of the product is 297 bps

used for this. Cells were processed with tripsin, and a suspension with 25,000 cells/ml was prepared. Fifty drops of 20 microliters containing 500 cells each were placed on the lid of a Petri cup (Nunc, Denmark) 60 mm in diameter. In order to get a humid atmosphere, Hanks solutions (2 ml) were added to the cups and then they were placed into the CO_2-incubator (5% CO_2). On the third cultivating day, the produced EBs were transferred to the 4-well substrates (the diameter of one well is 15 mm) precoated with gelatin for further differentiation.

AN IMMUNOCYTOCHEMICAL ANALYSIS OF THE PRESENCE OF THE GDNF HUMAN GENE PROTEIN PRODUCT IN THE TRANSFECTED LINES OF EMBRYONIC SCS Three-day-old EBs were cultivated in the 4-well tray with a 15-mm well diameter (Nunc, Denmark) (3–4 bodies per one well) processed with 0.01% gelatin in 700 microliters of embryonic SCs cultivating medium not containing LIF. On the 7th cultivation day, differentiated cells were fixed by 4% paraformaldehyde in PBS for 30 min at room temperature. After being washed three times, PBS cells were pre-incubated in the PBS solution containing 0.1% Triton X-100 and 5% FBS for 15 min at room temperature. Chicken polyclonal antibodies were used as primary antibodies against human GDNF (Promega, United States) in a 1 : 30 dilution in the PBS-0.1% Triton-5% FBS solution. Incubation was conducted for one night at +4°C. After washing PBS three times, secondary biotynilated rabbit antibodies were brought on against chicken immunoglobulins (Imtek,

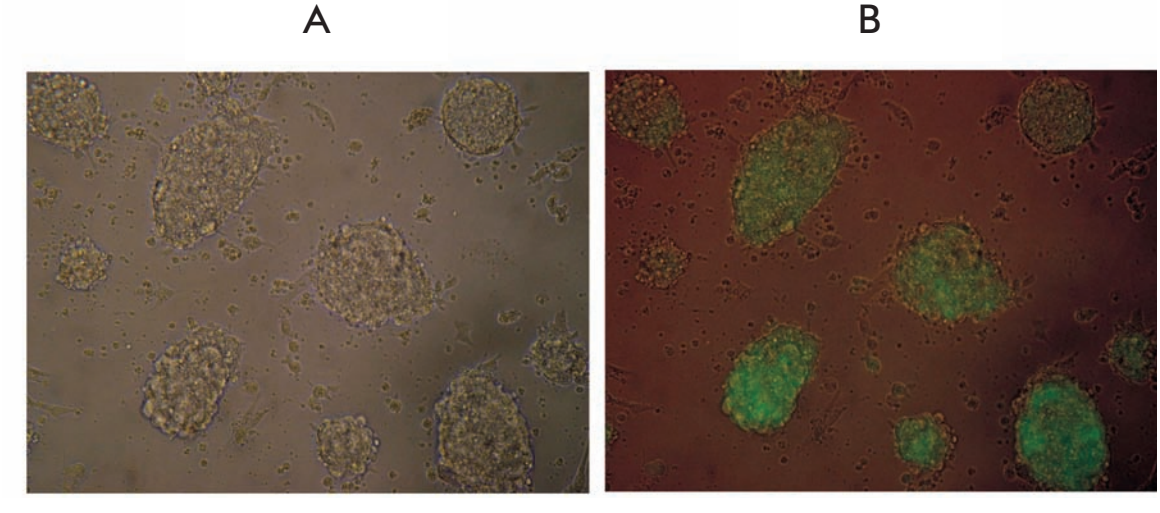

Fig. 3. es-GDNF line cells colonies in (A) the light microscope and (B) fluorescent microscope (x 100).

Russia) in a dilation of 1 : 500 and incubated for 2 h at room temperature and then processed in a peroxidase conjugate with streptavidin (Imtek, Russia) in a dilution of 1 : 400 for 1 h at room temperature. The reaction was developed by the use of a 0.015% solution of 3-amino-9-ethylcarbasol (Sigma, United States) in 50 mM Tris-citrate pH 7.0 buffer containing 0.06% H_2O_2. Coloring development was visually controlled with using an Olympus CKX41 (Olympus, Japan) microscope. The reaction ended with the cells being washed three times with distilled water.

STATISTICAL ANALYSIS Results were processed with the help of Sigma Plot (Jandel Scientific, United States) software. The validity of the group average differences were estimated with the help of dispersion analysis (one-way ANOVA). Results were represented in the form of standard error (mean ± SEM). Values of differences at $p < 0.05$ were considered valid.

RESEARCH RESULTS

During the initial stages of work, the presence of en expression of the receptors for GDNF - RET and GFRα1 on the мРНК level was determined in the embryonic SCs of the initial R1 line mouse with the help of RT (PCR). The findings are presented in Figs. 2A and 2B. It is seen from this information that embryonic SCs are expressed by both GFRα1 and RET, though with different efficiencies.

Two lines were obtained as a result of transfection with corresponding recombinant plasmids and further selection of the embryonic SCs: es-GDNF (the line bearing the GDNF gene fused with the "green protein" gene) and es-GFP (the control line only, with the "green protein" gene). The presence of the GFP gene insertion makes detecting the cell colonies easier after the transfection has been carried out; it also makes it easier to investigate the ways and patterns of the embryonic SCs differentiation, especially in *in vivo* tests. The green glow was observed in more than 80% (Fig. 3B) of transfected cells in the received cells' lineages. With the help of northern hybridization, coupled with the use of the labeled GDNF fragment, its expression in the es-GDNF line cells (Fig.4) was shown. Besides, as can also be seen from this picture, the expression of the endogenous GDNF gene happens in the embryonic SCs. To detect the protein product synthesis in this gene, the differentiated cells of the es-GDNF and es-GFP were colorated with the help of polyclonal to human GDNF antibodies. The results of the experiments are presented in Figs. 5 A and 5B. The slight pink coloring (Fig. 5A) is due to the fact that embryonic SCs seemingly synthe-

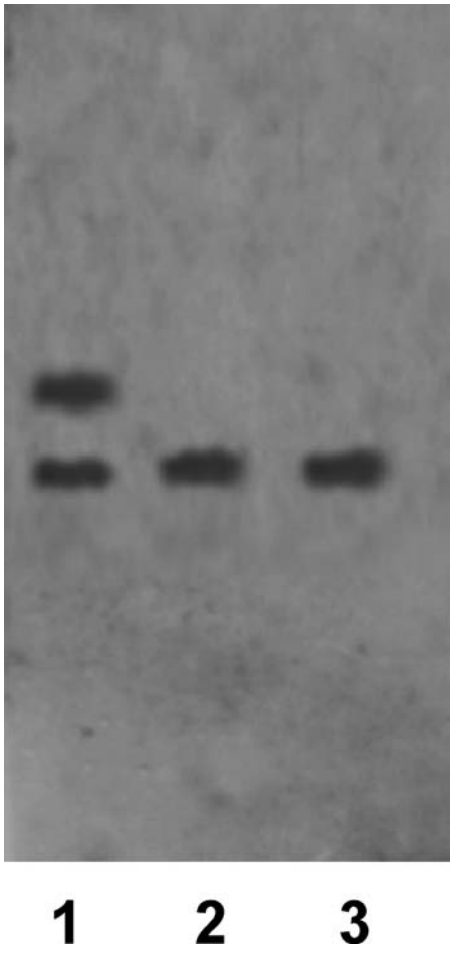

Fig. 4. GDNF gene expression analysis in embryonic SCs with the help of northern hybridization. (1) RNA from embryonic SCs transfected with a *LP14g* plasmid carrying the human GDNF gene fused with a GFP gene. (2) RNA from embryonic SCs transfected with a pEGFP-*N1*plasmid carrying the GFP gene. (3) RNA from non-transfected embryonic SCs.

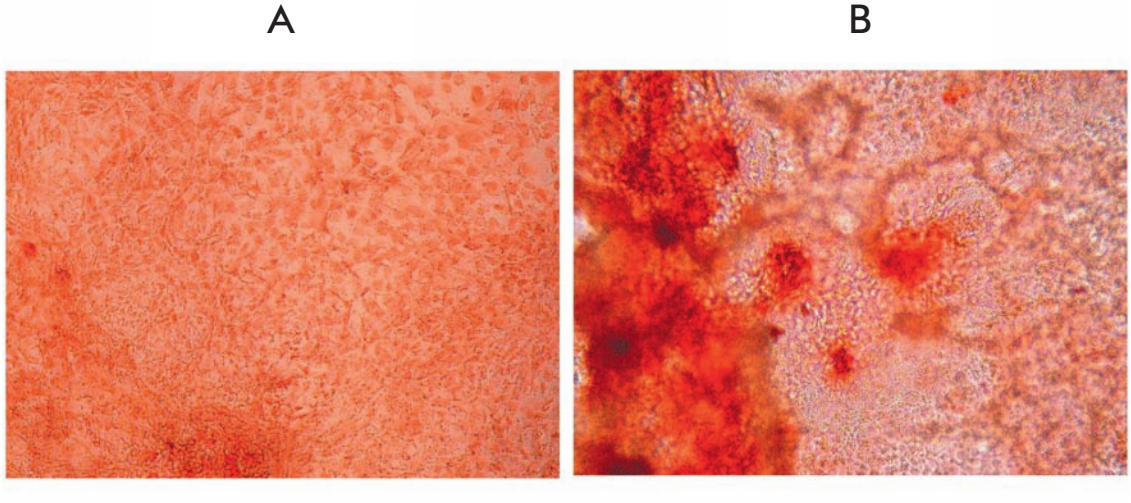

Fig. 5. Immunocytochemichal coloring of the es-GFP line cells (A) and es-GDNF (B) by the antibodies to human GDNF (x 100).

size their own GDNF in small amounts. Indirect confirmation of this was, as has already been mentioned, obtained with the help of northern hybridization (Figs. 4 *2* and 4 *3*). Brightly colored cell clusters are evidence of human GDFN synthesis in the es-GDNF line cells (Fig. 5B).

The influence of the human GDNF gene expression on the proliferative activity of the embryonic SCs was studied during the next stage. The proliferative activity of the control and experimental cell comparisons did not detect valid differences between them (this data has not been cited). These results allow us to suggest that the human GDNF gene product does not play an essential part in the cell-cycle regulation of these cells.

The analysis of this genetic expression's influence on the initial stage of the differentiation of the transfected embryonic SCs–EBs formation was examined. During research, the time of the EBs formation and their number was determined. The results of these experiments (EBs calculation was done on the 3rd and 4th days after the cell seeding) showed that the es-GDNF line cells form EBs almost concurrently with the control ones; however, the number of the EBs in the es-GDNF line cells was 40—45% lower than in the es-GPF line (Fig.6). These findings point to the fact that there is a verifiable decrease in the EB number in the es-GDNF line cells when compared to the controls, which testifies to the inhibiting GDFN influence on the earlier stages of the embryonic SC differentiation stage.

Consequently, as a result of the experiments, the embryonic mouse SCs that express the human GDNF gene fused with the GFP gene were obtained and partly characterized. The increased expression of the given gene leads to the slowing-down of the EB formation without influencing the morphology and proliferative activity of the transfected embryonic SCs. In the future, the given cells' line may be used both to investigate the way the human GDNF gene influences further stages of embryonic SCs *in vitro*, especially in the neuronal direction, and for *in vivo* experiments with the purpose of correcting several brain pathologies connected with neurodegenerative illnesses such as Parkinson disease. ●

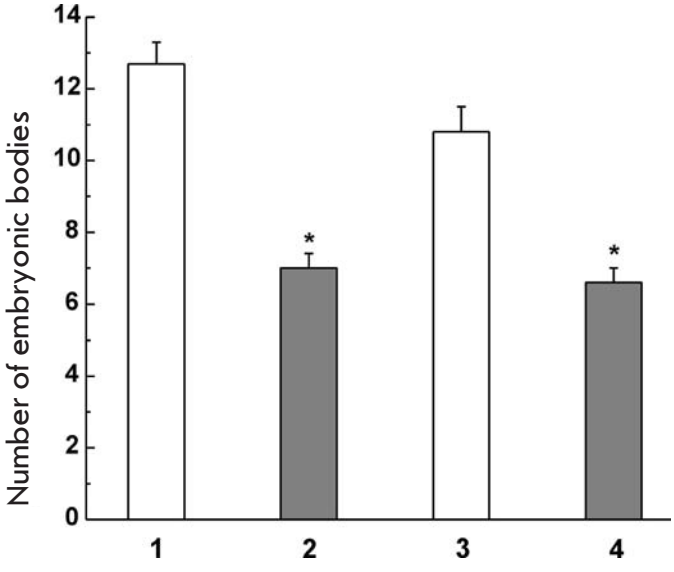

Fig. 6. hGDNF gene expression influence on the formation of the embroid bodies transfected by embryonic SCs in vitro.
(1, 2) number of formed EBs on the 3rd day after cell seeding.
(3, 4) number of formed EBs on the 4th day after cell seeding.
White columns indicate the (es-GFP) control. Grey columns are the (es-GDNF) test
* $p < 0,05$, $n = 30$.

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Direct Matrix-Assisted Laser Desorption–Ionisation (MALDI) Mass-Spectrometry Bacteria Profiling for Identifying and Characterizing Pathogens

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ABSTRACT This study examines the features and limitations of direct Matrix-Assisted Laser Desorption–Ionisation (MALDI) mass-spectrometry profiling of bacterial cells for investigating a microbial population. The optimal laboratory protocol, including crude bacteria lyses by a solution of 50% acetonitrile, 2.5% trifluoroacetic acid, and using α-cyano-4-hydroxy cinnamic acid as the MALDI matrix, has been developed. Two different bacteria species were under investigation, and representative mass spectra from 278 strains of Neisseria gonorrhoeae and 22 strains of Helicobacter pylori have been analyzed. It's known that both bacteria demonstrate a variable degree of polymorphism. For N. gonorrhoeae, the MALDI mass spectra that was collected possessed about 70 peaks, 20 of which were good reproducible ones. In spite of the fact that three peaks were found with differing spectra in some strains, little diversity in the N. gonorrhoeae population was revealed. This fact indicates the prospects in using direct MALDI mass-spectrometry profiling for gonococcus identification. In the case of H. pylori strains, the variety in the collected mass-spectra was shown to be essential. Only five peaks were present in more than 70% of strains, and a single mass value was common for all spectra. While these data call into question the possibility of the reliable species identification of H. pylori using this approach, the intraspecies differentiation of strains was offered. Good association between MALDI profile distributions and the region of strain isolation have been found. Thus, the suggested direct MALDI mass-spectrometry profiling strategy, coupled with special analysis software, seems promising for the species identification of N. gonorrhoeae but is assumed insufficient for H. pylori species determination. At the same time, this would create a very good chance for an epidemiological study of such variable bacteria as H. pylori.
Key words: MALDI mass spectrometry, bacteria profiling, Neisseria gonorrhoeae, Helicobacter pylori.

INTRODUCTION

Modern microbiology and its applied branches require the development of new rapid and precise methods for identifying clinically significant pathogens and for describing their characteristic features such as virulence, antibiotic sensitivity, and strain group. The relative tolerance of Matrix-Assisted Laser Desorption–Ionisation (MALDI) to contamination with salt and other impurities allows one to conduct a direct mass-spectrometry analysis of the microbial cell content (direct profiling) without the fractionation and purification of some components.

Generally, the considered method involves analyzing the complex mixtures of cell components such as proteins, peptides, lipids, and nucleic acids. However, the matrix formulation applied and parameters of mass-spectra establishment make it possible to register protein molecules that are of great worth, because a cell contains a significant quantity of variable proteins.

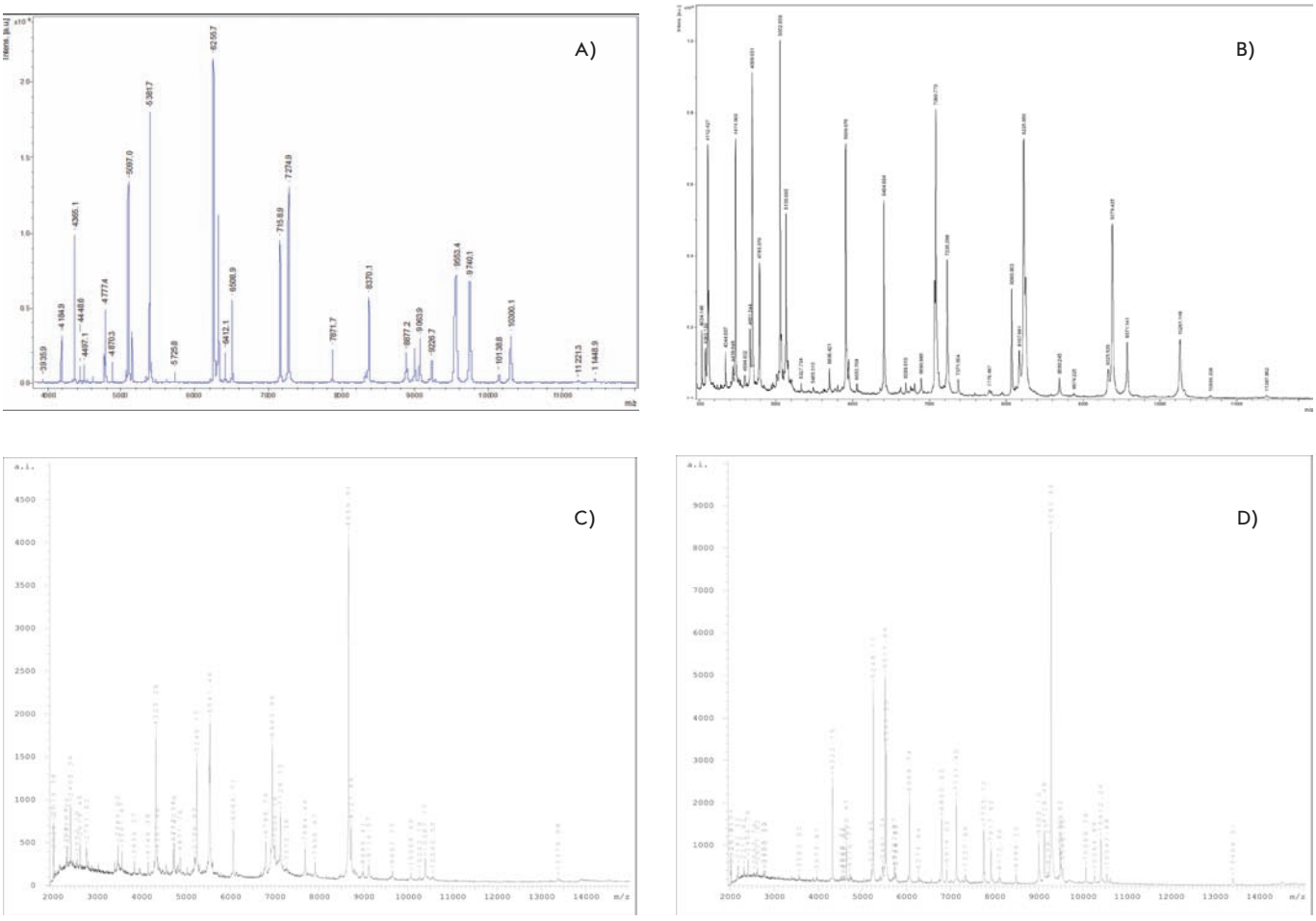

Fig. 1. MALDI mass spectra of E. coli DH-5α (A), N. gonorrhoeae ATCC 49226 (B), H. pylori J99 (C), and H. pylori 26695 strains registered using the α-CHCA matrix.

The registrability of mass-spectra, unique and reproducible for families, genera, species, and subspecies of microorganisms, makes it possible to use mass-spectrometry for bacteria identification and typing, which was demonstrated for the first time in 1975 [1]. Despite the fact that a "whole cell" spectrum (without any separation of the cell components) reflects only an insignificant part of the cell proteome, it is demonstrative enough to characterize the cell taxonomic features, which was established for a number of bacteria [2, 3, 4, 5, 6]. It should be noted that this method does not involve the identification of separate microbial proteins and allows the application of a unique mass-profile for characterizing any microorganism on the "fingerprint pattern" principle [7]. Specific features and, at the same time, disadvantages of the considered approach when compared to the traditional methods are as follows: (1) a quite high sensitivity (10^5–10^6 of cells or 0.5 μg of cell culture), (2) simple sample preparation, (3) high measurement rate, and (4) the possibility of automating and robotizing all investigation stages.

This study examines the advantages and disadvantages of using MALDI mass spectrometry to profile bacterial cells using such microbial populations as *Helicobacter pylori* and *Neisseria gonorrhoeae*, which are commonly referred to as microorganisms with a high genetic flexibility of genomes, as examples. Moreover, we set the task of estimating the variability of MALDI mass-profiles which were photographed according to the developed protocol within each bacterial population.

METHODS

BACTERIAL STRAINS The following laboratory strains were used in the investigation: *Escherichia coli* DH5α, *Neisseria gonorrhoeae* ATCC 49226, *Helicobacter pylori* J99, and *Helicobacter pylori* 26695; 278 *N. Gonorrhoeae* clinical strains from different regions of Russia (Moscow, St. Petersburg, Samara, Ekaterinburg, Murmansk, and Irkutsk); and 22 *H. pylori* clinical strains from Mongolia, Tuva, Yakutia, and the Moscow Region.

Fresh cultures of the stationary growth phase produced by the standard microorganism cultivation methods were used in the mass-spectrometry analysis.

PREPARING SAMPLES FOR MASS-SPECTROMETRY ANALYSIS The lysis of bacterial cells involved the trituration of the bacteria culture (single colony) in 50 mcl of 50% acetonitrile (ACN, Sigma–Aldrich, Germany) and 2.5% trifluoroacetic acid (TFA, Sigma–Aldrich, Germany). Supernatant produced in the course of the following centrifugation (1 min at 14,000 r/min) was used for MALDI mass-spectrometry analysis. The saturated solution of α-cyano-4-hydroxycinnamic acid mixed with 50% CAN and 2.5 TFA acted as a matrix (α-CHCA, Bruker Daltoniks, Germany). All the chemical agents that were applied were absolutely pure or meant exclusively for mass-spectrometry analysis.

MASS-SPECTROMETRY ANALYSIS The mass-spectrometry analysis was carried out on a time-of-flight MicroflexTM MALDI mass spectrometer (Bruker Daltonics, Germany) equipped with a 337-nm nitrogen laser. All measurements were performed in linear conditions with the detection of positive ions. To accumulate the mass spectra, the laser radiation power was set to the level of the minimum threshold limit value, which was sufficient for the desorption and ionization of the sample. The optimal mass-spectrometer parameters were set in the m/z 2,000-20,000 range. External calibration was based on the precise mass values of well-known *E. coli* proteins. The sample was applied to three cells in the plate. The spectrum obtained after summing up 10 spectral series per 50 laser bursts was recorded for each cell. The software of the Bruker Daltonics Company (Germany)—flexControl 2.4 (Build 38) and flexAnalysis 2.4 (Build 11)—were used for recording, processing, and analyzing the mass spectra. The accuracy of the mass measurements attained was ±2 Da.

INTERPRETATION OF MASS-SPECTRA The interpretation process assumed that most registered peaks corresponded to the protein molecules and the masses determined were the masses of non-fragmented proteins. Proteins were identified by comparing the experimental protein masses with the protein mass values annotated in the corresponding databases (SwissProt/TrEMBL) using the ExPASy-server resources (http://ca.expasy.org/srs5/). The experimental mass value, measured with an accuracy of up to ±2, was used for setting the "Molecular weight" parameter. If the attempt was unsuccessful, we carried out a second search with a mass value corresponding to the loss of N-tail methionine, taking into consideration the posttranslation protein modifications.

Statistical data processing The program resources of Microsoft Office Excel 2003 were used for creating intermediate tables, performing elementary calculations and descriptive statistics, and constructing diagrams. The cluster analysis was carried out with Statistica 6.0.

RESULTS AND DISCUSSION

The protocol of direct MALDI mass-spectrometry bacteria profiling was optimized using the laboratory *E. coli* DH5α strain, whose protein and nucleotide sequences were studied thoroughly. The method was based on the acid lysis of bacterial cells, which causes the extraction of the major ribosomal proteins that make up 20% of the total pool of *E. coli* proteins [8].

Solvent systems with different ACN and TFA concentrations and ratios, as well as the three most ubiquitous matrices used for sample ionization in MALDI mass-spectrometry analysis, were tested in the course of experiments to obtain the cell lysate. Estimation criteria were as follows: the reproducibility of MALDI mass-profiles within one strain, the resolution of mass-spectra peaks, the signal/noise ratio, the number of peaks (the representativeness of mass-spectrum), their intensity, and the range of m/z values registered in the course of analysis. The optimal solution (50% ACN, 2.5% TFA) for the lysis of bacterial cells was chosen on the basis of experimental data for further stages of investigation. This solution was suggested to help in creating the most qualitative mass spectra using any matrix substance involved in the investigation.

Table 1. The most reproducible peaks of laboratory *N. gonorrhoeae* ATCC 49226 strain mass-spectrum. Peaks, which are suggested to correspond to ribosomal proteins, are displayed in bold type

№	M (m/z)	M[1] (Да)	Ion type[2]	Description
1	4474	4473	$M+H^+$	RL36
2	4511			
3	4689	9377[3]	$M+2H^+$	RS20
4	4784	9570	$M+2H^+$	RL27
5	5010			
6	5052	5051	$M+H^+$	RL34
7	5130	10259[3]	$M+2H^+$	RS19
8	5484			
9	5908	5907	$M+H^+$	RL33
10	5946			
11	6053			
12	6404	6402[3]	$M+H^+$	RL32
13	7080	7078	$M+H^+$	RL29
14	7227	7226[3]	$M+H^+$	RL35
15	8068			
16	8167	8165	$M+H^+$	RL31
17	8225	8224[3]	$M+H^+$	RS21
18	9379	9377[3]	$M+H^+$	RS20
19	9570	9568[3]	$M+H^+$	RL27
20	10260	10259[3]	$M+H^+$	RS19

[1] Protein mass annotated in the SwissProt/TrEMBL databases
[2] Ion types are indicated only for peaks that correspond to certain proteins
[3] Protein mass with loss of N-tail methionine

The chosen conditions of direct MALDI mass-spectrometry profiling allowed us to obtain an *E. Coli* spectrum analogous in qualitative composition to the spectrum obtained earlier for the *E. coli* K-12 strain [9] (except for some peaks). This inconsistency is quite logical, because we analyzed another *E. Coli* strain: namely, DH5α.

Typical MALDI mass spectra of the laboratory *E. coli* DH5α, *N. gonorrhoeae* ATCC 49226, *H. pylori* J99, and *H. pylori* 26695 strains that were obtained according to the developed protocol are presented in Fig. 1. Though the mass spectra were established in the m/z 2000–20 000 range, the m/z 2000–12 000 area was the most informative from a visual standpoint. Moreover, a detailed analysis of the mass spectra showed that peaks on the m/z 2000–4000 area corresponded to the divalent ions of molecules whose monovalent ions were registered in the same spectra. Therefore, special attention was paid to the MALDI mass-spectrum area in the m/z 4000–12 000 range. As a whole, the peak list obtained for the laboratory *E. coli* DH5α strain corresponds to that obtained previously for the *coli* K-12 strain [9].

During each measurement, about 70 peaks were registered for the laboratory *N. gonorrhoeae* ATCC 49226 strain, 20 of which were easily reproducible. The identification procedure established that 14 of those peaks corresponded to gonococcus ribosomal proteins (Table 1). Analogous data were obtained for the laboratory *H. pylori* J99 and *H. pylori* 26695 strains: 14 out of 20 reproducible proteins were ribosomal.

Comparing the *H. pylori* J99 and 26695 strains spectra revealed some differences (displacement and presence or absence of peaks) (Fig. 1, Table. 2). Differences in peaks regarding ribosomal proteins may be explained on the basis of annotation to the genomes of these two strains, in particular, taking into account the data on amino-acid sequences of the corresponding proteins (RL32, RL29, RL24, RS16). Other differences of the spectrum peaks defy explanations at this level of investigation.

The developed methods for obtaining the reproducible MALDI mass-profiles of bacterial strains allowed us to analyze geographically heterogeneous groups of such clinical strains as gonococcus and *H. pylori*.

We carried out the MALDI mass-spectrometry profiling of 278 *N. gonorrhoeae* clinical strains taken from different regions of Russia. According to a comparative analysis of the mass spectra (peak lists) obtained, three peaks have the following m/z values: 4473, 5051, and 8165. These values correspond to such ribosomal proteins as RL36, RL34, and RL31, according to the *N. gonorrhoeae* ATCC 49226 mass-spectrum, and can occasionally vary to 4487, 5081, and 8146, respectively. Four combinations of variable m/z values of those proteins were revealed among the group of strains investigated. Taking into account the invariability of other m/z values in the spectrum, we distinguished four types of MALDI mass profiles (prototypes) of gonococcus. The investigated gonococcus strains (n = 278) were distributed as follows: 236 strains (84.9%) corresponded to type 1 (m/z 4473 / 5051 / 8165), i.e., control strain of *N. gonorrhoeae* ATCC

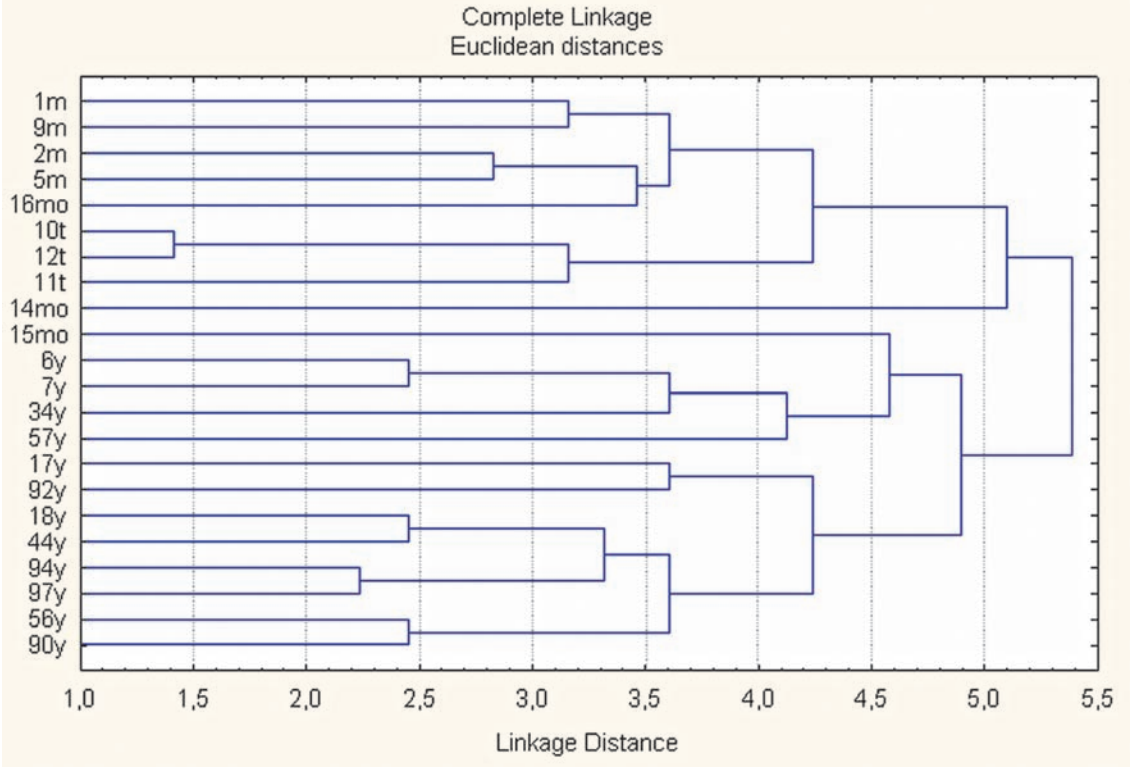

Fig. 2. Distribution of H. Pylori strains from Mordovia (m), Tuva (t), Yakutia (y), and Moscow Region (mo) according to the results of a cluster analysis of MALDI mass-profiles.

Table 2. The most reproducible peaks of the mass spectra of laboratory *H. pylori* J99 and *H. pylori* ATCC 26695 strains. Peaks, which are suggested to correspond to ribosomal proteins, are displayed in bold type

H. pylori J99		H. pylori ATCC 26695		M[1] (Да)	Ion types[2]	Description
№	M (m/z)	№	M (m/z)			
1	4322	1	4322	4320	$M+H^+$	RL36
2	5247	2	5247	5246	$M+H^+$	RL34
3	5517			5515	$M+H^+$	RL32
		3	5530	5529	$M+H^+$	RL32
4	5541	4	5541			
5	6068	5	6068	6066	$M+H^+$	RL33
6	6799	6	6799	6798[3]	$M+H^+$	RL28
7	6912	7	6912			
8	6947	8	6947	6946	$M+H^+$	Hpn[4]
9	7130	9	7130	7129[3]	$M+H^+$	RL35
10	7654	10	7654	7652	$M+H^+$	RL31
		11	7684	7683	$M+H^+$	RL29
11	7753			7752	$M+H^+$	RL29
		12	7906	7905	$M+H^+$	RL24
12	7917			7915	$M+H^+$	RL24
13	8484	13	8484	8482	$M+H^+$	RS21
		14	8657			
14	8972			8971	$M+H^+$	RS16
		15	8986	8985	$M+H^+$	RS16
		16	9114			
15	9129					
16	10067	17	10067	10065	$M+H^+$	RS20
17	10260	18	10260			
		19	10384			
18	10414					
19	10450	20	10450	10448	$M+H^+$	RS18
		21	10544	10543	$M+H^+$	RS19
20	10557					

[1] Protein mass annotated in the SwissProt/TrEMBL databases
[2] Ion types are indicated only for peaks that correspond to certain proteins
[3] Protein mass with loss of N-tail methionine
[4] Histidine-rich metal-binding polypeptide (Hpn), which is known to bind Ni^{2+} and Zn^{2+}, but its function in the bacterial cell's vital activity is still unclear [14].

49226; 26 strains (9.4%) were referred to type 2 (m/z 4487 / 5051 / 8165); 15 strains (5.4%), to type 3 (m/z 4487 / 5051 / 8147); and 1 strain from Irkutsk (0.4%), to type 4. Simpson's diversity index [10] for typing with the direct mass-spectrometry profiling of the gonococcus strain collection was 0.27.

Twenty-two *H.* pylori clinical strains recovered in different regions of Russia (Yakutia, Tuva, Mordovia, and Moscow oblast) were subject to typing. A comparative analysis revealed numerous variations and differences in those strains. If the registered peak displacement is suggested to be a variation in the m/z values, which group around a certain average value, and to reflect the mass and structure change of the same protein, it may be concluded that only 2 out of 20 spectrum proteins retain a constant mass in the group investigated. Nevertheless, the cluster analysis of the peak lists that were obtained allowed us to group the strains studied into three big groups correlating to the geographical specificity of the strains (Fig. 2). Taking into consideration the small volume of the sample collection (less than 30 samples), the Simpson's diversity index was not estimated.

In spite of the rich mass spectra with up to 70 peaks, direct MALDI mass-spectrometry profiling of laboratory (n = 1) and clinical (n = 278) *N. gonorrhoeae* strains revealed the homogeneity of profiles in the group investigated. It should be noted that the MALDI mass spectra photographed for gonococcus according to the protocol developed are much richer than those annotated previously [11]. Insignificant differences characteristic for the mass-spectra qualitative composition of clinical gonococcal strains are consistent with our ideas about the high conservatism of ribosomal proteins.

The low heterogeneity of the mass-spectra qualitative composition makes this approach inappropriate for gonococcus typing. However, these results have a reverse side and they establish the stability of the mass-spectra qualitative composition, which offers great opportunities for the species identification of a causative agent using this method. The marker mass profile composed of 20 stable displayable peaks may be used for identifying an individual species by comparing it with the experimental mass-spectrum of an unknown microorganism.

On the contrary, the direct MALDI mass-spectrometry profiling of laboratory (n = 2) and clinical (n = 22) *H. pylori* strains demonstrated a well-pronounced heterogeneity of the mass profiles obtained. Both qualitative and quantitative compositions varied. The mass spectra of laboratory strains were quite rich (up to 30 peaks), whereas the mass spectra of the clinical strains were rather poor (7–13 peaks). Similar results were presented in previous investigations [12, 13], which testifies to the regularity of this phenomenon. Only five peaks remained unchanged in the mass profiles of the strains investigated. Four of them are supposed to correspond to ribosomal proteins, and the fifth is referred to as a histidine-rich metal-binding polypeptide (Hpn). Hpn is known to bind Ni^{2+} and Zn^{2+}, but its function in the bacterial cell's vital activity is still unclear [14].

It should be noted that significant variability within one species is consistent with such well-known characteristic features of *Helicobacter* as the expressed macro- and microheterogeneity of the genome [15, 16, 17]. Establishing intraspecies heterogeneity by this method gives grounds to assume that this approach may be used for the same intraspecies classification and typing of microorganisms that was demonstrated with the help of cluster-analysis means.

CONCLUSIONS

The consolidated experience of using direct MALDI mass-spectrometry bacteria profiling gives grounds to assume that this approach may be used for the specific identification of the causative agent that was demonstrated when analyzing the voluminous group of clinical *N. gonorrhoeae* strains. On the contrary, intraspecies classification and typing of bacteria with this method is likely to be uninformative for species with a low intraspecies variability.

On the other hand, identifying bacteria characterized by a high intraspecies variability, for instance, *H. pylori*, is still a matter of debate and requires additional study. The data obtained demonstrate prospects for using direct MALDI mass-spectrometry profiling for strain differentiation and bacteria typing. ●

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The Genes of Antimicrobial Peptides for the Therapy of Intracellular Infections

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Resistance to antibiotics is of great social and economic importance and is regarded as a threat to the national security of any country and the global community as a whole. Among the bacterial agents of different infections, resistance to some antibiotics can reach 98%. Infections caused by antibiotic-resistant strains are distinguished by their significant duration, they often require hospitalization, they increase the length of hospital stay, and they often worsen the prognosis for a disease [1]. If the chosen medicines turn out to be ineffective, the doctors have to use second- or third-order medicines, which are often rather expensive, less safe, and not always available. All these facts increase direct and indirect economic expenditures, as well as cause a risk of antibiotic-resistant strain propagation. Causative agents of intracellular infections such as mycoplasmas and chlamydiae are characterized by high antibiotic resistance. Treating mycoplasmosis and clamidiosis with a wide range of antibiotics is almost ineffective due to the quick formation of resistance to these medicines, and, as a result, the development of virus persistence in the organism.

In connection with this, it is essential to create alternative therapeutic agents which will not cause or limit antibiotic resistance. Antimicrobial peptides (AMPs) may be such therapeutic agents. They represent a unique and quite diverse group of compounds which make up a major component of the natural immunity of all organisms [2]. Compared to antibiotics, antibacterial peptides have the following advantages: a wider range of antibacterial action, functional activity at micromolar concentrations, the absence of virus resistance to antimicrobial peptides, and the synthesis capability of natural peptide analogues with altered biological properties. The causative agent cannot become resistant to AMPs because of the unique mechanism of their action, which consists in the formation of channels and the following fragmentation of the bacterial cell membrane. However, to date, all investigations devoted to the study of AMPs have focused on exogenic (synthesized) peptides, while the mechanism of AMPs synthesized directly in the infected cell is still unclear. We chose melittine as a model peptide, which is an amphipathic α-helical peptide (a major component in bee poison) [3].

In that review, we were the first to show the inhibition of such experimental infections as mouse *Mycoplasma hominis* and *Chlamydia trachomatis* and broiler chicken *Mycoplasma gallisepticum*.

In this review, we used a pBI/mel2/rtTA plasmid vector containing the melittine gene under the control of the tetracycline-dependent CMV promoter and the transacting rtTA protein gene controlled by the early constitutive CMV promoter [4].

Using this plasmid construction allows the expression level of the antimicrobial peptide genes in the organism to be accurately regulated with the help of different inducer

Table 1. Influence of the recombinant pBI/mel2/rtTA plasmid vector injection on the *C. trachomatis* content in the vaginas of mice infected.

Observation period	C. trachomatis titer in vaginal lavages of mice infected (number of C. trachomatis inclusions/ml)						
	2 days	6 days	9 days	13 days	16 days	20 days	27 days
Group 1	12950	8490	4250	5220	2510	1070	1570
Group 2	12600	9750	3930	4850	2140	980	1470
Group 3	6850	2710	1920	2090	1080	370	350

Note: Differences between Group 3 and Groups 1 and 2 are reliable ($P < 0.05$).

Table 2. *M. gallisepticum* extraction from different parts of respiratory tract and internal organs.

	Organ	Group 1	Group 2	Group 3	Group 4
Respiratory tract	Windpipe	0/14 [a]	14/14	14/14	14/14
	Air pockets	0/14	12/14	10/14	14/14
	Lungs	0/14	10/14	6/14	11/14
	Total quantity of reisolations	0	36 [b]	30	39
Internal organs	Liver	0/14	4/14	0/14	3/14
	Milt	0/14	3/14	0/14	4/14
	Kidneys	0/14	8/14	6/14	7/14
	Heart	0/14	3/14	0/14	3/14
	Total quantity of reisolations	0	18	6 [c]	17

[a] Quantity of mycoplasma reisolations/total number of chickens
[b] Differences between Group 2 and Groups 3 and 4 are not statistically reliable.
[c] Differences between Group 3 and Groups 2 and 4 are statistically reliable, $P \leq 0.01$.

doses, which is of great importance when the products of the expressed genes are toxic.

We used female mice of the BALB/c line (6–8 weeks old and weighing 18–22 g).

Before contamination with *M.hominis*, the mice were injected subcutaneously with estradiol (Intervet UK, Great Britain) in doses of 0.5 mg per mouse (0.1 ml four times, with a week interval). Progesterone (Depo-Provera, Great Britain) was injected subcutaneously in a dose of 2.5 mg per mouse (0.1 ml, four days before contamination with *C. trachomatis)*.

M. hominis suspension (10^9 cell/ml titer) was injected (50 mcl) into the mice intravaginally after the second estradiol injection. A fraction of *C. trachomatis* elementary bodies (10^6 IFU/ml titer; IFU, inclusion-forming unit) was injected into the mice intravaginally (50 mcl) after progesterone injection. Recombinant pBI/mel2/rtTA plasmid vector was injected intravaginally using the Effectene Transfection Reagent (Qiagen GmbH, Germany). The recombinant vector was injected twice: 24 h before infection with *M. hominis* or *C. trachomatis* and 14 days after infection in doses of 2 μg per DNA/mouse (25 mcl) with addition of 25 mcl of cacao oil to increase the suspension viscosity. Doxycycline hydrochloride (ICN Pharmaceuticals, Moscow, Russia) was used as inducer of melittine gene transcription. The medicine was injected intramuscularly into the mice infected with *M. hominis* and *C. trachomatis* in doses of 2 μg per mouse and 1 μg per mouse, respectively, (50 mcl) at the moment of vector injection.

The animals were subdivided into three groups (six mice in each group, two independent experiments). Group 1 was infected with *M. hominis* or *C. trachomatis* without pBI/mel2/rtTA plasmid vector or doxycycline. Group 2 was injected with doxycycline in the corresponding dose with the following infection of *M. hominis* or *C. trachomatis*. Group 3 was injected with pBI/mel2/rtTA plasmid vector and doxycycline followed by *M. hominis* or *C. trachomatis*.

To determine the *M. Hominis* titer after the pBI/mel2/rtTA plasmid vector injection, we prepared ten-fold diluted lavages from the upper urogenital tracts of the mice. To determine the *C. trachomatis* titer, we used the direct fluorescence reaction and infected the McCoy cell line with the vaginal lavages.

The injection of the recombinant pBI/mel2/rtTA vector and the following contamination of mice were finished by the *M.hominis* infection inhibition. The results may be seen in Fig. 1. The *M. hominis* titer in the vaginal lavages of Group 1 mice varied, decreasing from 5.9 to 2.4 $\log_{10}$ ccu/ml (ccu, color change unit) in four weeks. In the Group 3 mice, which were injected with the recombinant pBI/mel2/rtTA vector and doxycycline before infection, the *M. hominis* titer was within 4.1–1.8 $\log_{10}$ ccu/ml.

In the case of the pBI/mel2/rtTA plasmid vector injection and the following infection of mice with *C. trachomatis*, the infection inhibition level was 45–80% (Table 1).

In spite of the fact that we did not achieve complete recovery of the mice from mycoplasmas and chlamydiae in the observation period, the rate of causative agent elimination was higher in Group 3 than in the control groups. Three mice of Group 3 infected with *M. hominis* recovered from the virus on the 21st day after infection; in the control groups 1 and 2, all mice had *M. hominis*. As for the mice infected with *C. trachomatis*, four mice from Group 3 were free from the virus on the 27th day after infection.

It should be noted that we did not obtain reliable statistical differences in the titers of mice from Groups 1 and 2 infected with *M. hominis* or *C. Trachomatis*, which, firstly, testifies to the absence of an uncontrolled expression of the melittine gene, and, secondly, to the fact that the chosen inducer (doxycycline) concentration does not influence the infection process development.

To investigate the influence of recombinant vector injection on the development of the *Mycoplasma gallisepticum* infection, 60 21-day-old Ross broiler chickens were marked and subdivided into four groups consisting of 15 chickens in such a way that the chickens' average weight was analogous in each group on the basis of the Student *t*-test.

Group 1 was not infected with *M. gallisepticum* or injected with the recombinant pBI/mel2/rtTA plasmid vector. Group 2 was infected with *M. gallisepticum*, but the re-

combinant pBI/mel2/rtTA plasmid vector was not injected. Group 3 was injected with the plasmid vector 5 h before infection with *M. gallisepticum*. Moreover, the mentioned chickens were injected intramuscularly with doxycycline (ICN Pharmaceuticals, Moscow, Russia)—which acted as an inducer of melittine gene transcription—24 and 5 h before the infection in doses of 0.1 per chicken (in the volume of 100 mcl). Group 4 was injected with doxycycline (in the same dose and with the same intervals), followed by infection with *M. gallisepticum*. Chickens of that group were not injected with the pBI/mel2/rtTA plasmid vector.

All chickens were subject to clinical, postmortem, immunologic, and biological examinations.

Nine days after infection, the Groups 1 and 3 did not have any respiratory symptoms. At the same time, Groups 2, 4, and 5 were revealed to have respiratory rale. The second group was characterized by a reliable statistical decrease in average weight. *M. gallisepticum* extraction from the chickens' internal organs is of special interest (Table 2).

In spite of the fact that we did not obtain reliable differences in the frequency of *M. gallisepticum* reisolation from the chickens' respiratory tracts in Groups 2 and 3 (Table 4), *M. gallisepticum* was detected only in 6 out of 56 internal samples. The livers, spleens, and hearts of that group of chickens did not contain *M. gallisepticum*.

Undoubtedly, the most important mechanism of membrane-active antimicrobial peptides, which leads to the inhibition of mycoplasmosis and clamidiosis infections in the cell culture and *in vivo*, is their direct cytotoxic action on these bacteria [5].

Moreover, the *in vitro* processing of mycoplasmas with amphipathic peptides such as cecropin A, melittine, and magainin 2 depolarizes their plasmamembranes, alters their morphology, and decreases their mobility [6]. As was shown previously, the melittine gene expression in the HeLa cell culture results in a reduction of the transmembrane potential of the transfected cell [7], which is followed by a breakdown in the process of mycoplasma and chlamydia adhesion in the cell and, as a consequence, an interruption of the normal cycle of their development [8]. Moreover, it is quite possible that melittine expression alters the cell's cytoskeleton, and, as a consequence, breaks down the traffic of chlamydia inclusions.

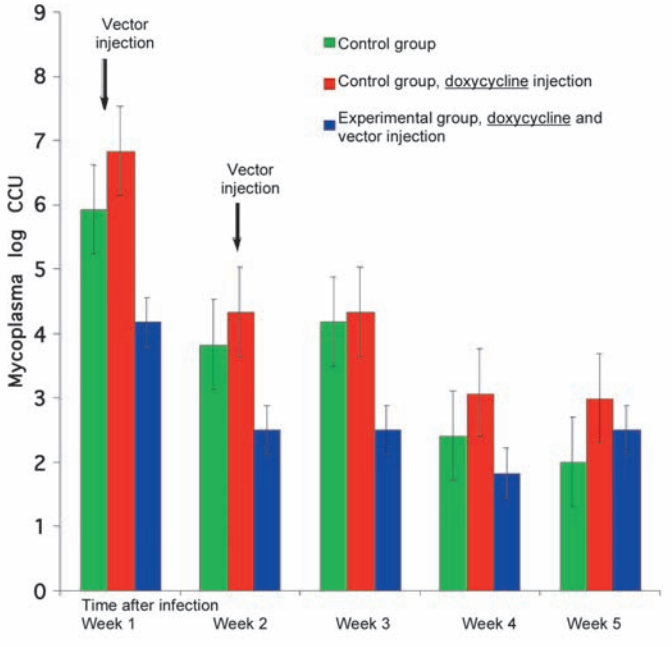

Fig. 1. Influence of the recombinant pBI/mel2/rtTA plasmid vector injection on the *M. hominis* content in the vaginas of mice infected.

In spite of the fact that we did not manage to completely eliminate the virus from the urinogenital and respiratory tracts in our experiments, these data allow us to suggest that the recombinant plasmid vectors expressing the antimicrobial peptide genes may be considered as potential agents for preventing and treating micoplasmosis and clamidiosis. ●

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UDK 577.32

The Role of Stacking Interactions in Complexes of Proteins with Adenine and Guanine Fragments of Ligands

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INTRODUCTION

The biological function of proteins is closely connected to interactions with their ligands and substrates. Proteins acting as receptors and enzymes bind these small molecules. Knowledge of the molecular mechanisms of protein-ligand interactions, particularly in the spatial structure of the protein-ligand complex, is a prerequisite for understanding the structure-functional properties of proteins and their role in biochemical pathways in the living cell. The availability of such a structure serves as a basis for rational drug design projects and greatly assists the search for new inhibitors (the ligands of certain protein-targets in an organism).

Experimental tools for determining the spatial structure of proteins and their complexes with ligands (such as X-ray crystallography or NMR spectroscopy) have particular limitations. Even if the structure of a protein is available, determining the structure of its complex with ligands may be experimentally demanding. Problems with purification and crystallization become especially difficult in studies of transmembrane proteins, which include a biologically important class of G-protein coupled receptors. However, recent successes in determining the structure of beta-adrenergic and adenosine receptors [1] are cause for optimism.

The technical difficulties restraining experimental methods stimulated computational molecular modeling. One of them (molecular docking) is a method aimed at predicting the spatial structure of a protein-ligand complex by docking a ligand molecule into the known atomic-resolution structure of a protein-binding site and estimating the reliability of the results. Nowadays, molecular docking has become an integral part of both fundamental studies aimed at understanding the structure-functional role of protein amino acids and applied drug-design programs [2,3].

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).

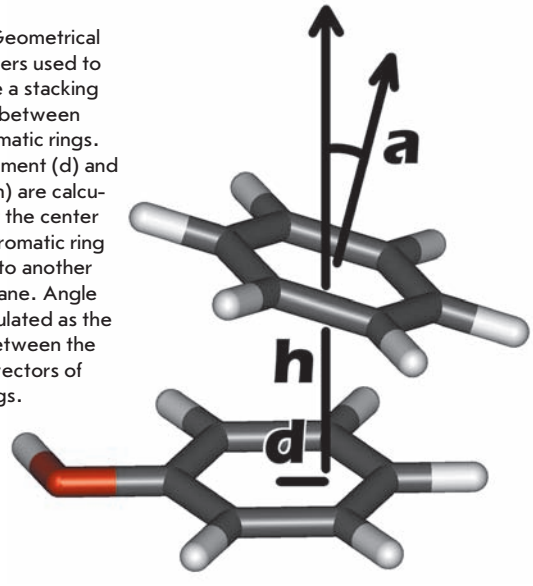

Fig. 1. Geometrical parameters used to describe a stacking contact between two aromatic rings. Displacement (d) and height (h) are calculated for the center of one aromatic ring relative to another ring's plane. Angle α is calculated as the angle between the normal vectors of both rings.

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.

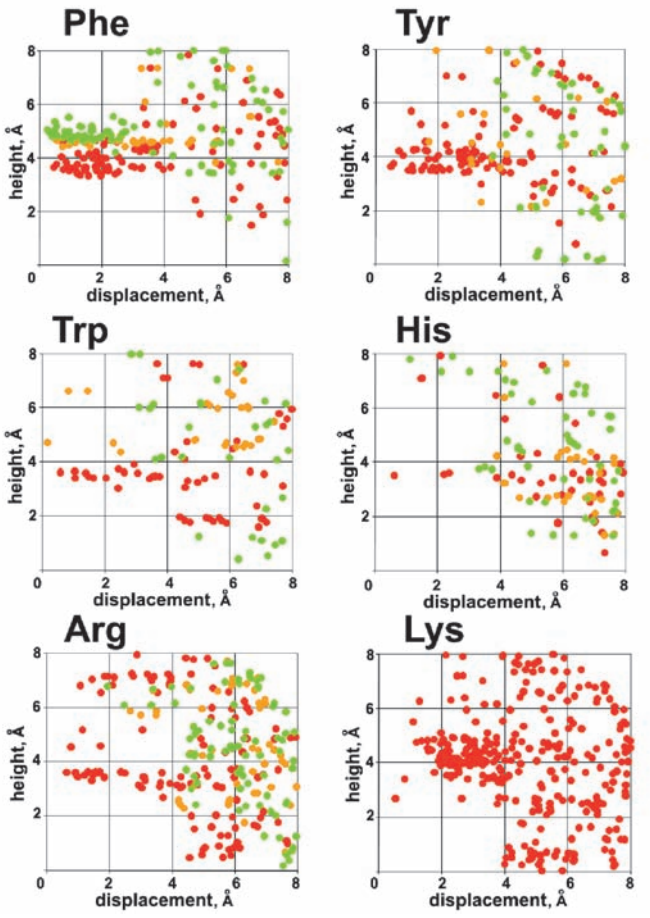

Fig. 2. The distribution of aromatic rings and positively charged side chain groups of amino acids around the guanine moiety of various ligands in complexes with protein receptors. The color red corresponds to $\cos^2\alpha = 0.6 - 1.0$ (parallel orientation), green corresponds to $\cos^2\alpha = 0.0 - 0.4$ (T-shaped orientation), and yellow corresponds to intermediate geometry. Here, α is the angle between the planes of both rings. For Lys this value is not defined.

Interestingly, the distribution for the planar guanidine group of Arg very much resembles that of Tyr, Trp, and His, where the orientation parallel to guanine is predominant. For the amino group of the Lys side chain, two modes of contacts with guanine were observed: above the plane (cation-ϖ interaction) and in the plane. The latter corresponds to the formation of a hydrogen bond to the heteroatoms of the guanine ring.

Distributions for adenine are similar to those obtained for guanine (data not shown). The results of the presented analysis may be used in developing scoring criteria and applied to rescoring the results of docking or even during the docking procedure.

GUANINE-SPECIFIC SCORING FUNCTION

We demonstrated the efficiency of an explicit account of stacking interactions in a scoring function along with reranking the results of GTP docking to the 14 different proteins that bind this ligand. All structures of GTP-protein complexes, which were generated with the docking procedure, were labeled as either correct or misleading by the value of root-mean-square deviation (rmsd) of the guanine atoms from the reference X-ray structure (see Methods). To estimate the validity of a docking pose, we constructed a number of scoring criteria in the form of a linear combination of the interaction terms. To do that, all GTP-protein complexes were divided into two equal groups: the training and the test sets. The weighting coefficients for these terms were fitted by the linear regression procedure to the binary function, which took on the value of 1 for the correct docking poses and 0 for the seven complexes of the training set. To test the robustness of the new scoring functions, the leave-one-out cross-validation procedure was performed. The relative error of all weighting coefficients of interaction terms was <30%, thus indicating the reliability of the results.

New scoring functions were used to analyze the GTP docking poses generated for each complex. The efficiency of the *goldscore* [18] function implemented in the docking algorithm achieved approximately 50%; it ranked correct poses at the top for only four and three complexes out of seven for the training and test sets, respectively (Table 1). Ranking by the value of $T_{stacking}$, which describes stacking contacts, was better than by *goldscore;* their combination (*SF1*) yields even better results. A similar effect was observed when the term $T_{stacking}$ was added to the criterion based on lipophilic contacts and hydrogen bonds between the protein and the guanine moiety of the ligand (*SF2* and *SF3*, Table1).

Of the proposed scores, the *SF3* is the most efficient. The number of complexes for which the correct pose was ranked at the top by *SF3* considerably exceeds that of *goldscore*. Also, the average best rank of the correct docking pose improves; i.e. the quality of ranking increases uniformly for all complexes. This can also be seen from the results of ranking for each complex (Fig. 3).

CONCLUSIONS

The analysis of structural data for complexes of proteins with ligands containing adenine or guanine moiety yielded a more accurate definition of the geometrical parameters of stacking interactions with aromatic side chains of receptor amino acids. Reranking the results of GTP docking demonstrated that an explicit account of stacking in scoring criteria provides a more efficient estimation of the reliability of the structure of the protein-ligand complex predicted with molecular modeling approaches. The obtained results can be further applied to a broader class of nucleobase-containing ligands.

METHODS

The structures of complexes of proteins with adenine- and guanine-containing ligands were taken from the Brookhaven Protein Data Bank (PDB) [19]. The PDBlig web server [20] was used to identify those PDB entries that contain a ligand with a purine nucleobase (adenine or guanine as a substructure). Structures with modified nucleobases and entries that contain nucleic acids other than simple nucleotides or nucleosides were omitted. Finally, to reduce the redundancy

Table 1. Ranking of the results of GTP docking.

Ranking method	Training set, 7 complexes		Test set, 7 complexes	
	Number of complexes for which correct pose was ranked at top	Average best rank of correct pose	Number of complexes for which correct pose was ranked at top	Average best rank of correct pose
goldscore	4	4.7	3	12.1
T_{stack}	5	2.3	3	7.0
$SF1 = -1.3 + 0.21 \times T_{stack} + 0.016 \times goldscore$	5	1.7	4	6.9
$SF2 = 0.06 + 0.007 \times T_{lipophilic} + 0.43 \times T_{h\text{-}bond}$	5	2.3	4	6.9
$SF3 = 0.05 + 0.004 \times T_{lipophilic} + 0.39 \times T_{h\text{-}bond} + 0.22 \times T_{stack}$	6	2.0	5	6.4

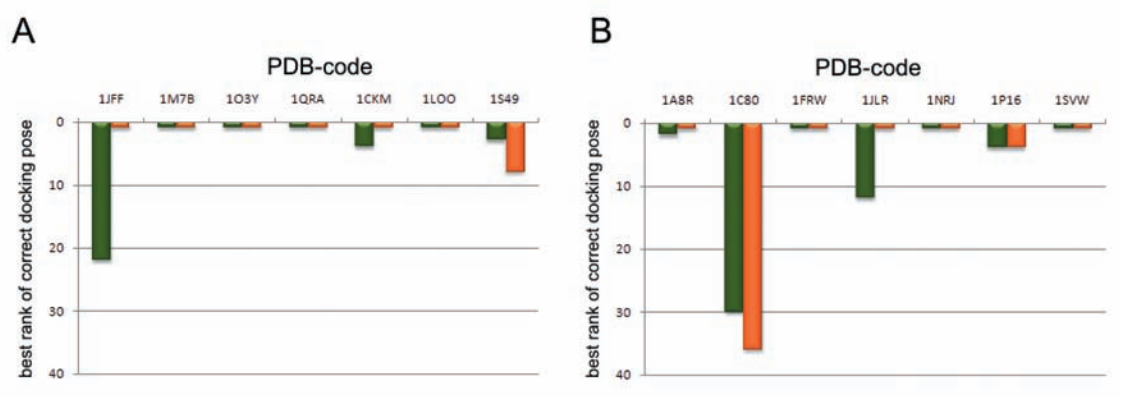

Fig. 3. Efficiency of ranking the docking poses for GTP-protein complexes by the *SF3* (red) and by *goldscore* (green) for the training (A) and the test (B) sets. The best rank of a correct GTP pose is shown.

of the set of protein-ligand complexes, a multiple sequence alignment was carried out using the Clustalw program [21]. After that, all complexes with the same ligand were clustered according to similarity in a protein sequence and the structure with the best resolution from each cluster was retained.

GTP docking was performed using the GOLD [18] program with the *goldscore* scoring function. The parameters of the docking procedure were taken as default. For each complex, 60 docking poses were generated. An rmsd cut-off 2.5 Å over the coordinates of guanine atoms was used to judge whether a pose was correct or misleading.

The surface area of the hydrophobic GTP-protein contact (as a measure of hydrophobic interactions, $T_{lipophilic}$) was calculated based on the concept of Molecular Hydrophobicity Potential (MHP) using the PLATINUM web server [22]. A MHP-scale shift of +0.2 was applied to the ligand surface to achieve a more realistic distribution of the hydrophobic/hydrophilic properties of GTP.

The term $T_{h\text{-}bond}$ is a binary function that takes on the value of 1 when a hydrogen bond network of guanine and a particular motif is formed; if not, it takes on a value of 0. Such motifs were hydrogen bonds between the guanine atoms N1, N2, and O6 and residues *i*, *i*, *i*, or *i*, *i*, *i*-2. ●

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