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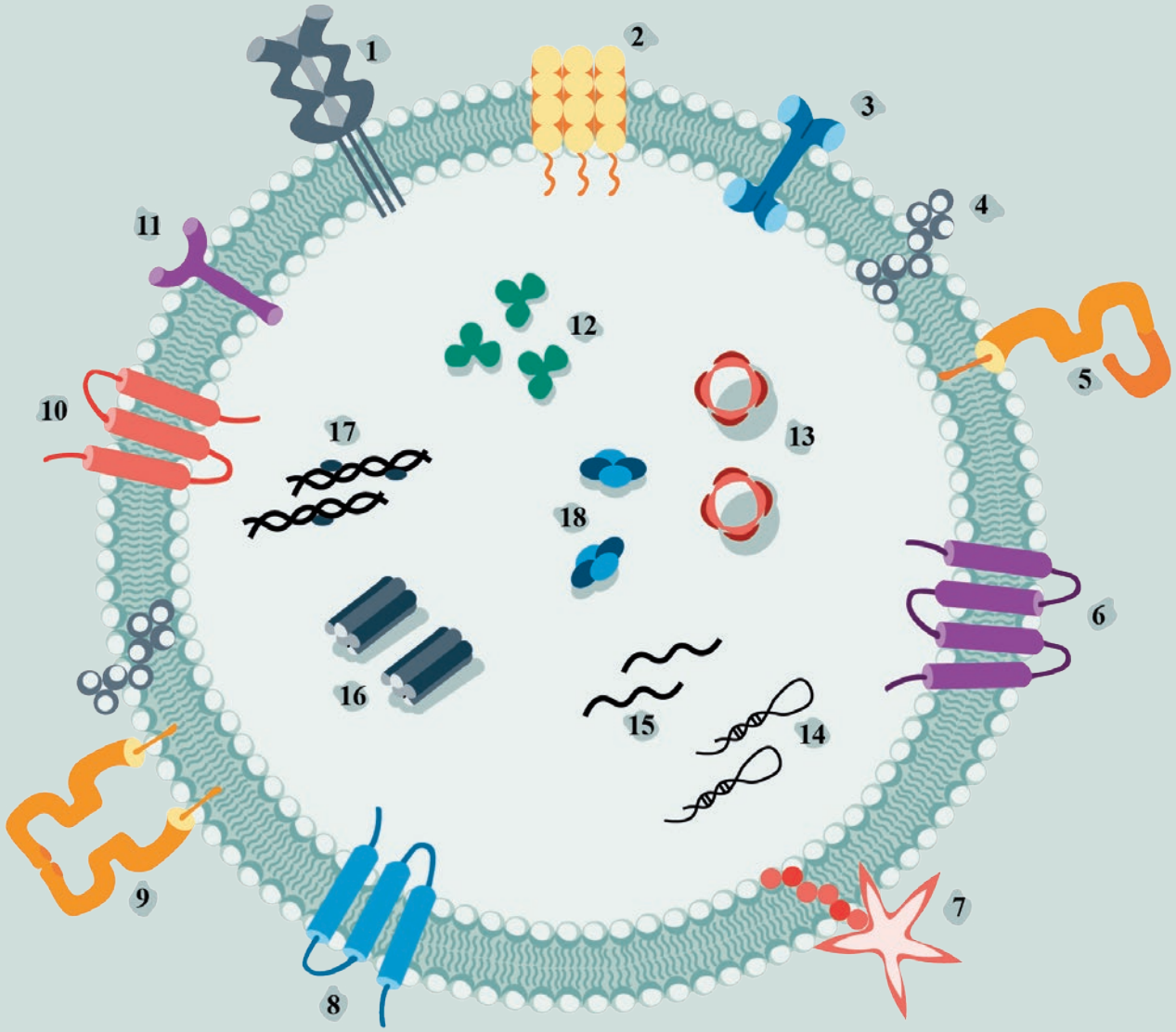
The Role of Tumor-Derived Vesicles in the Regulation of Antitumor Immunity



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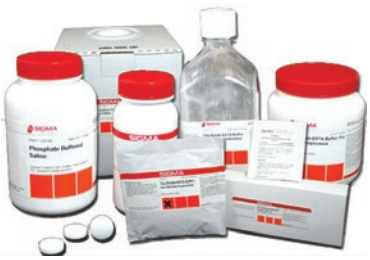

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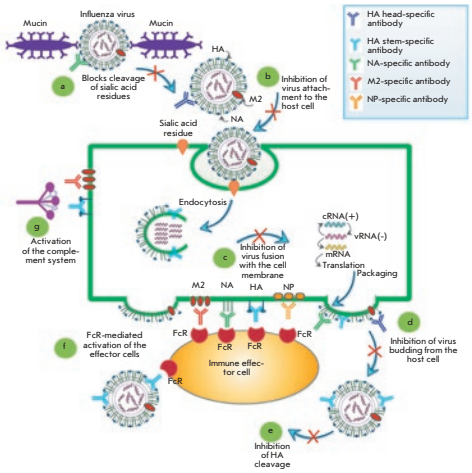
Non-neutralizing Antibodies Directed at Conservative Influenza Antigens

E. S. Sedova, D. N. Scherbinin, A. A. Lysenko, S. V. Alekseeva, E. A. Artemova, M. M. Shmarov

In this review, we focused on the mechanisms of anti-influenza action of non-neutralizing antibodies, such as antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and antibody-mediated complement-dependent cytotoxicity. The influenza virus antigens that trigger these reactions are hemagglutinin and neuraminidase, as well as highly conserved antigens, such as M2 (ion channel), M1 (matrix protein), and NP (nucleoprotein). In addition, the mechanisms of action and methods for detecting antibodies to neuraminidase and to the stem domain of hemagglutinin of the influenza virus are considered.



Mechanisms of action of anti-influenza antibodies

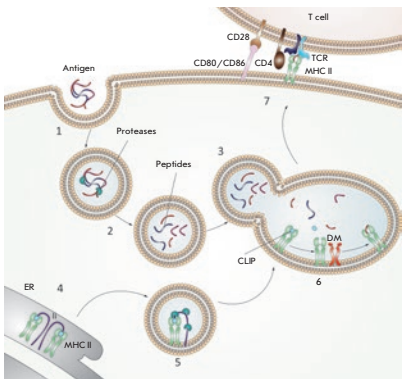


Diagram showing antigen presentation by MHC II molecules

The Contribution of Major Histocompatibility Complex Class II Genes to an Association with Autoimmune Diseases

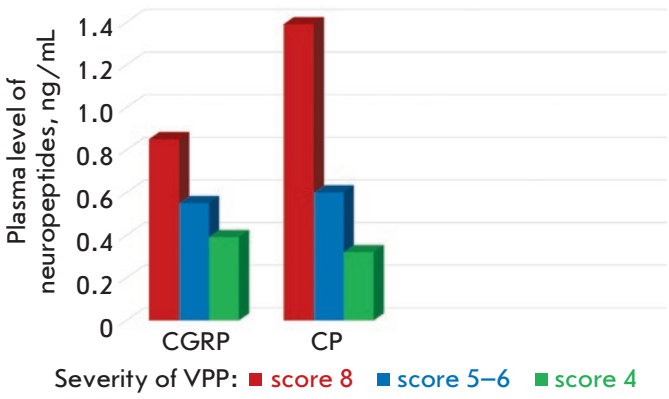
M. Yu. Zakharova, T. A. Belyanina, A. V. Sokolov, I. S. Kiselev, A. E. Mamedov

This review dwells on the most relevant aspects of this problem: namely, the correlation between carriage of certain MHC II alleles and an increased (positively associated allele) or reduced (negatively associated allele) probability of developing the most common autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, autoimmune thyroiditis, etc. The most universal haplotypes, DR3-DQ2 and DR4-DQ8, are positively associated with many of these diseases, while the universal allele HLA-DRB1*0701 is protective.

Calcitonin Gene-Related Peptide and Substance P as Predictors of Venous Pelvic Pain

S. G. Gavrilov, G. Yu. Vasilieva, I. M. Vasiliev, O. I. Efremova

The purpose of this work was to study the contents of calcitonin gene-related peptide (CGRP) and substance P (SP) in blood plasma of patients with pelvic varicose veins. The CGRP and SP levels in blood plasma highly correlate with the presence of pelvic venous pain.



The CGRP and SP levels and the severity of venous pelvic pain in group 1 patients

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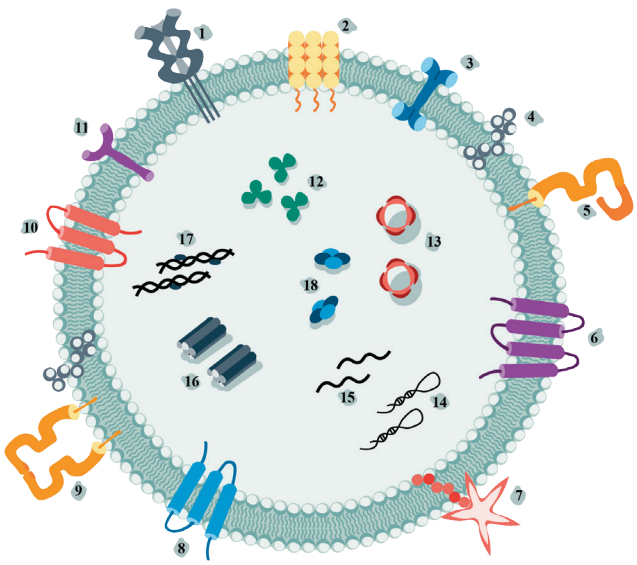

IMAGE ON THE COVER PAGE
(see the article by *V. M. Ukrainskaya et al.*)

The Contribution of Major Histocompatibility Complex Class II Genes to an Association with Autoimmune Diseases

M. Yu. Zakharova[1,2*], T. A. Belyanina[1], A. V. Sokolov[3], I. S. Kiselev[2], A. E. Mamedov[1]
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ABSTRACT **Genetic studies of patients with autoimmune diseases have shown that one of the most important roles in the developing of these diseases is played by a cluster of genes of the major histocompatibility complex (MHC), as compared with other genome areas. Information on the specific contribution of MHC alleles, mostly MHC class II ones, to the genetic predisposition to autoimmune diseases is crucial for understanding their pathogenesis. This review dwells on the most relevant aspects of this problem: namely, the correlation between carriage of certain MHC II alleles and an increased (positively associated allele) or reduced (negatively associated allele) probability of developing the most common autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, autoimmune thyroiditis, etc. The most universal haplotypes, DR3-DQ2 and DR4-DQ8, are positively associated with many of these diseases, while the universal allele HLA-*DRB1**0701 is protective.**
KEYWORDS **antigen presentation, autoimmune diseases, human leukocyte antigen, major histocompatibility complex, multiple sclerosis, rheumatoid arthritis, type 1 diabetes.**
ABBREVIATIONS **AD – autoimmune disease; MHC – major histocompatibility complex; HLA – human leukocyte antigen; APC – antigen-presenting cells; MS – multiple sclerosis; SLE – systemic lupus erythematosus; T1D – autoimmune type 1 diabetes; RA – rheumatoid arthritis; GD – Graves' disease; N – narcolepsy; AT – autoimmune thyroiditis; LD – linkage disequilibrium; IC – invariant chain.**

INTRODUCTION

The major histocompatibility complex (MHC), or human leukocyte antigen (HLA), contains several gene clusters that encode surface heterodimeric proteins, which are anchored to the plasma membrane and are responsible for antigen presentation to T cells, a stage that is followed by the development of an adaptive immune response. MHC proteins are subdivided into class I, class II, and class III (the complement system) [1]. MHC class I proteins occur in almost all cell types and are involved in the presentation of self-antigens fragments, which trigger the $CD8^+$ T cell-mediated immune response. MHC class I molecules are found on the surface of professional antigen-presenting cells (APCs) and mostly present fragments of foreign antigens (bacterial, viral, etc.) captured by APCs. The MHC II–peptide complex interacts with $CD4^+$ T cells (*Fig. 1*).

MHC proteins are heterodimers that consist of two chains: the long α chain containing a transmembrane domain and a short universal β2-microglobulin chain (for MHC I), or long α and β chains carrying extracellular, transmembrane and short cytoplasmic domains (for MHC II). The peptide-binding groove is an essential structural element of MHC, because its structure is responsible for peptide binding and further triggering of the immune response. HLA molecules need to be highly polymorphic to ensure presentation of a large number of variable peptides.

MHC genes are located on chromosome 6 (except for the gene for the light chain of MHC I (β2-

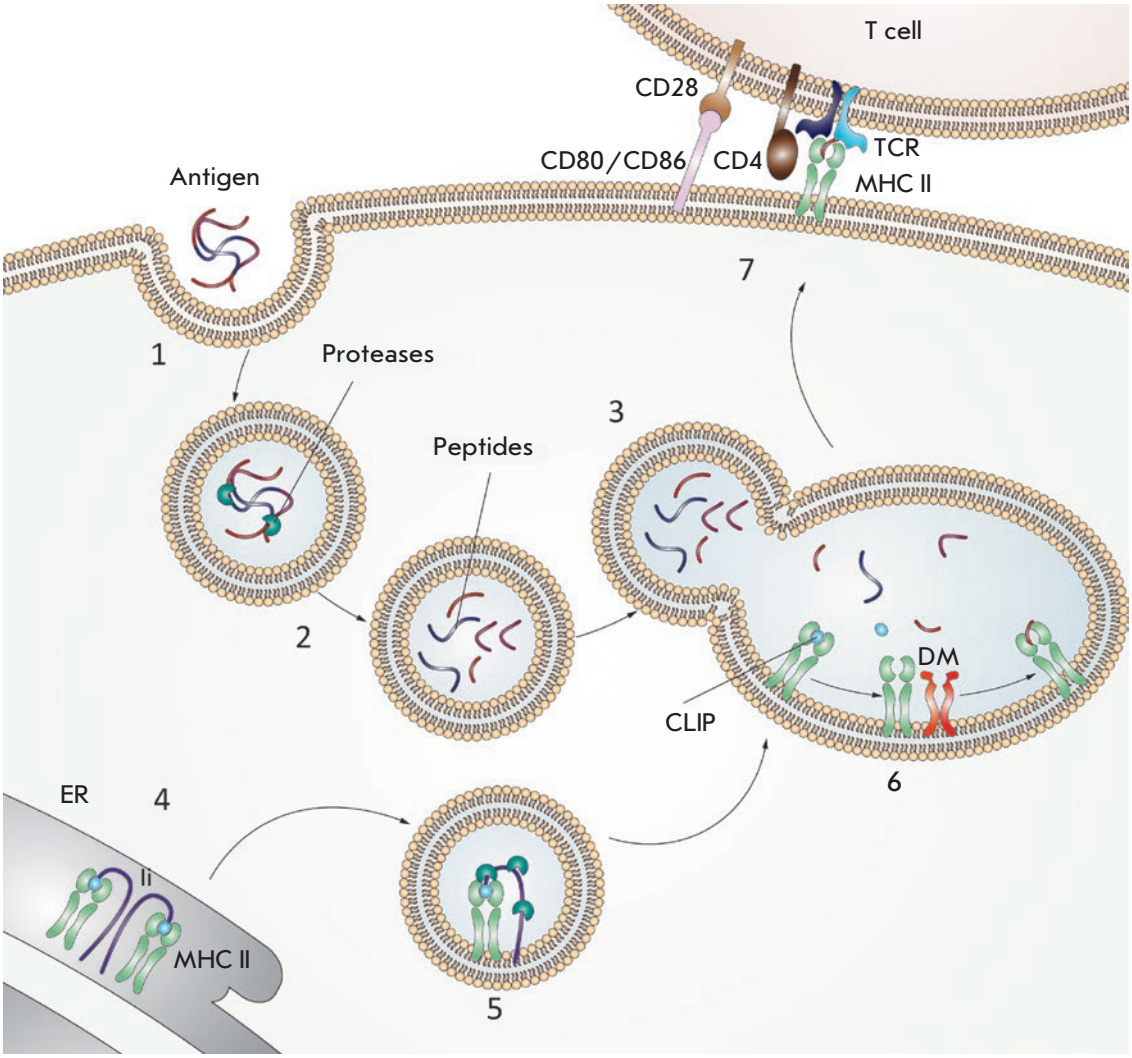

Fig. 1. Diagram showing antigen presentation by MHC II molecules. (1) An antigen enters intracellular vesicles. (2) Acidification of vesicles activates proteases that hydrolyze the antigen into peptide fragments. (3) Vesicles containing the peptide fragments merge with vesicles containing MHC II molecules (green). (4) The invariant chain (Ii) (violet) binds to the newly synthesized MHC II molecules and partially occupies the peptide-binding groove. (5) The invariant chain undergoes proteolytic degradation; as a result, the CLIP peptide (blue) remains bound in the groove. (6) DM (orange) binds to the MHC II molecules and catalyzes the peptide exchange. (7) The MHC II molecules, loaded with an antigenic peptide (red), are transported to the cell surface where they can be recognized by a CD4 + T cell receptor TCR (cyan blue). The CD4 co-receptor molecule (brown) present on T cells also binds to the MHC II molecules. For T-cell activation to occur, the CD80 or CD86 co-stimulating molecules (pink) expressed on the antigen-presenting cell must bind to the CD28 co-stimulating molecule (beige) expressed on the T cells

microglobulin), which resides on chromosome 15) and form extensive clusters (*Fig. 2*). Class I genes include *HLA-A*, *HLA-B*, and *HLA-C*, which encode the α chains of the heterodimer. MHC class II molecules are mainly encoded by the genes of the HLA-DR, HLA-DQ, and HLA-DP loci; each of them includes an α and β chain gene (e.g., the *DRA1* gene coding for the α chain and the *DRB1*, *DRB3*, *DRB4*, and *DRB5* genes coding for β chains in the HLA-DR locus). This nomenclature has evolved through historical sequences of discovery of HLAs: they were named using Roman numerals and English alphabet letters as they were progressively discovered.

The MHC locus is the most polymorphic in the human genome [2]. It is responsible for the existence of a vast diversity of MHC protein forms. To classify the products of these genes' expression, MHC molecules were subdivided into groups, in accordance with

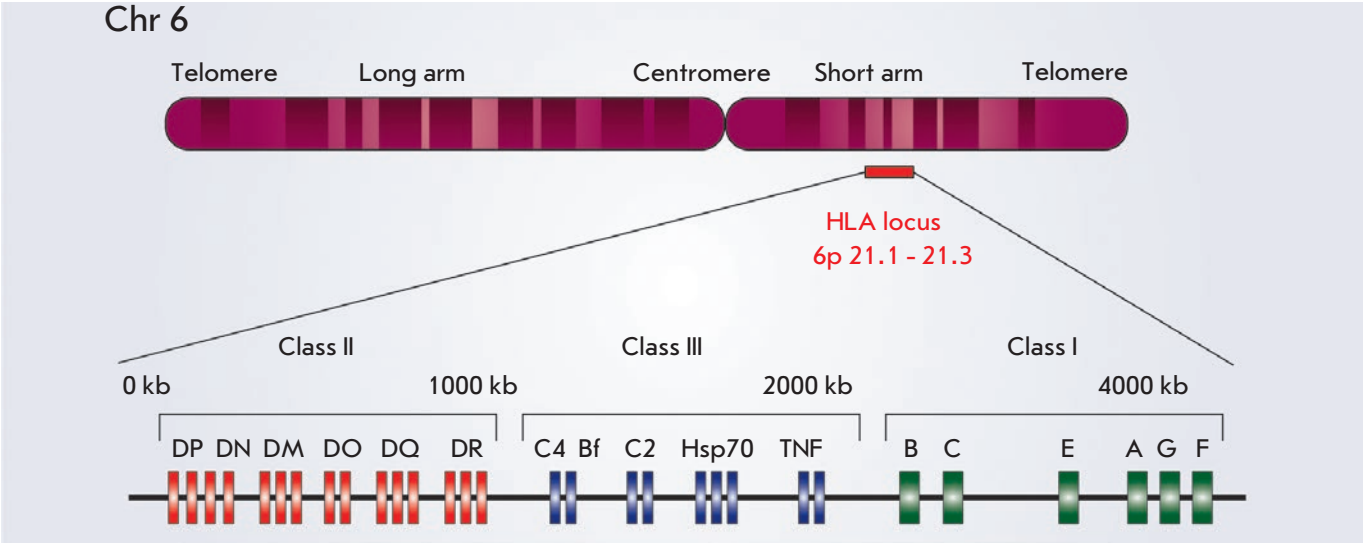

Fig. 2. Schematic representation of the HLA locus on human chromosome 6. The HLA region is located on the short arm of chromosome 6 from 6p21.1 to p21.3 and is shown with a red stripe. The length of class II (red), class III (blue), and class I (green) genes (from the centromeric to the telomeric end) is shown. The class II region includes genes for the α and β chains of the MHC class II molecules HLA-DR, HLA-DP and HLA-DQ. In addition, the genes encoding the DMα and DMβ chains, as well as the genes encoding the α and β chains of the DO molecule (DOα and DOβ, respectively), are also located in the MHC class II region

their serotypes (e.g., the HLA-DR1 serogroup). The advances in molecular genetic methods have made it possible to refine the nomenclature and identify the groups of *HLA* alleles that correspond to serogroups of their protein products (DRA*01 + DRB1*01, respectively) and subsequently even the specific gene alleles (DRA*01 + DRB1*0101, *0102 or *0103, respectively) [3]. The *HLA-B* gene is the most polymorphic MHC class I gene (with 1,077 alleles reported); the *HLA-DRB1* gene, with 669 alleles, is the most polymorphic among MHC class II genes [4, 5]. Extensive linkage disequilibrium (LD) regions (up to 500 kb) were found within the *HLA* genomic region [6]. These extensive inherited gene clusters complicate the identification of specific disease-associated alleles, since they often cannot be differentiated from the full inherited haplotype.

To date, many biomedical studies have focused on the role of MHC II in the initiation of autoimmune responses, since they can present both exo- and endogenous peptides to $CD4^+$ T cells under pathological conditions. Recently, there have been reports of a lot of association examples between certain MHC II alleles and the risk of developing autoimmune diseases (ADs) (*Table*). This fact is one of the main reasons behind the development of an autoimmune process and explains the phenomenon of "autoimmunity" at the molecular level.

In patients with autoimmune diseases (such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), type 1 diabetes (T1D), rheumatoid arthritis (RA), Graves' disease (GD), etc.), auto-antibodies are synthesized and lymphocytes often penetrate into the target organ, leading to inflammation and partial destruction. These diseases are mostly chronic. Although the AD patienthood can often be stabilized for prolonged time periods, researchers still need to gain a detailed understanding of the disease mechanisms in order to develop an effective treatment strategy. Antigen presentation and further T cells activation are considered key components of the immune response in many diseases [7] and are often the therapy targets. Therefore, studying the peculiarities of antigen presentation (and the structure and features of MHC I and II proteins in particular) is of utmost importance.

Interestingly, the autoimmune diseases accompanied by autoantibody production are typically associated with MHC II, while the diseases not accompanied by this phenomenon are more commonly associated with certain MHC I alleles [8]. An association with haplotypes including gene clusters is also observed in many diseases, which is likely to be the result of a linkage disequilibrium of these genes that occurs during inheritance. Thus, many diseases are known to be associated with the DR3-DQ2 MHC II haplotype and with the MHC I HLA-B8 and HLA-A1 alleles, which are components of the extensive, conserved haplotype [8].

The association between the HLA-DRB1, HLA-DQA1, and HLA-DQB1 alleles and autoimmune diseases

HLA-*DRB1**	MS	T1D	RA	GD	N	SLE	AT	Odds ratio	
								Positive association	Negative association
0101								1.5 – 3.0	0.5 – 1.0
0102								3.0 – 5.0	0.1 – 0.5
0103								5.0 +	0 – 0.1
0301									
0401	Sardinia/Japan					Japan			
0402									
0403									
0404									
0405									
0701									
0801	15/08								
0901	Japan								
1001									
1101									
1102									
1103									
1201									
1301	Sardinia/Israel								
1302						Japan			
1401									
1403						Japan			
1501									
1601									
HLA-*DQA1** HLA-*DQB1**								DQ	
0102 0601								DQ6.1	
0102 0602								DQ6.2	
0201 0303								DQ9.2	
0301 0302								DQ8.1	
0501 0201								DQ2.5	

MS – multiple sclerosis; T1D – type 1 diabetes; RA – rheumatoid arthritis; GD – Graves' disease; N – narcolepsy, SLE – systemic lupus erythematosus; AT – autoimmune thyroiditis.

THE POTENTIAL MECHANISMS OF A LINK BETWEEN MHC II ALLELES AND THE RISK OF DEVELOPING CERTAIN ADS

MHC II molecules are involved in the presentation of antigens, including autoantigens (*Fig. 1*). The immune response develops after the 13–18 amino-acid antigenic peptide is presented by APCs using a MHC II molecule and recognized by the respective T-cell receptor on the $CD4^+$ T-cell surface. Dendritic cells, B cells, and macrophages can act as APCs [9].

MHC II is synthesized in the endoplasmic reticulum and leaves this compartment as a complex with the invariant chain (IC) [10]. The IC catalyzes the release of MHC II from the endoplasmic reticulum and prevents its aggregation. The IC undergoes proteolysis in late endosomes, and only its small fragment (CLIP) remains bound to MHC II. It is most likely that CLIP impedes the interaction between MHC II and nonspecific peptides by blocking their access to the binding pockets [9].

In late endosomes, CLIP is exchanged for a MHC-specific antigenic peptide [11]. HLA-DM, a nonconventional MHC class II molecule [12], plays a crucial role in this process. This molecule is not polymorphic and cannot interact with antigenic peptides; however, its structure is similar to that of other MHC II molecules. HLA-DM catalyzes the binding of antigenic peptide to HLA-DR and substantially increases the rate of this reaction [13, 14]. Hence, HLA-DM contributes to the specific binding of MHC II to high-affinity peptides. The MHC II–peptide complexes are then delivered to the plasma membrane to present peptides to $CD4^+$ T cells. The interaction with these cells determines whether the immune response is started or not. If the immune response is initiated, $CD4^+$ T cells activate naïve B cells for a subsequent production of specific antibodies/autoantibodies (for presentation of self-antigens) and contribute to the recruitment of macrophages to the immune response. Autoreactive $CD4^+$ T cells against a number of self-antigens have been identified in patients with AT, GD, and MS [15].

Several hypotheses exist regarding the emergence of autoimmune diseases mediated by a number of MHC II alleles. Thus, a positive or negative association between autoimmune diseases and various HLA alleles can be determined through the structural features of the antigen-binding groove of the MHC molecule, which is encoded by a certain allele and is responsible for peptide binding (particularly, the arrangement of certain amino acids in definite positions within this groove, such as the positions 11, 71, and 74 in the MHC β-chain) [8, 16]. These MHCs, with point substitutions, vary in their efficiency of binding and presentation of self-peptides [17, 18]. The structural MHC elements indirectly related to the initiation of an autoimmune response can reside not only in the peptide-binding groove, but also in its proximity, within the area directly in contact with the T-cell receptor. The MHC polymorphism in this region may cause either binding of autoreactive effector T cells or weak selection of regulatory T cells [6].

The autoimmune response can also be initiated through molecular mimicry, when exogenous viral or bacterial peptides share a structural similarity with endogenous self-peptides. Certain MHC alleles structurally adapted for presentation of these exogenous infectious peptides can also present structurally similar self-peptides, followed by the initiation of an autoimmune response [19].

Some examples have been reported when medication (abacavir in the treatment of HIV [8, 20] or even low-molecular-weight compounds such as Be^{2+} [21]) could be bound to the peptide-binding groove of a specific MHC allele, thus changing the specificity of peptide presentation and enabling presentation of self-peptides.

In some cases (e.g., in patients with the celiac disease [22] or RA [23]), the antigen peptides undergo post-translational modification and are preferentially presented on risk alleles.

The data on a positive or negative link between a number of MHC alleles and the risk of developing ADs are of exceptional importance in enabling successful target immunotherapy using drugs targeted at the stage of MHC II antigen presentation.

THE DIVERSITY OF MHC II ALLELES ASSOCIATED WITH THE RISK OF DEVELOPING THE MOST COMMON AUTOIMMUNE DISEASES

Multiple sclerosis

Multiple sclerosis is a chronic neurodegenerative disease of the central nervous system (CNS) which is diagnosed in 0.1% Europeans and North Americans [24]. The risk factors for MS include polygenic inherited predisposition and a number of external factors, such as some infectious diseases, diet, and various social and climatic factors [25].

The disease has been shown to be clearly associated with the carriage of various genetic variants of MHC II. The most significant association between an extensive DR15-DQ6 haplotype (HLA-*DRB1**1501/HLA-*DRB5**0101/HLA-*DQA1**0102/HLA-*DQB1**0602) and MS has been revealed in Caucasians [26]. Since all these alleles are characterized by a substantial linkage disequilibrium, it remained unclear for a long time which allele contributes the most to the predisposition to MS. A study focused on the association between MHC II genes and MS in African-Americans has allowed researchers to achieve some progress toward solving this problem. Linkage disequilibrium is less pronounced in this population, and the HLA-*DRB1**1501 allele is the one most typically associated with MS, thus indicating that this allele plays the most important role among the three alleles in the haplotype [27]. Today, the HLA-*DRB1**1501 allele has been recognized as the major risk allele for MS in Caucasians; its association with the disease has been demonstrated in most of the populations analyzed [28].

Multiple sclerosis has conventionally been regarded as a disease that affects women more often than men, since the female-to-male ratio between patients with relapsing-remitting (or secondary progressive) MS is 2.5 : 1. Interestingly, Hensiek et al. [29] have reported that women are also more likely to carry the HLA-*DRB1**15 allele.

In addition to *DRB1**1501, the universal risk allele for MS, other variants of the *HLA-DRB1* gene positive-

ly associated with MS have been reported for different populations (*Table*). An association between *DRB1**03 and MS has been revealed in many European populations; the risk of developing MS is significantly higher in homozygous carriers [30]. In patients from Sardinia and Japan, the HLA-*DRB1**04 allele cluster was found to be also positively associated with MS, in addition to HLA-*DRB1**03 [31, 32]. The HLA-*DRB1**13 allele, which was also detected within the HLA-*DRB1**1303/HLA-*DQB1**0301 haplotype in MS patients from Sardinia (Italy), was also found to be associated with MS in Israelis [33]. A strong positive association between carriage of the HLA-*DRB1**08 variant and the risk of developing MS was found to exist in Caucasians with the *HLA-DRB1**15/08 genotype [34, 30].

The HLA-*DRB1**14 variant was found to be a major protective allele (i.e., the allele negatively associated with the disease and that reduces its risk compared to the average population risk) in Northern Europeans [34]. The HLA-*DRB1**01, *07, and *11 allele clusters are also regarded as protective, albeit to a lesser extent, in Caucasians [30, 35, 36]. The HLA-*DRB1**11 variant also exhibits a pronounced protective effect in African-Americans [37] and residents of Sardinia [32]. The *DRB1**0901 allele can be considered as protective against MS in Japanese natives; its frequency across Asian countries is normally higher than it is in other countries [31].

Recent studies have demonstrated that the effect of protective and risk alleles can mutually compensate in heterozygous carriers. Thus, the effect of the HLA-*DRB1**15 and *03 risk alleles was found to be mitigated in the presence of the protective HLA-*DRB1**14 or *11 variant [30, 34].

It should be mentioned that carriage of certain HLA alleles is associated with the age of onset in multiple sclerosis. Thus, carriage of HLA-*DRB1**1501, the major risk allele for MS, is associated with earlier onset of the disease in Caucasians [38], while carriers of the *DRB1**0405 allele display an earlier onset of the disease in the Japanese population [31]. It is known that in patients with MS, the immune system attacks the components of the myelin sheath formed by oligodendrocytes [39]. A number of autoantigens in MS have been identified: the myelin binding protein (MBP), proteolypid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and the myelin-associated glycoprotein (MAG). Today, MBP is considered to be the most important of these autoantigens. MBP-specific $CD4^+$ T cells have been revealed in the brain and spinal cord tissues of MS patients [40], while APCs presenting the main encephalitogenic peptide MBP (a fragment consisting of amino acids 85–99) have been detected directly in demyelination foci [41, 42]. MHC II molecules encoded by HLA-*DRB1**1501, the universal risk allele for MS that binds to the MBP_{85-99} fragment, play a crucial role in the presentation of this peptide on the surface of APCs. An autoimmune response to this complex in humanized mice has been reported [43], which can be regarded as the main mechanism explaining the observed association.

Type 1 diabetes

Type 1 diabetes (T1D), found in 0.06–0.15% of the population, is caused by an autoimmune inflammation of pancreatic tissue, resulting in impaired insulin secretion [41, 44]. It has been demonstrated that autoreactive T cells are derived from normal cells in patients with T1D, due to the presentation of insulin fragments on MHC II molecules. The association between T1D and the *DRB1**03 and *DRB1**04 allele clusters was described earlier [6, 45]. Later, an association between this disease and *DQB1* variants was revealed; the alleles of this gene (e.g., HLA-*DQB1**0302 (DQ8) or HLA-*DQB1**0201 (DQ2)) are associated with a high risk of T1D only when encoding a neutral amino acid (e.g., Ala) at position 57. If this position is occupied by the negatively charged aspartic acid as is the case for the *DQB1**0602 (DQ6.2) and *DQB1**0303 (DQ9) alleles [46], the respective allele will exhibit a protective activity [6, 47]. It has been shown that the amino acid residue 57 is located in the P9 pocket of the peptide-binding groove and is responsible for the formation of the DQA1–DQB1 heterodimer [47]. Probably, if aspartic acid is substituted for a neutral amino acid at this position, the specificity of the MHC molecule will be changed and it will become able to present insulin fragments. Interestingly, the HLA-*DRB1**0301, HLA-*DRB1**0405, and HLA-*DRB1**0401 alleles are positively associated with T1D, while the very similar HLA-*DRB1**0403 allele is negatively associated with T1D [46, 48]. It is possible that in patients with this disease, the antigen-binding grooves in the positively and negatively associated MHC II molecules are structurally similar and have only a single-point mutation affecting the specificity of peptide binding. Furthermore, the HLA-*DRB1**0701, HLA-*DRB1**1401, and HLA-*DRB1**1501 alleles also exhibit a strong protective activity [46].

Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease that affects the joints. Almost all RA patients carry the HLA-*DRB1**0401, HLA-*DRB1**0404, HLA-*DRB1**0405 or HLA-*DRB1**0101 allele [49–51]. Interestingly, the β chains of MHC II are products of these alleles and share an amino acid motif inside the peptide-binding groove at positions 67–74, which forms the so-called "degenerate epitope" [41, 18]. It has been demonstrated

that the point mutations within the degenerate epitope change the charge, affect the association with RA, and are often the only differences between the risk and protective alleles *DRB1**0103, *DRB1**0402, *DRB1**0701, *DRB1**1102, and *DRB1**1301 [6, 7, 49, 52]. A positive association between *DQB1* variants and RA has also been demonstrated [53], although this association is probably caused by a linkage disequilibrium with *DRB1* alleles [54].

Graves' disease

Graves' disease (GD), also known as toxic diffuse goiter or Basedow's disease, is an autoimmune disorder caused by excessive secretion of thyroid hormones by the diffuse tissue of the thyroid gland, resulting in thyroid hormone poisoning (thyrotoxicosis). This disease is eight times more likely to affect women than men. It typically develops in middle-aged adults (usually between 30 and 50 years of age). The observed significant familial predisposition to GD indicates that the genetic component substantially contributes to the development of this disease. It has been demonstrated that predisposition to both GD and RA is associated with the degenerate motif in the *DRB1* gene product (namely, with the amino acid at position 74 in the β chain of MHC II). Thus, the MHC molecule encoded by the GD-associated *DRB1**03 variant and the product of the protective variant *DRB1**07 carry Arg and Glu, respectively, at position 74 [55, 56]. It is worth mentioning that the protective MHC II alleles and the risk alleles also differ for T1D and RA in terms of the amino acid residing at position 74 [6]. The position 74 in the β chain of MHC might be exceptionally important, since this amino acid residue is located within the P4 pocket, where the peptide-binding motif of MHC overlaps with the T-cell receptor docking site [57].

Narcolepsy

Narcolepsy is a chronic neurodegenerative disease characterized by excessive daytime sleepiness and disturbed nighttime sleep [41, 58]. It is a complex disease whose etiology has yet to be fully elucidated. Its presumably autoimmune nature has been attributed to an explicit association with the *DQB1**0602 MHC II allele, as almost 100% of patients diagnosed with narcolepsy carry this allele [59]. Findings of an autoimmune T-cell response in patients with this disease have also been reported [60]. Since the structurally very similar HLA-*DQB1**06011 allele (differing from the HLA-*DQB1**0602 by only 9 codons in the β-chain gene) is protective for this disease [61], it is likely that the association/protection mechanism is also related to variations in the binding strength of the presented peptide and T-cell receptor docking in this case. An antigen whose fragments can be presented by the HLA-DQ6.2 product (*DQA1**0102/*DQB1**0602) has not yet been conclusively identified; however, a hypothesis has been put forward that this antigen could be orexin (hypocretin), a neurotransmitter that is involved in sleep regulation and synthesized in the hypothalamus [51]. The crystal structure of an HLA-DQ6.2 molecule bound to the peptide (a hypocretin derivative) has been deciphered [62].

Interestingly, the accumulation of data on a link between MHC II and a risk of developing ADs has revealed that the same variants are associated with several other diseases. These variants are often found in extensive haplotypes that involve the *DRB1*, *DQA1*, and *DQB1* genes and are inherited together due to a strong linkage disequilibrium. The DR3-DQ2 and DR4-DQ8 alleles within the so-called extended haplotypes (*DRB1**03/*DQA1**0501/*DQB1**0201 and *DRB1**04/*DQA1**0301/*DQB1**0302, respectively) are associated with T1D [41, 63]. Meanwhile, DR3 is also associated with MS, GD, SLE, and AT; therefore, it is referred to as the "autoimmune haplotype" [6]. DR4 is also associated with a number of diseases, including RA and AT. On the other hand, it is worth mentioning that the HLA-*DRB1**0701 allele exhibits a protective effect in many diseases, such as MS, T1D, RA, GD, and AT (*Table*).

Recent studies have demonstrated that the extent of any association between a certain MHC allele and autoimmune diseases is also dependent on the regulated level of expression of such an allele. Furthermore, it has been revealed that increased expression of a particular MHC II allele may change the T-cell receptor repertoire during T-cell maturation in the thymus gland and affect the survival and expansion of mature T-cell clones. It has been shown that the MHC expression can be regulated at both the transriptional and post-transcriptional levels [64].

CONCLUSIONS

Most autoimmune diseases are caused by a number of factors (including genetic, social, and climatic ones) and depend on a patient's age and sex, smoking, past history of infections, etc. However, the risk of developing an AD significantly increases in patients with genetic predisposition, which is often dependent on carriage of certain MHC II genes. The MHC II variants whose carriage may lead to the development of an autoimmune disease in a particular person have been characterized. A number of MHC II alleles exhibiting protective activity against specific diseases have been reported. A cluster of MHC II genes, either positively or negatively associated with the diseases, can vary depending on a person's ethnicity. More and more structural data on

autoantigen presentation on MHC II molecules is becoming available each year. Information on the structures of several trimolecular MHC II–peptide–T-cell receptor complexes has been obtained. An integrated approach is needed for a comprehensive understanding of the mechanisms of AD induction and for developing novel therapeutic modalities. Such an approach should include an in-depth investigation of the elemental stages of MHC II antigens presentation mechanism, the basis of the protective activity exhibited by different MHC II alleles, the different characteristics of MHC II autoantigen loading, including the kinetic peculiarities, and the eliciting of a further autoimmune response involving activated $CD4^+$ T cells. ●

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Double-Stranded RNAs in Plant Protection Against Pathogenic Organisms and Viruses in Agriculture

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ABSTRACT **Recent studies have shown that plants are able to express the artificial genes responsible for the synthesis of double-stranded RNAs (dsRNAs) and hairpin double-stranded RNAs (hpRNAs), as well as uptake and process exogenous dsRNAs and hpRNAs to suppress the gene expression of plant pathogenic viruses, fungi, or insects. Both endogenous and exogenous dsRNAs are processed into small interfering RNAs (siRNAs) that can spread locally and systemically through the plant, enter pathogenic microorganisms, and induce RNA interference-mediated pathogen resistance in plants. There are numerous examples of the development of new biotechnological approaches to plant protection using transgenic plants and exogenous dsRNAs. This review summarizes new data on the use of transgenes and exogenous dsRNAs for the suppression of fungal and insect virulence genes, as well as viruses to increase the resistance of plants to these pathogens. We also analyzed the current ideas about the mechanisms of dsRNA processing and transport in plants.**
KEYWORDS **RNA interference, double-stranded RNA, hairpin RNA, transgenic plants, exogenous dsRNA, regulation of pathogen genes, plant resistance.**
ABBREVIATIONS **RNA – ribonucleic acid; RISC – RNA-induced silencing complex; siРНК – small interfering RNA; dsRNA – double-stranded RNA; hpRNA – hairpin RNA; HIGS – host-induced gene silencing; SIGS – spray-induced gene silencing.**

INTRODUCTION

RNA interference is an evolutionarily conserved intracellular process that encompasses a dedicated strategy for regulating gene expression. The most important aspect of the RNA interference mechanism is that it does not change the primary chromosome structure of the target genes but is able to significantly attenuate gene expression and lead to a number of changes in the phenotype of cells and whole organisms [1, 2]. The idea of using RNA regions complementary to a specific region of the mRNA of the target gene to suppress the expression of this gene was first described in 1984 [3] as an alternative to classical genetic analysis, i.e., to the generation of mutants that alter the primary structure of the genetic locus. However, the first experiments on the use of antisense RNA to suppress gene activity failed to yield reliably positive results and the mechanisms of this suppression remained poorly understood [4–6]. The term "RNA interference" was first introduced in 1998, when Tabara et al. showed that the process could be initiated by incubation of nematodes in a solution of gene-specific double-stranded RNA fragments [7–9]. However, by that time, explicit indications of the role of complementary RNAs in the regulation of the expression of endogenous eukaryotic

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genes had already been revealed in transgenic plants and fungi [10–12].

A fundamentally important result was reported in a paper published in 1993 and devoted to the resistance of transgenic tobacco to the tobacco etch potyvirus [13]. A relationship between the detected resistance and RNA interference was proved, because there was co-suppression of both the transgene encoding a viral genome fragment and the virus RNA genome. Therefore, this process should function precisely at the RNA level. During the 1990s, numerous studies reported on RNA interference in many organisms, including fungi, animals, and plants [14, 15]. These studies showed that the RNA interference process is initiated by the Dicer-like enzyme (DCL) that cuts long molecules of viral or cellular double-stranded RNA into short fragments of 21–25 nucleotides, called siRNAs. One of the two chains of each fragment is called a guide strand, because it is further included in the RISC complex. Under the action of this complex, a short single-stranded RNA fragment forms hydrogen bonds with the complementary sequence of an extended RNA molecule and causes cleavage of the latter by a RISC complex protein called Argonaute (AGO). This ensures high specificity of the cleavage. These events lead to the suppression (silencing) of the cell gene or virus replication [1, 16].

The movement of siRNAs in the plant is subdivided into intercellular (local) and systemic (long-distance) transport [17]. This movement occurs through the symplast: i.e., from the place of initiation to neighboring cells through intercellular channels called plasmodesmata, as well as systemically over large distances through conducting tissue of the phloem. Systemic movement of the silencing signal occurs within a few days after initiation and is usually directed from photosynthetic sources (i.e. leaves) to roots and the apical meristem [18, 19]. The systemic silencing signal was identified in plants by direct sampling of the phloem sap [20, 21] and by detection of the signal in grafted parts of the plant [22–24]. Mobile silencing signals include double-stranded siRNA molecules (21–24 nucleotides) [20, 21, 24, 25]. In this case, Dunoyer et al. [26] directly showed that chemically synthesized exogenous, fluorescently labeled siRNAs actually move from cell to cell and over long distances.

Beginning with studies that proved that artificial double-stranded RNAs cause RNA interference [9], the efficiency of this strategy for the protection of plants from pathogenic organisms and viruses has been convincingly proved [27, 28]. In this review, we describe examples of potential practical application of RNA interference in the protection of plants from pathogens.

EXPRESSION OF DOUBLE-STRANDED RNAs IN TRANSGENIC PLANTS TO SUPPRESS PATHOGENS

At present, it is obvious that RNA interference may be used to achieve desired pathogen resistance in crop plants by manipulating the expression of the genes of viruses, bacteria, fungi, nematodes, and insects [29, 30]. The method of double-stranded RNA delivery, which was previously widely used for plant protection, is based on the use of transgenic cultures producing pest-specific dsRNAs. The transgene-mediated pathogen suppression method generally involves identification of the pathogen target gene(s) to be

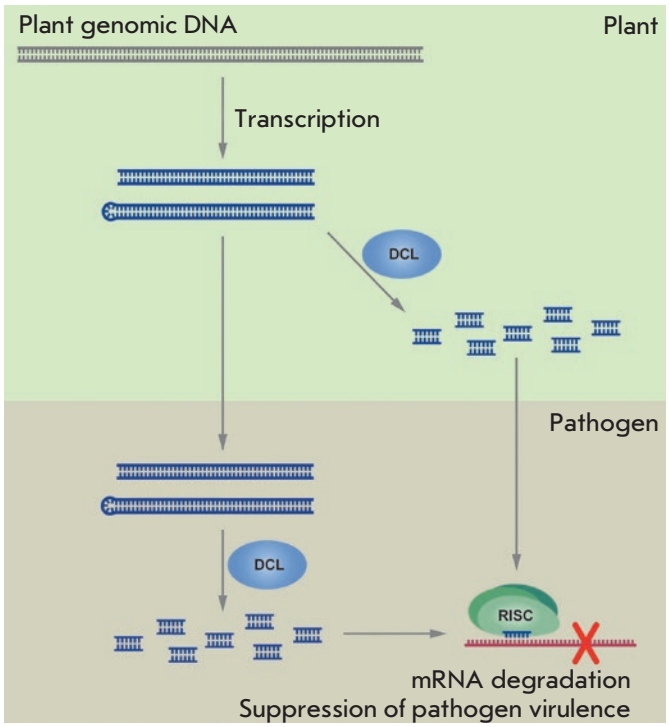

Fig. 1. Schematic representation of the use of transgenic dsRNA for RNA interference in plants. Artificial dsRNA is produced from transgenic constructs. Endogenous long dsRNAs are either transported directly into the pathogen's cytoplasm through an undefined mechanism, or dsRNA molecules (dsRNA or hpRNA) are recognized in the plant by DICER ribonuclease (DCL) that cleaves long dsRNAs into short interfering RNAs. The latter are then transferred to pathogen cells, where they are incorporated into the RNA-induced silence complex (RISC) that directs specific degradation or translational repression of pathogen mRNAs. Interfering RNAs and the RISC complex can form directly in the pathogen cells. Arrows indicate different steps of short interfering RNA induction and dsRNA/siRNA movement between plant cells and phytopathogens

suppressed, followed by the generation of a construct producing a hairpin dsRNA, using a genetically engineered cassette containing the target gene (or its fragment) in sense and antisense orientations, as well as a relatively short spacer separating complementary segments, plant transformation, and, finally, screening and evaluation of transformant traits [31, 32] (*Fig. 1*). Transgenic construct-based expression of these dsRNAs in the appropriate host plant often leads to protection against infection. This biotechnological method, called host-induced gene silencing (HIGS), has emerged as a promising alternative to other plant protection methods, because it is highly selective relative to the target organism's genes. In addition, this method has minimal side effects compared, e.g., with protein-producing transgenes or chemical protective treatment [29, 33].

Over the past 10 years, a number of studies on the use of HIGS to combat fungal diseases have been published [29, 33, 34]. The efficiency of HIGS in fighting against phytopathogenic fungi was proved in an important study published in 2010 [35]. Expression of an interference cassette for the *GUS* marker gene encoding beta-glucuronidase (hairpin (hp)GUS) in tobacco plants was shown to suppress the expression of this gene in *Fusarium verticillioides* fungal cells. However, the efficiency of HIGS against the rust pathogen varied, depending on the gene used. For example, in transgenic wheat plants producing double-stranded RNAs to the *MITOGEN-ACTIVATED PROTEIN KINASE 1* (*PtMAPK1*), *CYCLOPHILIN* (*PtCYC1*), or *CALCINEURIN B* (*PtCNB*) gene of the rust fungus *Puccinia triticina* [36], disease symptoms decreased by 51–68% and fungus biomass dropped by 59–69% compared with control vector constructs. In wheat leaves expressing these constructs, symptoms of *Puccinia graminis* infection also decreased slightly. Therefore, meticulous selection of the target genes may obviously enhance the efficiency of HIGS and provide a wider range of resistance to rust fungi.

An obvious effect of HIGS was also demonstrated in cereals infected with the powdery mildew pathogen *Blumeria graminis* [37]. A reduction in powdery mildew symptoms and a decrease in the number of functional haustoria inside epidermal cells were found in barley or wheat plants with HIGS-mediated suppression of the Avra10 effector gene. Suppression of fatty acid metabolism genes using the HIGS strategy has revealed the efficiency of this method in generating disease tolerance in some other crop plants. HIGS-mediated suppression of a rice gene, *OsSSI2*, led to enhanced resistance to the fungus *Magnaporthe grisea* and the leaf blight bacterium *Xanthomonas oryzae* [38]. Enhanced resistance against *M. grisea* in rice plants was achieved through the suppression of two genes: namely, *OsFAD7* and *OsFAD8*, which encode Ω-3 fatty acid desaturase [39]. Furthermore, suppression of the genes involved in lignin production led to enhanced resistance of soybean plants to the phytopathogen *Sclerotinia sclerotiorum* [40].

In contrast to the presented data, HIGS-mediated silencing of the genes of the oomycete *Phytophtora parasitica* failed to initiate an obvious protective response in transgenic arabidopsis plants expressing *PnPMA1* dsRNA [41]. However, other examples indicate the possibility of a successful use of HIGS against oomycetes. For example, transgenic tobacco plants expressing glutathione S-transferase gene dsRNA developed noticeable resistance to a tobacco phytophthora strain [42].

The problems related to using transgenic plants expressing dsRNAs to the genes of parasitic nematodes are summarized by Lilley et al. [43]. They noted that complex relationships between the plant and the parasite remain not fully understood. In particular, the inability to transform parasitic nematodes and generate their mutant lines obstructs our understanding of gene functions, which, in turn, complicates the identification of genes that may be effective targets for RNA interference. However, data for other cellular pathogens, in particular the soybean nematode *Heterodera glycines* and fungi, can be used for this purpose. For example, Youssef et al. [44] used the HIGS strategy to suppress the *HgALD* gene (fructose-1,6-diphosphate-aldolase), which reduced the number of female offspring by 58%.

Silencing of the housekeeping genes of the root nematode by the expression of dsRNA in the host plant also enhanced anti-nematode resistance [45]. Ibragim et al. [46] were able to successfully reduce the formation of *Meloidogyne incognita* galls in soybean roots by suppressing the genes encoding tyrosine phosphatase and fructose-1,6-diphosphate aldolase, a key glucogenesis enzyme.

An alternative HIGS strategy, which is aimed at combating nematodes, involves the genes necessary for parasitism [47, 48]. Genes *flp-14* and *flp-18* of the gall nematode *M. incognita* encode neuropeptides that are involved in nematode migration and host root invasion [47]. HIGS-mediated silencing of either of the two genes in transgenic tobacco plants reduces the infection of most lines with this nematode. Fertility of females decreases by ~50–80%. Parasitism can also be disrupted by HIGS-mediated silencing of the genes encoding the nematode effector proteins that play an important role in establishing successful parasitic relationships with the host. Transgenic *Arabidopsis thaliana* plants expressing dsRNAs for regions of the conserved root-knot nematode effector gene, *16D10*,

encoding a small secretory peptide assisting in the selection of feeding sites develops a wide spectrum of resistance to *M. incognita* [48]. Reduced susceptibility to *M. incognita* was also detected in the roots of transgenic grape plants expressing constructs based on a hairpin of a *16D10* gene sequence fragment [49]. Sindhu et al. [50] used suppression of four different genes involved in the parasitism of the sugar beet nematode (*Heterodera schachtii*) in the *A. thaliana* host expressing dsRNAs. Although total resistance was not achieved, the number of mature female nematodes decreased to 23–64% in different transgenic plant lines.

RNA interference is also used to control insect pests causing significant crop losses [51–53]. Mao et al. [54] developed a strategy that controls an insect's sensitivity to plant phytotoxins. After an insect attack, plants synthesize a variety of secondary metabolites aimed at reducing the viability of pests. In response, some insects have developed the ability to detoxify these compounds, which is often associated with the activity of cytochrome P450 monooxygenase. According to a genetic and biochemical analysis, expression of cytochrome P450 (*CYP6AE14*) in cotton worm larvae (*Helicoverpa armigera*) is necessary to initiate resistance to gossypol, a cotton phytotoxin [54]. Furthermore, expression of CYP6AE14 dsRNA in larvae grown on transgenic arabidopsis, tobacco, or cotton plants reduces synthesis of the appropriate protein and enhances sensitivity to gossypol [54, 55]. Later, the same authors showed that the protection level may be increased by co-expression of CYP6AE14 dsRNA and cysteine protease [56]. The host-plant-induced dsRNA causing cytochrome P450 silencing was also used to enhance sensitivity to deltamethrin pyrethroid that is used to control cotton pests [57]. These results suggest that cytochrome P450-targeted enzymatic systems are an effective pathway to reducing resistance to pyrethroids.

Crop plants encoding heterologous proteins or overexpressing these proteins are fundamentally different from crops that encode cassettes for the synthesis of interfering dsRNAs. RNAs are known to be non-toxic to humans, while foreign proteins produced by transgenic plants can, in some cases, be toxic or allergenic [58]. Therefore, transgenic crops with RNA-based resistance genes are much safer for humans than crops with excessive expression of proteins and do not require a determination of acute oral toxicity and assessment of the digestibility of an administered RNA component. Some biosafety problems are associated with the use of transgenic plants expressing dsRNAs, because transcriptional gene silencing by chromatin modification can lead to hereditary changes that have an adverse effect. This fuels public concern about the safety of genetically modified organisms [59]. Furthermore, many countries have put legislative restrictions on the cultivation of transgenic plants (Law Library of Congress (US): Global Legal Research Directorate; Restrictions on Genetically Modified Organisms; Global Legal Research Center: Washington, DC, USA, 2014, p. 242). Therefore, the development of new, environmentally friendly approaches to enhancing pest resistance without significant modifications in the plant genome is an important undertaking. One of these approaches is genome editing using the CRISPR/Cas system. First, CRISPR/Cas systems can be used to introduce point mutations or small deletions into specific genes of the host plants in order to block the mechanisms promoting the spread of the pathogen in the plant. Second, CRISPR/Cas systems can be developed for the mutagenesis of pathogen genomes. For example, CRISPR/Cas9 systems can be targeted directly at DNA- or RNA-containing viruses [60].

METHODS FOR DELIVERY OF ARTIFICIAL DOUBLE-STRANDED RNAs IN PLANTS: DIRECT TREATMENT OF PLANTS WITH DSRNA

RNA interference-based methods have been proved to be an effective strategy for protecting plants from the diseases caused by viral and cellular pathogens. However, the possibility of a widespread use of HIGS remains very doubtful because the development of genetically modified crop plants takes a lot of time and is still widely mistrusted by people in many European countries.

The search for alternative strategies was facilitated by the results of earlier studies, which showed that dsRNA solutions may be used for RNA interference of the nematode *Caenorhabditis elegans* [7]. Furthermore, successful experiments on the suppression of the growth and reproduction of parasitic plant nematodes *in planta* proved that RNA interference in this case may be a promising method for reducing the viability of pests [43]. At the moment, these studies are innovative and may lead to significant progress in the development of an RNA interference-based approach to plant protection by direct introduction of exogenous dsRNA complementary to the pathogen genome. These studies will clarify the following important issues: (i) the methods and mechanistic basis for the introduction of dsRNAs into plants; (ii) solving the problems of transport, processing, and stability of dsRNAs in the external environment and in cells; and (iii) implementation of large-scale production and purification of exogenous dsRNA to make this approach economically viable. Several alternative methods for dsRNA delivery, which do not involve plant transformation, have been

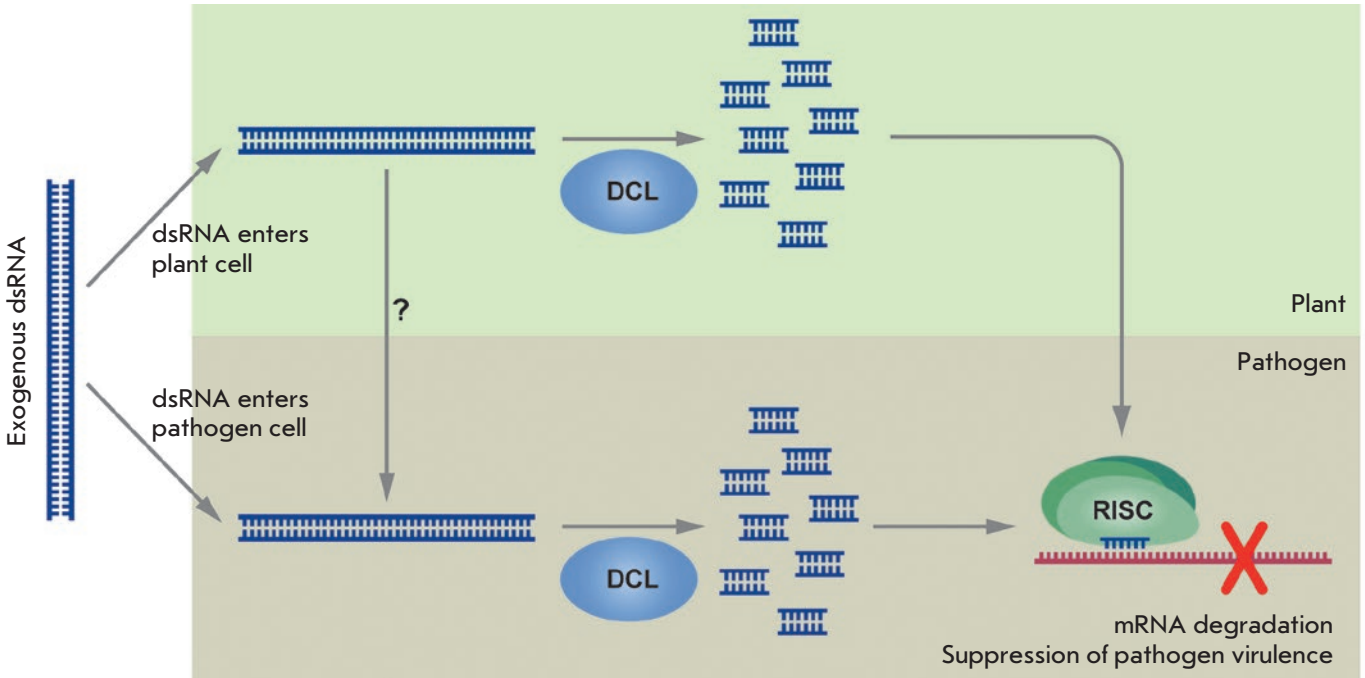

Fig. 2. Schematic representation of the use of exogenous dsRNA for RNA interference induction and degradation of target plant pathogen mRNAs. Exogenous artificial dsRNA is dissolved and applied to plant leaves, flower buds, roots, or seeds. Uptake and transport of exogenous dsRNAs occur through an undefined mechanism. dsRNA or hpRNA molecules are recognized by DICER-like (DCL) ribonuclease that cleaves long dsRNAs into siRNAs. siRNAs are then incorporated into the RNA-induced silencing complex (RISC) that guides sequence-specific degradation or translational repression of homologous pathogen mRNAs. Arrows depict different steps of the RNAi induction process and dsRNA / siRNA movement between plant cells and plant pathogens

proposed. In particular, dsRNA can be translocated into the plant vascular system (xylem and phloem) through roots or by direct injection of RNA molecules into a tree trunk [28, 61–66].

However, the spraying of plants (mainly leaves) is currently considered the most promising method. This method is called spray-induced gene silencing (SIGS). Exogenous interfering dsRNAs can either be directly uptaken by pest cells or transferred first to plant cells and then to pathogen cells (*Fig. 2*) [64, 67, 68]. In this regard, it is important to note that locally sprayed RNAs also inhibit pathogen virulence in distal untreated leaves [68, 69]. Obviously, these dsRNAs, or shorter products of their processing, are capable of systemic spread in plants.

Initial "naked" dsRNA preparations have been shown to protect plants from microbial pathogens for 10 days after spraying [64, 67, 68]. However, incorporation of dsRNAs into hydroxide nanolayers, called BioClay, was recently shown to increase the duration of the protection against infection by more than 20 days [69]. BioClay nanolayers prevented degradation of dsRNA by RNase or sunlight. Because these nanoparticles and incorporated RNA are non-toxic and easily decomposed, this method is considered environmentally friendly. Moreover, it increases the efficiency of SIGS in combating plant diseases in the field [70]. Thus, advances in nanoparticle technology have markedly improved the potential efficiency of SIGS for plant protection. In addition, chitosan polymers were also used to encapsulate dsRNA and achieve RNA interference. Chitosan nanoparticles were produced by self-assembly of the polymer with dsRNA using electrostatic interactions between the positive and negative charges of amino groups in the chitosan and phosphate groups in nucleic acid, respectively. This method is well-suited for long dsRNAs. Chitosan nanoparticles, when applied to plants, can enter a pest's body, along with food. This system is very inexpensive and highly efficient. In addition, chitosan polymers are non-toxic and readily biodegradable [63, 69, 71, 72].

The length of exogenous dsRNA is very important for an efficient suppression of the genes of plant

pathogens. The dsRNA length required to achieve a pronounced effect varies depending on the pathogen type and taxon. Insect studies have shown that the dsRNA length required for successful RNA interference ranges from 140 to 500 nucleotides in most cases. For viruses, this length is more than 200–300 nucleotides [28]. In general, each particular gene is believed to require screening for several dsRNA types of different lengths and locations. In addition, dsRNA can be either very specific to the target gene of a particular pathogen type or designed for a wider range of closely related species [69, 71, 72].

The efficiency of RNA interference induction by exogenous dsRNAs also depends on their optimal (sufficiently high) concentration, which in practical applications requires the production of large amounts of dsRNA [73–75]. In RNA interference experiments, dsRNAs have been produced *in vitro* by bidirectional transcription using T7 polymerase [76, 77]. However, it is obvious that such a system is unsuitable for large-scale production, for economic reasons. Therefore, it has been proposed to use an inducible cassette with the T7 phage RNA polymerase promoter, which expresses dsRNA in the RNase III-deficient *Escherichia coli* strain HT115, M–JM109, or M–JM109lacY [78–81]. In addition, stable and efficient systems for dsRNA production in *Pseudomonas syringae* bacteria [82] and *Saccharomyces cerevisiae* yeast [83] were recently developed. Obviously, the listed microbiological expression systems can potentially be used for large-scale and inexpensive production of dsRNAs for practical applications of SIGS in agriculture.

In recent years, the SIGS system has been shown to be effectively usable to control plant pathogenic fungi. Application of dsRNAs, which were synthesized *in vitro* and directed against a number of fungal genes, to the leaf surface was found to reduce the spread of infection by blocking growth, altering morphology, and reducing pathogenicity and lead to less pronounced manifestations of the disease [67, 68, 84, 85]. The use of exogenous dsRNA on plant surfaces is currently regarded as an innovative strategy for protecting plants from fungal infection [28, 63, 64, 67]. It is supposed that there may be two ways for dsRNAs deposited on the plant surface to occur in fungal cells: (i) after the spraying of plants, dsRNAs immediately penetrate fungal cells and undergo processing into siRNAs; and (ii) RNAs enter plant cells and form short siRNAs that are translocated to fungal cells (*Fig. 2*) [67, 68, 86]. The effect of *Myo5* gene-silencing in *Fusarium asiaticum* is found to linger only if dsRNA continuously enters fungal cells, because *F. asiaticum* cannot support the amplification of secondary siRNAs. The findings of Song et al. [86] indicate that dsRNAs entering plants are processed into siRNAs that are then amplified by plant RNA-dependent RNA polymerase (RdRP), resulting in the formation of secondary siRNAs. Interestingly, uptake of dsRNA through the wound surface of tip cut wheat coleoptiles was more efficient than through the intact surface. In addition, penetration of dsRNA was enhanced by a nonionic surfactant, Silwet L-77 [84, 86].

Over the past few years, numerous studies have shown that dsRNAs that are complementary to a number of important insect pest genes can become an effective inducer of SIGS and increase insect mortality, decrease their growth rate and fertility, and reduce their sensitivity to insecticides [87]. Treatment of leaves with artificially synthesized dsRNAs targeted at the genes involved in insect development significantly increases mortality and inhibits insect growth [88–92]. This effect can be achieved by irrigating the roots of plants by dsRNA, which leads to effective suppression of the target gene and abnormal development of insect pests [74, 76, 93]. RNA interference using exogenous dsRNAs can be used for a very wide range of insect genes. For example, suppression of the expression of two ATPase genes in *Diabrotica undecimpunctata* and *Leptinotarsa decemlineata* reduced insect survival by 40–50% [76]. Mortality of the cabbage moth (*Plutella xylostella*) on leaves sprayed with dsRNAs to *Pl. xylostella* acetylcholinesterase genes, *AChE1* and *AChE2*, reached 74 and 89%, respectively [94]. In addition, SIGS to the juvenile hormone acid O-methyltransferase (JHAMT) and vitellogenin genes significantly reduced the levels of these proteins (up to 85–90%) in several taxonomically distant insects [95].

At the moment, the subtle mechanisms of dsRNA penetration from plants into pest cells are not fully understood. Obviously, dsRNA directly penetrates fungal hyphae from plant cells and intercellular spaces. The mechanisms of dsRNA action on nematodes and insects are less understood. The natural pathway is initial RNA penetration from ingested plant sap into digestive tract cells. In this case, endocytosis probably plays an important role. For example, two genes required for efficient penetration of dsRNA during nutrition were found in nematodes. They were *Systemic RNAi-deficient* (*SID*) genes [64, 67]. The *SID-2* gene encodes a transmembrane protein involved in a rather slow uptake of dsRNA by endocytosis, while the *SID-1* gene product is necessary for fast, not related to endocytosis, transport and forms channels in the plasma membrane [64, 67].

The effect of exogenous dsRNAs on virus resistance in various species, including tobacco, tomato, corn, papaya, and orchids, has been analyzed in several experimental studies. In this case, plants were treated either

with RNA synthesized *in vitro* or with nucleic acid preparations purified from bacterial strains expressing dsRNA or hpRNA [79, 80, 96, 97]. dsRNAs targeted at virus replicase or coat protein genes was found to delay the development of disease, reduce infection symptoms and the number of infected plants, and decrease the virus titer [28, 65]. In addition, it was confirmed that the protective effect induced by dsRNA or hpRNA lingers for at least 20–70 days after inoculation of the virus [72, 98].

In conclusion, it should be noted that SIGS is a targeted and environmentally friendly strategy for plant protection both after and before harvest and, obviously, minimally harmful to the health of consumers. In addition, because the highly conservative pathogen genes necessary for their growth or virulence are often selected for SIGS, pathogens are not able to generate a sufficient amount of mutations in these important genes to avoid the influence of SIGS and simultaneously preserve their vital functions. Finally, it should be emphasized once again that the SIGS technology is much more acceptable to the public than, e.g., chemical treatments, and its development requires significantly less time than the creation of stable transgenic cultures. ●

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Non-neutralizing Antibodies Directed at Conservative Influenza Antigens

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ABSTRACT **At the moment, developing new broad-spectrum influenza vaccines which would help avoid annual changes in a vaccine's strain set is urgency. In addition, developing new vaccines based on highly conserved influenza virus proteins could allow us to better prepare for potential pandemics and significantly reduce the damage they cause. Evaluation of the humoral response to vaccine administration is a key aspect of the characterization of the effectiveness of influenza vaccines. In the development of new broad-spectrum influenza vaccines, it is important to study the mechanisms of action of various antibodies, including non-neutralizing ones, as well as to be in the possession of methods for quantifying these antibodies after immunization with new vaccines against influenza. In this review, we focused on the mechanisms of anti-influenza action of non-neutralizing antibodies, such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-mediated complement-dependent cytotoxicity (CDC). The influenza virus antigens that trigger these reactions are hemagglutinin (HA) and neuraminidase (NA), as well as highly conserved antigens, such as M2 (ion channel), M1 (matrix protein), and NP (nucleoprotein). In addition, the mechanisms of action and methods for detecting antibodies to neuraminidase (NA) and to the stem domain of hemagglutinin (HA) of the influenza virus are considered.**
KEYWORDS **influenza virus, broad-spectrum influenza vaccine, antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, antibody-mediated complement-dependent cytotoxicity**
ABBREVIATIONS **ADCC – antibody-dependent cellular cytotoxicity; ADCP – antibody-dependent cellular phagocytosis; CDC – antibody-mediated complement-dependent cytotoxicity; HA – hemagglutinin; NA – neuraminidase; NP – nucleoprotein; WHO – World Health Organization; DNA – deoxyribonucleic acid; NK – natural killers; ITAM – immunoreceptor tyrosine-based activation motif; ELISA – enzyme-linked immunosorbent assay; IFNγ – interferon gamma; TNFα – tumor necrosis factor alfa; ELLA – enzyme-linked lectin assay.**

INTRODUCTION

Influenza is a highly contagious infection; it is responsible for annual epidemics and periodical pandemics that appear at varied intervals. According to the WHO, 20–30% of children and 5 to 10% of adults are infected with influenza annually in the world and 250 to 500 thousand people die from severe complications of the influenza infection. In pandemics, the extent of complications and mortality increase significantly. For instance, according to various sources, around 50 to 100 million people died from influenza during the 1918–1919 flu pandemic [1].

The most potent protective measure against the influenza infection and its spread is vaccination. Modern influenza vaccines, as a rule, induce the formation of antibodies to the influenza HA and NA surface antigens. The surface proteins of the influenza virus undergo constant antigenic drift. Therefore, annual renewal of the strain composition of the vaccine is required [2].

To date, the development of new broad-spectrum influenza vaccines which would help avoid the necessity of annual changes in the strain composition of the vaccine remains urgency. In addition, the creation of new vaccines based on highly conserved influenza virus proteins would allow us to better prepare for potential pandemics and significantly reduce the damage they cause.

The key to evaluating the effectiveness of influenza vaccines is to determine the level of humoral response after vaccination. Neutralizing antibodies to the globular head domain of hemagglutinin are produced during viral infection and undergird the protective mechanisms of all the influenza vaccines available to date [3].

Most virus-neutralizing antibodies bind to the head domain of HA, inhibit the binding of HA to the sialic acid residue and prevent the virus from entering the cells (*Fig. 1,* b). These antibodies are determined by conventional hemagglutination inhibition and neutralization reactions [4–6]. Moreover, many HA head-specific antibodies are also able to inhibit the release of the virus from the cell (*Fig. 1,* d). This defense mechanism cannot be evaluated by conventional hemagglutination inhibition and neutralization inhibition assays; it is detected by adding antibodies to cells that have been previously infected with the influenza virus [7].

Antibodies against various conserved antigens of the influenza virus (such as NP, M1, M2) are generally non-neutralizing in nature and cannot prevent the development of the viral infection. However, they are able to exert a protective function through various immune mechanisms. Thus, the study of the mechanisms of action of various antibodies, including non-neutralizing ones, as well as the development of methods for evaluating the level of such antibodies after immunization with new influenza vaccines, is relevant for the development of novel broad-spectrum influenza vaccines.

ANTIBODIES TO CONSERVED ANTIGENS OF THE INFLUENZA VIRUS PARTICIPATING IN THE REACTIONS OF ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY, ANTIBODY-DEPENDENT PHAGOCYTOSIS, AND ANTIBODY-MEDIATED COMPLEMENT-DEPENDENT CYTOTOXICITY

The ability of antibodies to neutralize the influenza virus has traditionally been considered the most important mechanism of protection against influenza. However, recent studies have shown the importance of other antibody-mediated effects, which also contribute to antiviral protection [3]. The following mechanisms of anti-influenza action are realized by non-neutralizing antibodies: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-mediated complement-dependent cytotoxicity (CDC) [8]. The influenza virus antigens that trigger these reactions are HA, NA, and highly conserved antigens such as the M2 ion channel, M1 matrix protein, and nucleoprotein (NP).

Unlike neutralizing antibodies, the functions of which are implemented by the variable regions, the effect of non-neutralizing antibodies depends on the conserved Fc region. The Fc region is able to interact with various components of the immune system, while the variable part of the antibody binds to the antigen. The most significant antibody isotypes for the implementation of the effector functions of non-neutralizing antibodies are IgG and IgM, with IgG3 possessing the highest functional potential [9].

Neutralizing antibodies can bind with their Fc region to the specific Fc receptors exposed on the surface of most immune cells, including NK cells, macrophages, and neutrophils (*Fig. 1,* f). After binding to antibodies, these immune cells are activated and become involved in the defense response against a pathogen. A total of six different receptors involved in the activation (FcγRI, IIA, IIC, IIIA, and IIIB) or inhibition (FcγRIIB1/B2) of human immune cells have been described. Non-neutralizing antibodies can also activate the complement system (*Fig. 1,* g) [9].

Antibody-dependent cellular cytotoxicity (ADCC)

Influenza virus-infected cells carry viral proteins on their surface – mainly HA and NA – since new virions are formed by budding from the cell membrane. Anti-influenza IgG can bind viral proteins on the cell surface, thus opsonizing the infected cells. The Fc gamma receptor IIIa (FcγRIIIa) exposed on the surface of many cells of the innate immune response, such as NK cells, monocytes, and macrophages, binds to the Fc region of IgG. The interaction between FcγRIIIa and IgG bound to the infected cell leads to phosphorylation of the tyrosine-based activation motif (ITAM) and activation of the Ca^{2+}-dependent signaling pathway. As a result, NK cells begin to produce cytotoxic factors (perforins and granzymes), which lead to the death of the infected cell, and antiviral cytokines (IFNγ, TNFα) and chemokines (*Fig. 2*) [10].

One of the main targets for the antibodies involved in ADCC is the conserved stem of HA, which is one of the most represented surface proteins of the influenza virus. For instance, it has been shown that antibodies with a broad spectrum of activity against the conserved HA stem protect mice from a lethal influenza infection through a mechanism that involves an interaction with Fc-FcγR. On the contrary, the protective activity of antibodies in relation to the variable head domain of HA has manifested itself both in the presence and absence of an interaction with FcγR [11].

Furthermore, re-infection of macaques with the influenza virus has led to a rapid appearance of ADCC responses. Antibodies capable of inducing activation of NK cells were found in the bronchoalveolar lavages of the macaques, which correlated with a reduced virus shedding and decreased disease duration [12]. In humans, high titers of antibodies capable of participating in ADCC were also shown to correlate with a decrease in the incidence of an experimental infection [13]. Moreover, elderly people who had previously been infected with viruses close to the strain that caused the 2009 swine influenza pandemic and who retained a significant amount of titers of the antibodies participating in ADCC but had no neutralizing antibodies were

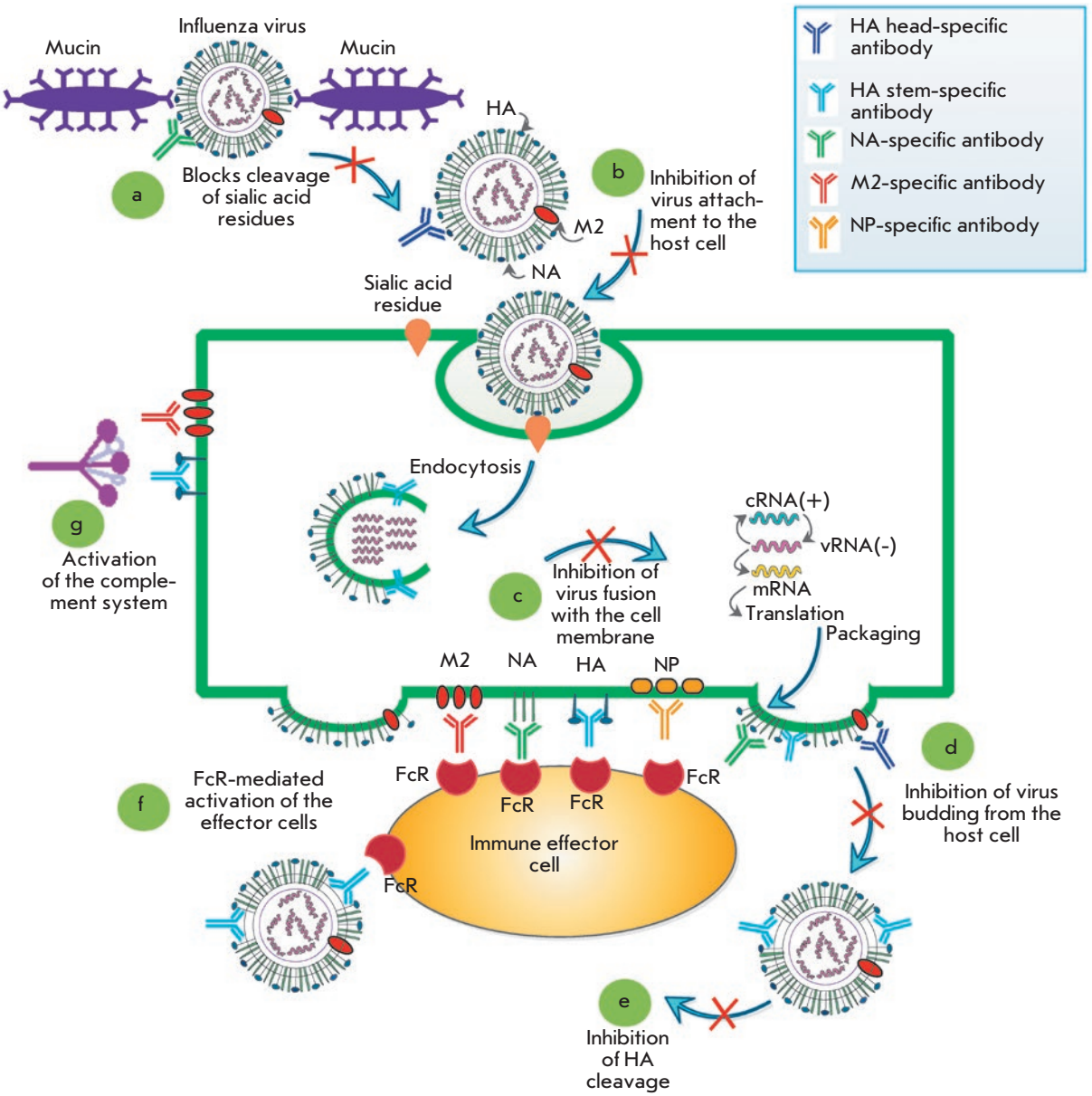

Fig. 1. Mechanisms of action of anti-influenza antibodies. The influenza virus enters the body through respiratory tract mucosa, where viral hemagglutinin (HA) binds to the terminal sialic acids of mucin. Neuraminidase (NA) releases the virus by cleaving the terminal sialic acid residues. Antibodies to neuraminidase can inhibit the reaction, and the virus would not be able to penetrate the mucous layer **(a)**. After penetrating the mucous layer, the influenza virus binds to the sialic acids on the surface of the target cells and enters the cell by endocytosis. Neutralizing antibodies bind to influenza HA and block this process **(b)**. The endosomes of the target cells become acidified, thus triggering the fusion of the endosomal and viral membranes via HA, which results in the release of the viral genome into the cell cytoplasm. Antibodies to the stem domain of HA can inhibit this process **(c)**. After the synthesis of viral proteins, the internal proteins are packed into viral particles containing HA, NA, and the M2 ion channel molecules on the virion surface. On the cell surface, the HA, NA, and M2 proteins can be bound by antibodies that block the budding of viral particles. Maturing viral particles are covered by the host cell membrane as a result of the interaction between HA and sialic acids. Meanwhile, NA cleaves terminal sialic acids from the virus, while antibodies to NA can inhibit this process **(d)**. Finally, in the matured viral particles, HA0 is cleaved into the HA1 and HA2 subunits by the host proteases that are present in the respiratory tract. Antibodies directed to the HA stem domain can block this process **(e)**. In addition, viral antigens exposed to the surface of an infected cell (including the internal protein NP, which is detected on the surface of the infected cell) are targets for antibodies that activate effector cells via the Fc-FcR interaction **(f)**. Antibodies directed to the viral antigens exposed on the cell surface can also activate the complement system **(g)**

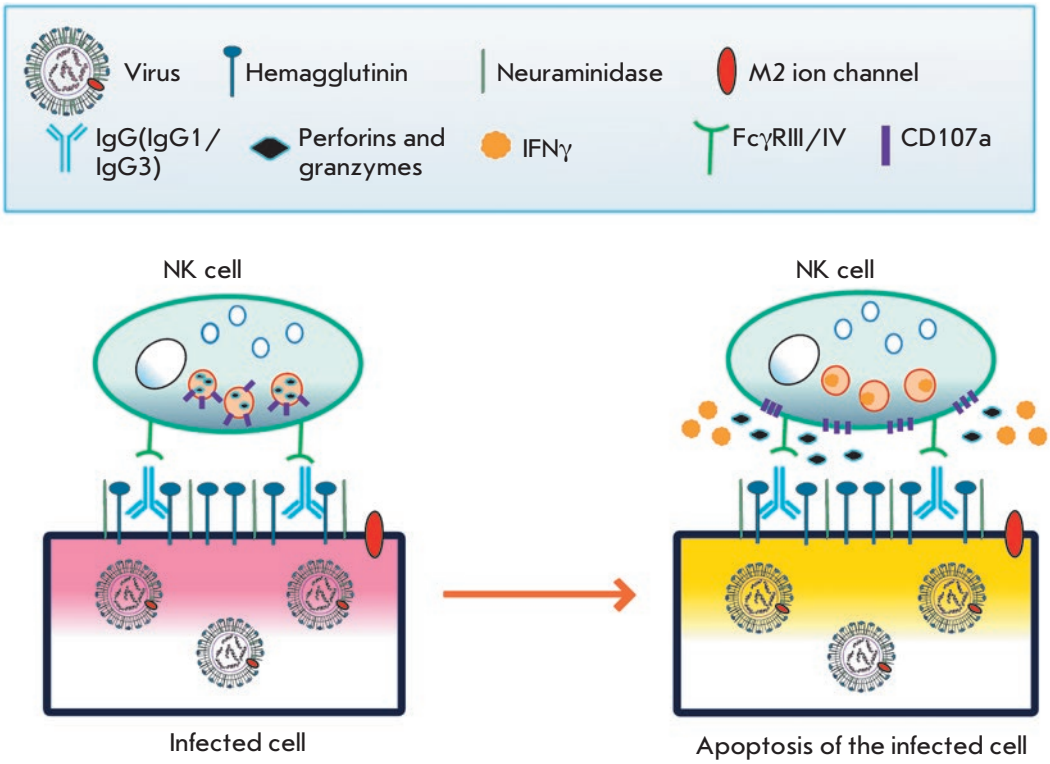

Fig. 2. The mechanism of antibody-dependent cellular cytotoxicity (ADCC) in a cell infected with an influenza virus. IgG binds to the viral antigens on the surface of the infected cell. NK cells recognize the infected cells via Fc-FcR interactions and then release cytotoxic granules and secrete antiviral cytokines

protected from the pandemic influenza virus. Thus, non-neutralizing HA stem-specific antibodies capable of inducing ADCC are directly related to the level of protection against an influenza virus [14]. In addition, according to published data, vaccines against seasonal influenza viruses weakly induce the production of antibodies that can participate in ADCC, while the presence of NK cell-activating antibodies with a broad spectrum of activity in elderly people suggests that these antibodies accumulate over a lifetime as a result of re-infection with various influenza virus strains [15].

Using a panel of 13 monoclonal antibodies to the influenza virus HA protein (both neutralizing and non-neutralizing ones, both stem- and head-specific ones), DiLillo et al. [16] showed that Fc-FcγR interactions are necessary for all broad-spectrum antibodies in order to ensure *in vivo* protection. A similar result was obtained by comparing two NA-specific antibodies, one of which had a broad spectrum of action; the other was strain-specific. This suggests that the spectrum of action of not only certain HA-specific antibodies, but also antibodies to other influenza antigens exposed on the surface of an infected cell, depends on the Fc-FcγR interaction. Moreover, the dependence of some antibodies on the Fc-FcγR interaction can be circumvented by significantly (8–10-fold) increasing the amount of the antibody involved in the interaction with the influenza virus. It should be noted that, during viral infection, broad-spectrum antibodies are generated in much smaller quantities than strain-specific ones. Thus, the Fc-FcγR interaction apparently can increase the efficiency of the broad-spectrum antibodies, thereby compensating for their small quantity [16].

Antibodies to conserved viral proteins, such as nucleoprotein (NP), also contribute to the ADCC response. For instance, the influenza infection and vaccination induce the production of antibodies to the NP, M1, and M2 proteins involved in ADCC [17, 18]. It has been shown that influenza virus NP is expressed on the surface of infected cells for some time and, therefore, can serve as a target for ADCC [19–21]. Carragher et al. demonstrated that vaccination of laboratory mice with soluble recombinant NP of the influenza A virus induces high titers of antibodies to NP and an extremely weak T cell response. At the same time, vaccination reduced the manifestation of disease symptoms and decreased the influenza virus titers in the lungs of the influenza-infected animals infected. Passive transfer of the sera of immunized mice to naive animals also provided protection against an influenza infection [22]. Subsequent studies have demonstrated that the protective effects of the serum of mice immunized with influenza A recombinant NP upon passive transfer to animals with B cell deficiency and mice with a normal

number of B cells manifest themselves through the mechanism that includes FcγR [23]. Macaque studies have shown that NP-specific antibodies have the ability to activate NK cells *in vitro* [18, 24].

The serum of healthy children and adults (but not infants) contains antibodies to various proteins of the H7N9 influenza A virus that are involved in the ADCC response, with the level of NP-specific antibodies being significantly higher than those to HA and NA. The level of antibodies to NP of the seasonal influenza A viruses that are involved in ADCC correlated with the level of antibodies to NP of the H7N9 influenza A virus. Therefore, production of these antibodies that cross-react with H7N9 is assumed to be triggered by vaccination and infection with seasonal influenza A viruses [25]. The antibodies to influenza A virus NP involved in ADCC were found in children vaccinated with seasonal inactivated influenza virus vaccines. NP-specific antibodies that can interact with FcγRIIIa and activate NK cells have been identified in healthy and influenza-infected volunteers. Healthy donor serum containing NP-specific antibodies were shown to induce NK cell activation against virus-infected cells expressing NP [13, 26].

Another conserved influenza protein found on the surface of infected cells is the M2 ion channel. Antibodies to this protein can protect mice from an influenza virus infection in laboratory experiments. Moreover, in the immunization of animals with the M2 ectodomain both in soluble form and as conjugated to various carriers, the protective ability depends mainly on the antibodies. Notably, the presence of NK cells was critical to protection. [27]. Experiments on passive immunization of both wild-type mice and mice with the FcRγ-/-, FcγRI-/-, FcγRIII-/-, and (FcγRI, FcγRIII)-/- phenotypes showed that FcR (more specifically, FcγRIII) is required for the protective effect of anti-M2e antibodies [28, 29]. The human monoclonal antibody against the influenza A (Ab1-10) virus M2 protein was able to activate NK cells and trigger ADCC *in vitro*, with ADCC against both target cells expressing M2 and cells infected with influenza [30].

To accurately determine the level of anti-influenza antibodies involved in ADCC, a reaction with the participation of the target antigen and effector cells (usually NK cells) is required. The antigen can be either a recombinant target protein, influenza-infected cells, or target cells expressing the desired antigens. If the antigen is a recombinant protein, it is treated with the test serum and then effector cells are added to the resulting antigen–antibody complex. When conducting this reaction, one can assess ADCC by measuring the activation of the effector cells and their expression of surface and secreted marker proteins (as a rule, these are surface activation marker CD107a and interferon gamma) (*Fig. 3,* a) [31]. If the antigen is infected cells or target cells, ADCC can also be analyzed by assessing the death rate of antibody-treated target cells after their interaction with effector cells (*Fig. 3,* b) [32].

Antibody-dependent cellular phagocytosis (ADCP)

Phagocytosis is a crucial immunological process in which phagocytes engulf microbial and infected cells. The first step of ADCP includes opsonization of a microbial or infected cell by antibodies. After opsonization, phagocytes recognize the antibodies bound to foreign antigens, mainly via the Fcγ receptors CD32 (FcγRIIA) and CD64 (FcγRIA), as well as the Fcα receptor CD89 [33]. The phagocytes involved in ADCP include monocytes, macrophages, neutrophils, and dendritic cells (*Fig. 4*) [17, 34]. ADCP is one of the most important antibody-induced effector defense mechanisms against the influenza virus. FcγR-/- mice have been shown to be highly sensitive to influenza even in the presence of influenza antibodies obtained from FcγR+/+ mice. Moreover, the absence of NK cells was not crucial for the defense response. It has also been shown that FcγR+/+ mouse macrophages actively engulf opsonized viral particles [35]. Dunand et al. showed that some non-neutralizing human broad-spectrum monoclonal antibodies protect mice from an influenza infection through Fc-mediated recruitment of effector cells, with the protection being associated exclusively with ADCP but not with ADCC or activation of the complement system [36].

According to He et al., alveolar macrophages are crucial for the induction of ADCP by human and mouse monoclonal antibodies both *in vitro* and in experiments on protecting animals from infection with homologous and heterologous influenza A virus strains [37]. Interestingly, the ability of alveolar macrophages to protect the lungs from damage during an influenza infection is reduced in elderly mice [38].

In addition to alveolar macrophages, other effector cell populations can also participate in the ADCP-mediated response to the influenza virus. For instance, neutrophils, which are the largest in number amongst blood leukocytes, express high levels of FcγRIa/b/c, FcγRIIa, and FcγRIIIb on their surface after activation. In addition, neutrophils constitutively express FcαRI, which binds IgA and activates the cytotoxic and phagocytic responses [15]. Analysis of the Fc-FcγR interactions between various IgG specific to the HA stem and effector neutrophils showed that monoclonal human and mouse HA stem-specific antibodies can induce the production of reactive oxygen species (ROS), which are further delivered to the neutrophil`s phagolysosomes. However, such an effect could not be

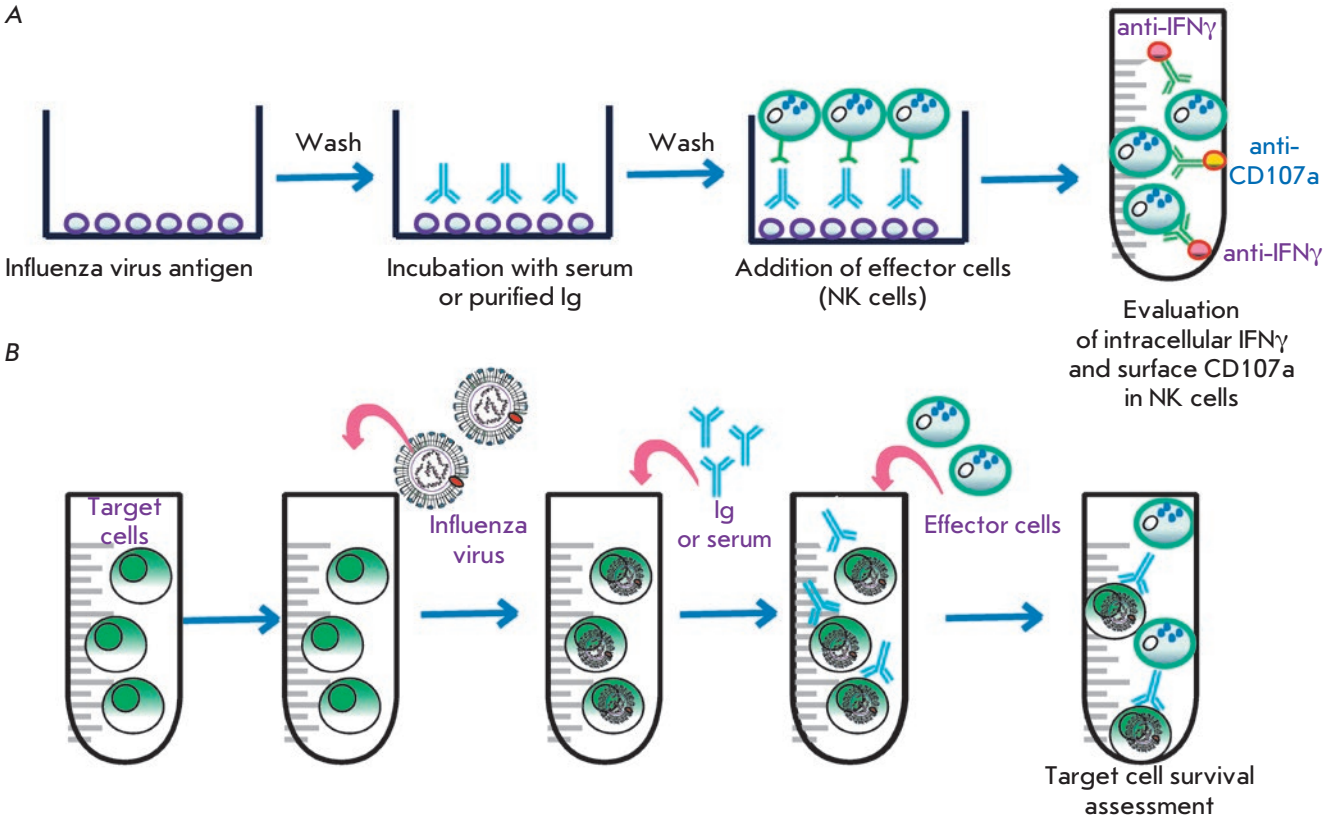

Fig. 3. Evaluation of ADCC in the laboratory. (a) Evaluation of ADCC using immobilized influenza virus antigens. Firstly, antigens are immobilized, washed, and incubated with serum or IgG isolated from the blood. Secondly, unbound IgGs are washed off and effector cells (peripheral blood monocytes or isolated NK cells) are added to the antigen-antibody complex. Thirdly, after incubation, activation of the effector cells is analyzed. Activation is evaluated by adding labeled antibodies to the surface and secreted marker proteins (as a rule, the surface membrane activation marker CD107a and interferon gamma are used). (b) Evaluation of ADCC using influenza virus-infected cells or target cells expressing the major viral antigens. Cells expressing viral antigens are incubated with serum or preliminarily purified IgG. Next, effector cells are added to the antibody-treated cells and ADCC is evaluated by counting dead target antibody-treated cells

detected in the case of HA head-specific antibodies [39]. The depletion of neutrophils resulted in a reduced survival rate of influenza-infected mice [40, 41].

A study of the ADCP mechanism in the influenza infection showed that both macrophages and neutrophils are quickly recruited to the lungs and are present in bronchoalveolar lavage, the respiratory tract, and alveoli, where they contribute to the rapid scavenging of infected and dead cells. Although the supernatant of influenza-infected cells can stimulate phagocytosis by monocytes regardless of the involvement of antibodies [40], antibodies contribute to the effective clearance of viral particles and infected cells by interacting with the FcγRIa and FcγRIIa on immune cells. Antibody-mediated viral phagocytosis causes a decrease in the infection spread and severity, as well as it symptoms, and a reduction in virus shedding [42]. It is assumed that each subsequent influenza infection, as well as influenza vaccinations, slightly induces the cross-reactive antibodies involved in ADCP, with their level increasing with each subsequent influenza infection [17].

Various anti-influenza antibodies, including antibodies to the hemagglutinin stem [36, 37, 39] and antibodies to the M2 ion channel [28, 43], can induce ADCP.

The activity of the antibodies responsible for ADCP is studied as follows: target cells expressing influenza antigens are labeled with an intravital dye, then the target antibodies and phagocytic cells are added, and the survival number of the target cells is assessed. [35].

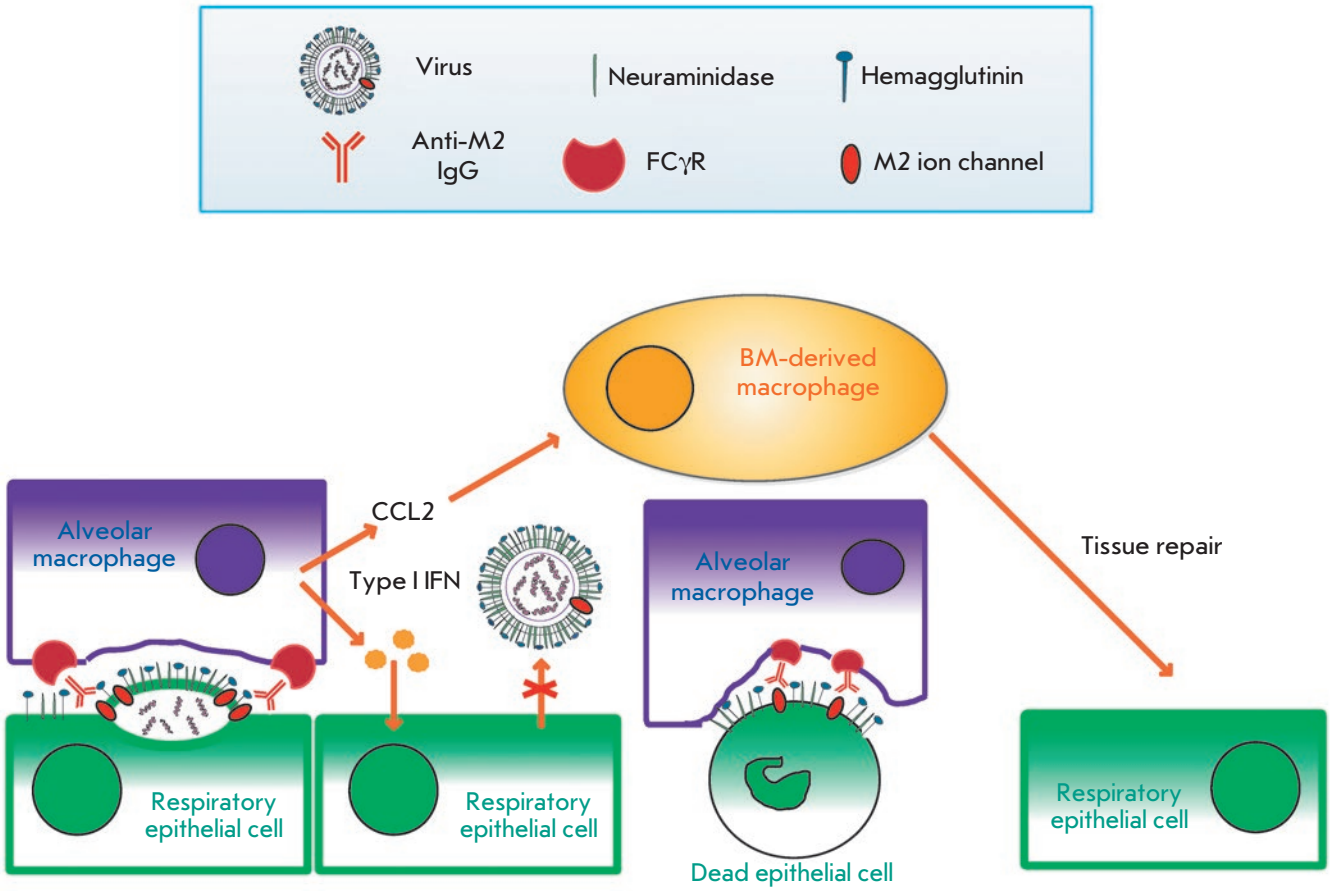

Fig. 4. The mechanism of the protective action of the antibodies binding to the ectodomain of the influenza M2 ion channel. Respiratory tract epithelial cells infected with the influenza A virus expose the HA, NA, and M2 viral proteins on their surface. New viral particles are budding from the infected cells. On the surface of the budding virion, antibodies bind to the ectodomain of the M2 protein and opsonize the viral particle. These antibodies activate alveolar macrophages via Fcγ receptors. Activated macrophages are able to phagocytize budding virions and fragments of the cell membrane containing the M2 protein. Dying infected cells can be also opsonized by antibodies to the M2 ectodomain and phagocytosed by alveolar macrophages via the FcγR-dependent pathway. Activated macrophages also produce type I interferons, which possess antiviral activity and regulate the expression of chemokine CCL2, which attracts bone marrow macrophages promoting tissue repair

Antibody-mediated complement-dependent cytotoxicity (CDC)

The complement system consists of soluble and membrane-bound proteins that are found in the blood and tissues of mammals. These proteins interact with each other and with other components of the immune system, resulting in the production of a number of effector proteins that contribute to the elimination of various pathogens [27].

As early as in 1978, it was shown that the complement system is necessary to protect mice from a lethal influenza infection [44]. In 1983, it was established that human serum contains antibodies capable of neutralizing the influenza virus by activating the classical complement pathway [45]. To date it is known that influenza virions can activate both the classical and alternative complement pathways, and that antibody opsonization is required for efficient lysis of virions [46].

In 2018, research was carried out to study the effectiveness of immunization of mice with a knockout of the C3 complement component with virus-like particles carrying the M2e proteins of human, porcine, and avian influenza A viruses, as well as virus-like

particles carrying HA of the H5-subtype influenza A virus. It turned out that immunization with the M2e vaccine did not protect C3 knockout mice from the influenza A viruses, while even low levels of antibodies to the M2e protein were enough to protect wild-type animals from the influenza A virus infection upon passive transfer. On the contrary, C3 knockout mice immunized with a HA vaccine, which induces the production of strain-specific neutralizing antibodies, were protected from infection with a homologous influenza virus despite the low level of antibody response [47]. Thus, one can state the ability of antibodies to the influenza virus M2 ion channel to protect against influenza A virus infection through the activation of the complement system.

The complement system is not only capable of neutralizing viral particles, but it is also involved in the lysis of infected cells. For instance, vaccination with a seasonal trivalent inactivated influenza vaccine led to an increase in the level of antibodies capable of activating the complement-dependent lysis of influenza-infected cells *in vitro*, although the effect was not pronounced [48]. CDC-inducing antibodies were detected among both influenza HA head-specific and stem-specific antibodies. Meanwhile, antibodies to the stem domain demonstrated a broad spectrum of action and were able to induce CDC against different influenza A strains [49].

Antibodies of the IgG1 and IgM classes have been shown to be involved in the activation of the complement system [46]. The level of antibodies involved in CDC correlated with the protection against a seasonal influenza virus in children [32].

The activity of antibodies in CDC is evaluated by the rate of death of target cells expressing the influenza antigen or infected with an influenza virus in solutions containing complement components and antibodies. Cell death is assessed using various metabolic dyes [50].

ANTIBODIES TO INFLUENZA VIRUS HEMAGGLUTININ AND NEURAMINIDASE

Influenza virus HA and NA are highly variable proteins. However, the broad-spectrum vaccines that are currently under development may also include HA and/or NA and their epitopes. For instance, the HA stem is a rather conserved part of the molecule. Various strategies exist for redirection of the immune response to this particular antigen during vaccination [51]. Most NA-specific monoclonal antibodies obtained from the serum of people who have been infected bind to different influenza strains, providing ground for the development of broad-spectrum vaccines based on this antigen [52].

Antibodies to the influenza virus HA stem

The HA stem is more conserved than the head domain. However, it is less immunogenic, possibly due to the fact that the bulky head region of the protein sterically hinders the access of antibodies to the HA stem. However, in addition to the non-neutralizing antibodies involved in the ADCC, ADCP, and CDC reactions, a certain amount of neutralizing HA stem-specific antibodies is detected in humans after an influenza infection or vaccination, with the infection being more effective in inducing the formation of this type of antibodies than vaccination.

HA stem-specific antibodies can interfere with the fusion of the virus with the endosomal membrane. Effective fusion requires the presence of 3–5 neighboring HAs with fusion peptides bound to the endosomal membrane (*Fig. 1,* c). Neutralizing HA stem-specific are able to prevent the pH-induced exposure of the fusion peptide and prevent the formation of a network of HAs interacting with the endosomal membrane. In addition, some antibodies to the HA stem can inhibit cleavage of the immature HA0 precursor into the HA1 and HA2 subunits (*Fig. 1,* e), which are required for a successful infection of cells with a newly formed viral particle [4]. HA stem-specific antibodies can suppress the release of viral particles from the cell (*Fig. 1,* d) [53], including the pathway involving steric inhibition of neuraminidase activity [54].

Neutralizing HA stem-specific antibodies cannot be revealed using a hemagglutination assay. The principal methods for an evaluation of these antibodies are the reactions of neutralization [5], microneutralization [6], and neutralization based on pseudotyped viral vectors [55]. The latter present chimeric viruses carrying influenza virus surface antigens that do not contain any genetic material and are not infectious. These pseudoviruses are usually derived from lentiviral vectors and the vesicular stomatitis virus. They allow avoidance of highly pathogenic influenza strains when performing a neutralization reaction (which is especially important when studying broad-spectrum antibodies). According to some reports, this method of evaluation is more sensitive and more suitable in the detection of neutralizing HA stem-specific antibodies than the conventional neutralization assay [55].

Antibodies to influenza virus neuraminidase

Influenza virus neuraminidase is involved in various stages of the infectious process. It cleaves the viral particles from the sialic acid residues of the respiratory tract mucins, thus allowing for the virus entry into the cell. NA allows the release of new virions from the host cell, thus preventing them from remaining bound to

the sialic acid residues on the cell surface. In addition, NA prevents the aggregation of virions, which is due to the interaction between the HA of a virion with the sialylated glycans of another one [56]. Antibodies to the influenza virus NA can interfere with any of these processes (*Fig. 1,* a,d).

It was shown that, in the absence of HA-specific antibodies, NA-specific antibodies can protect laboratory animals from an influenza infection [57]. Moreover, the presence of NA-specific antibodies also correlates with protection against an influenza virus in humans. The titer of anti-NA antibody has been shown to increase in human blood with age [56].

The production of antibodies to neuraminidase is induced by an influenza infection. However, their level is usually lower than that of the antibodies to HA. It is crucial that most NA-specific monoclonal antibodies derived from the serum of individuals who have suffered from an infection bind to a wide range of modern and historical influenza strains, inhibit NA activity, and protect laboratory mice in passive transfer experiments [52]. Several strategies for the development of broad-spectrum vaccines that induce the formation of antibodies to influenza NA exist. One of them is the creation of a pandemic vaccine based on a cocktail of several subtypes of NA (N1, N2, N6, N7, N8, N9, and etc.) associated with human and zoonotic influenza strains. It is also possible to include NA as an additional antigen in vaccines based on conserved influenza antigens: such as M2, NP, etc. [58].

ELISA is one of the easiest ways to evaluate the immune response to NA. To reliably assess the level of NA-specific antibodies, recombinant, tetrameric, glycosylated, and enzymatically active NA should be used as antigen. However, ELISA does not provide any information on the functionality of the measured level of antibodies. NA enzymatic activity inhibition assays are based on the cleavage of small molecules by neuraminidase, which generates the signal to be measured. However, unlike terminal sialic acids, which are attached to the glycans of large proteins, these small molecules more easily access the active center of the NA protein [59]. The assay that allows one to obtain the most realistic estimates of the anti-NA activity of antibodies is ELLA (enzyme-linked lectin assay), which uses the highly sialylated glycoprotein fetuin as a substrate. The method is based on measuring the amount of galactose, the penultimate sugar residue in fetuin, which is bound to the substrate. NA cleaves terminal sialic acids, after which galactose can be measured using horseradish peroxidase-conjugated peanut lectin. ELLA has been optimized for routine serology; it is now used to evaluate the titers of neuraminidase-inhibiting antibodies [58].

A key component of ELLA is enzymatically active NA, the inhibition of which is evaluated. NA can be used in the form of a purified protein or as part of a viral particle. When using a viral particle, it should be kept in mind that HA-binding antibodies can reduce NA activity due to the steric hindrance effect. Therefore, reassortant viruses of the H6NX and H7NX subtypes are commonly used in this assay. Although the use of reassortant viruses cannot completely exclude the effect of HA-specific antibodies on the NA activity, this assay is considered as the "gold" standard in the evaluation of the inhibitory anti-NA activity of antibodies [59].

CONCLUSION

In this review, the main mechanisms involving anti-influenza antibodies and the methods for the detection of these antibodies were considered. Antibodies can provide protection against influenza via Fc-independent or Fc-dependent mechanisms. Fc-independent antibodies directly neutralize the virus by preventing its entry into the cell, fusion, or budding from it. Antibodies to the head domain of hemagglutinin [5], which are usually strain-specific, are mainly involved in the direct neutralization of the influenza virus. As a rule, Fc-dependent antibodies are non-neutralizing but are able to activate antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, or the complement system [18, 34, 49]. Such antibodies can be targeted at the stem domain of the HA, NA, M2, or NP proteins of the influenza virus, and most of them are broad-spectrum antibodies [11, 21, 28, 58].

The influenza vaccines currently being developed are aimed at generating an immune response not mainly to the conventional HA and NA influenza antigens, but to various conserved viral antigens. When creating a broad-spectrum vaccine, it is necessary to know what effectiveness criteria should be considered in preclinical and clinical trials. The traditional methods for assessing the humoral immune response to influenza vaccines by hemagglutination and neutralization reactions will no longer be relevant for most newly developed vaccines. The development of methods for evaluating non-neutralizing anti-influenza antibodies and studying their mechanisms of action are necessary if we seek to create effective broad-spectrum vaccines that can provide protection against both seasonal and potentially pandemic influenza virus strains. ●

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The Role of Tumor-Derived Vesicles in the Regulation of Antitumor Immunity

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ABSTRACT **In this article, we present a comprehensive, updated, and elucidative review of the current knowledge on the function played by tumor-derived vesicles (TDVs) in the crosstalk between tumor and immune cells. Characterization of the structure, biogenesis, and the major functions of TDVs is reported. The review focuses on particular ways of suppression or activation of $CD4^+/CD8^+$ T cells by tumor-derived vesicles. Tumor-derived vesicles play an important role in the suppression of antitumor immunity. During the last 15 years, vesicle research has elucidated and improved our knowledge about the role of the vesicles in intercellular communication. Nevertheless, there are still blinds spots concerning vesicle heterogeneity and isolation methods, their uptake by target cells, and the role of mRNA in T-cell transformation or suppression. Along with the substantial progress in understanding of the role of tumor-derived vesicles in intercellular communication, novel antitumor therapy strategies based on vesicle inhibition in a tumor microenvironment are likely to appear very soon.**

KEYWORDS **vesicles, exosomes, immune response, $CD4^+/CD8^+$ T cells, tumor microenvironment.**

ABBREVIATIONS **CD – cluster of differentiation; EVs – extracellular vesicles; Hsp – heat shock proteins; TUBB4B, TUBA1C – tubulin beta; MHC – major histocompatibility complex; APC – antigen-presenting cell; N-SMase – sphingomyelin phosphodiesterase; ARF6 – ADP-ribosylation factor 6; ESCRT – the endosomal sorting complexes required for transport; MVBs – multivesicular bodies; PLD2 – phospholipase D2; Th – T helper; Treg – regulatory T cells; NK – natural killer; TRAIL – tumor necrosis factor ligand superfamily member 10; IL – interleukin; NKG2D – natural killer group 2 member D; PD-1 – programmed cell death 1; $A_{2A}R$ – adenosine A_{2A} receptor; cAMP – cyclic adenosine monophosphate; STAT – signal transducers and activators of transcription; TGFβ – transforming growth factor beta; IFN-γ – interferon gamma; JAK – janus kinase; MAPK – mitogen-activated protein kinase; LFA – lymphocyte function-associated antigen 1; ICAM – intercellular adhesion molecule 1; TCR – T-cell receptor.**

INTRODUCTION

New data on the origin, composition, and influence of extracellular vesicles (EVs) on cells have significantly changed their functions and significance. While being earlier regarded as "cellular debris," EVs have become a new means of intercellular communication. It turns out that these structures (typically the intracellular ones) are actively involved in the regulation of the immune response, as well as other processes that require intercellular communication [1, 2]. The observation that EVs can modulate the phenotype and function of target cells at the genetic and epigenetic levels by transferring genetic material (usually different types of RNA) was an extremely important step in the "biography" of vesicles [1]. The secretion of extracellular vesicles by both normal and tumor cells makes them an important component of the tumor microenvironment. It should be emphasized that tumor cells secrete more vesicles in comparison to the normal cells of the surrounding tissue, which can be attributed to the fact that they proliferate rapidly under constant stress conditions [3–5]. Many factors can influence EV production by

cells. Thus, a low pH in the tumor microenvironment is known to be important for maintaining the stability of the lipid/cholesterol composition of vesicles [6]. Changes in the number and composition of vesicles correlate well with the severity and prognosis of many diseases. This fact allows one to use EVs as a non-invasive diagnostic tool [7].

Being a component of the cellular environment, vesicles are apparently involved in cell differentiation, division, and maintenance/alteration of the cell phenotype both in normal cells and in various pathologies, including cancer [2]. Although tumor-derived vesicles (TDVs) suppress the immune system and contribute to tumor development, they simultaneously contain tumor antigens. This property of vesicles could potentially be used in immunotherapy for eliciting an antitumor immune response [8].

This review discusses how tumor-derived vesicles are involved in the immune response regulation and affect the function of $CD4^+/CD8^+$ T cells in the context of a tumor microenvironment.

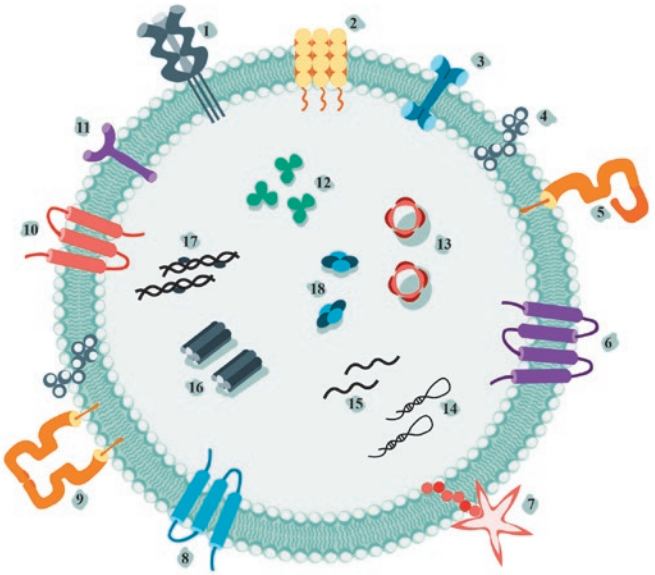

Fig. 1. Schematic representation of a typical vesicle with the most common components. Adhesion molecules (integrins (11), tetraspanins (6, 7, 8, 10)); signal transduction molecules (syntetins, annexin V(3)); major histocompatibility complex molecules (MHC class II (5) and MHC class I (9)); cytoskeletal proteins (actin, myosin (16)); heat shock proteins (12); lipids (ceramide (4)); proteins responsible for vesicle biogenesis (13); and proteins of metabolism (GAPDH(18)). 1 – FasL; 2 – ICAM-1; 3 – annexin V; 4 – ceramide; 5 – MHC I class; 6 – tetraspanin CD81; 7 – CD80; 8 – tetraspanin CD9; 9 – MHC class II; 10 – tetraspanin CD63; 11 – integrin; 12 – heat shock proteins; 13 – AUX/Alix; 14 – microRNA; 15 – mRNA; 16 – actin, myosin; 17 – DNA with histones; 18 – GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

1. CHARACTERISTICS OF EVs

The term "extracellular vesicles" is used to describe spherical cellular structures (30–1000 nm in size) enclosed in a lipid bilayer. According to their average size and biochemical profile (a combination of their components), vesicles are classified into different types. The difficulties associated with obtaining pure vesicles and physical isolation of their individual types make accurate vesicle classification rather challenging [9]. Extracellular vesicles can theoretically be classified according to their size or origin [9, 10].

1) Exosomes are structures of endosomal origin (30–150 nm in size) that carry characteristic markers belonging to the tetraspanin (CD9, CD63, CD81) and chaperone (Hsp70, Hsp90) families.

2) Microvesicles are cytoplasmic particles that actually are budded cell membrane fragments. Their size ranges from 100 to 1000 nm.

3) Apoptotic bodies are large (typically described as 1000–5000 nm) fragments of cells being formed during apoptosis.

In this study, we provide a detailed description of exosomes and microvesicles, which are further referred to using the general term "vesicles" (or EVs).

1.1. Vesicular composition

EVs contain a set of proteins that are characteristic markers of the cell line they have been derived from [11]. For example, tubulin proteins (TUBB4B and TUBA1C) are found in vesicles derived from lung cancer cells [12], while CD20 is present in B-cell lymphoma-derived vesicles [13]. The protein and lipid compositions of vesicles (shown in more detail in *Fig. 1*) has been studied in an attempt to understand the entire range of effects exerted by tumor-derived vesicles on immune cells [14–16]. Any tumor-derived vesicles are characterized by the presence of tetraspanins (CD63, CD81, and CD9), whose amount may vary depending on the tumor type and stage of progression [17]. Major histocompatibility complex (MHC) class I and II molecules can also be found on the vesicle membrane (*Fig. 1*), which is especially important for EVs secreted by antigen-presenting cells (APCs) [18, 19]. Along with proteins and lipids, vesicles can also contain genetic material (DNA [16, 20, 21], ribosomal RNA, messenger RNA, as well as microRNA and other non-coding

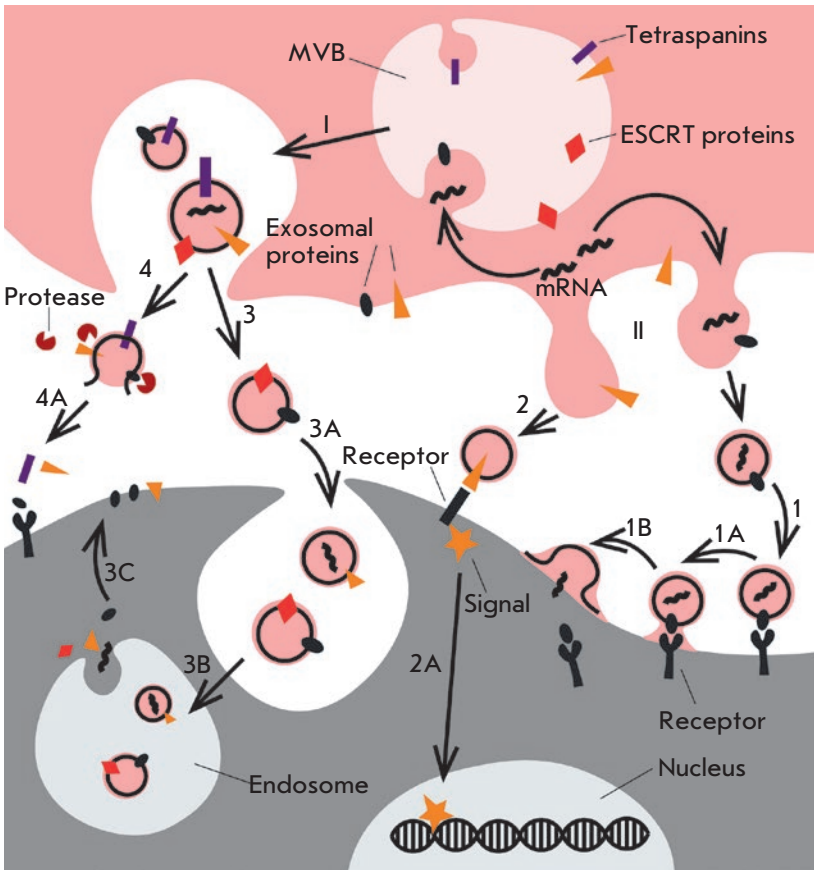

Fig. 2. Biogenesis of EVs and their uptake by the target cells. The EV biogenesis pathways (I, II). I – EV biogenesis in MVBs via the ESCRT-dependent/ESCRT-independent pathways: fusion of MVBs with the plasma membrane; II – EV formation by direct budding from the plasma membrane into the extracellular space. The interaction between the secreted membrane vesicles and recipient cells (1, 2, 3, 4). 1, 2 – Binding of secreted vesicles to the surface of a recipient cell involves interactions between exosomal ligands and cellular receptors, fusion with the plasma membrane (1A) and release of vesicle components into the cytoplasm (1B); 2A – activation of signal pathways; 3A – vesicle endocytosis; 3B – fusion of the endocytosed exosomes with the limiting membrane of the endosome; 3C – incorporation of exosome membrane proteins into the endosome membrane, which could then be recycled to the cell surface; 4 – exosome degradation by extracellular proteases; 4A – interactions between exosomal ligands and cellular receptors

RNAs). The mechanism via which nucleic acids are loaded into EVs has not been fully elucidated, but certain "barcodes" (short microRNA and RNA sequences specific to RNAs isolated from vesicles) have been detected [20–22]. Databases on the molecular composition of vesicles, such as EVpedia [23], Vesiclepedia [24], and Exocarta [25], provide a thorough description of the protein and lipid components found in different types of EVs.

1.2. Biogenesis of vesicles and how they crosstalk with target cells

Exosomes and microvesicles form in the cell via different pathways (*Fig. 2*) [26]. Microvesicle budding from the cell membrane is mediated by cytoskeletal proteins (actin, myosin, etc.), neutral sphingomyelinase N-SMase that is involved in ceramide formation, as well as ARF6 (ADP-ribosylation factor 6) and phospholipase PLD2 [27, 28]. The endosomal sorting complex (ESCRT), which sends ubiquitinated proteins into multivesicular bodies (MVBs), is involved in the formation of both microvesicles and exosomes [26, 29]. The ESCRT consists of four protein components (ESCRT-0, -I, -II and -III), which consecutively bind proteins to form intracellular vesicles [7]. In turn, the accessory proteins syndecan–syntetin–(ALG-2-interacting protein X) trigger exosome budding from the membrane into MVBs [30]. According to the available data, formation of glycolipoprotein microdomains (lipid rafts) containing neutral sphingomyelinase (N-SMase) is an alternative pathway of EV biogenesis. Ceramide synthesis by sphingomyelinase and its accumulation in the membrane cause raft merging and formation of an exosome in the MVB cavity [31]. The endosomes inside MVBs are released into the extracellular space via fusion between the cell membrane and MVBs; this process is regulated by GTPases belonging to the Rab and Ras families, as well as the SNAPE protein (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) [21, 32].

The existence of several pathways of EV biosynthesis has been confirmed in many studies [33]. Hence, formation of MVBs was observed in cells lacking ESCRT proteins, although the budded vesicles had a nonconventional composition and morphology [31]. The inhibition or knockout of sphingomyelinase N-SMase reduces

vesicle secretion by cells and suppresses the metastatic spread and angiogenesis in tumor [34]. However, there is doubt about a spontaneous formation of EVs via the ESCRT-independent pathway, since it has been proved that lipid rafts are not needed for exosome formation [35]. Although the pathways of EV biogenesis are theoretically subdivided into an ESCRT-dependent and ESCRT-independent one, formation of a given population may depend on each pathway to a different extent [9, 36].

EVs can possess various functions in a tumor microenvironment, but almost all of these functions are implemented when a vesicle interact with the target cell [2]. There are at least four different ways through which EVs carry protein molecules to the cell surface or deliver them inside cells (*Fig. 2*).

· contact between specific vesicle molecules exposed at the exterior of the membrane and the receptors of recipient cells, making activation of the intracellular signaling cascades possible [2, 19];

· cleavage of surface vesicle proteins by extracellular proteases, followed by crosstalk between vesicular proteins and membrane receptors;

· fusion of the vesicular and cell membranes, followed by either release of the intravesicular content into the cytoplasm or endosome formation [26]; and

· phagocytosis and the uptake of an entire vesicle by a recipient cell [5, 21, 37].

The crosstalk between vesicular tetraspanins, proteoglycans, lectins, and integrins and membrane receptors of the recipient cell triggers vesicle penetration of the cell, which can be blocked by an antibody specific to a given vesicle protein. For example, treatment of vesicles with the anti-CD81 or anti-CD9 antibody or blockage of proteoglycans by heparin sulfate reduces vesicle adhesion to the recipient cell. Vesicle endocytosis can also be blocked using cytochalasin B or latrunculin A, which inhibits cytoskeletal components (actin and fibronectin) [2]. Secretion of vesicles and their endocytosis are processes that have mostly been studied in vitro thus far. These processes need to be studied in vivo as well to elucidate the physiological role of their effect on surrounding cells.

Hence, a conclusion can be drawn that the diversity of EVs and their protein composition, as well as the multiple variants of crosstalk between EVs and target cells, suggest that EVs are a multifunctional component of any physiological or pathophysiological process. Vesicles can have various functions depending on their cellular origin: from regulating the immune responses and suppressing tumor invasion to being involved in intercellular communication. Studying the question of how these nano-sized structures in the cellular environment exhibit diametrically opposed effects could allow one to use vesicles as targets for anti-tumor therapy or as "liquid biopsy" for diagnosing tumor invasion [38].

2. TUMOR MICROENVIRONMENT: THE IMMUNE RESPONSE AND THE ROLE OF TUMOR-DERIVED VESICLES

Different cell populations forming the stroma (fibroblasts) and the immune environment (tumor-infiltrating lymphocytes, macrophages, myeloid-derived suppressor cells, etc.) are present in a tumor microenvironment. The complex of immune reactions is mediated by T cells, which not only trigger and stimulate ($CD4^+$ T cells, T-helper cells (Th)) or regulate (regulatory T cells (Treg)) the immune response, but also destroy infected or tumor cells ($CD8^+$ killer T cells, cytotoxic T cells). The eradication of tumor cells and memory cell formation is a reasonable result of the T-cell immune response [8]. In turn, tumor cells find various ways to "evade" the immune response. As suggested by the available data, releasing tumor-derived vesicles is one such way.

The following mechanisms via which tumor-derived vesicles can contribute to tumor evasion of immunosurveillance have been identified (*Fig. 3*):

(1) initiating apoptosis in cytotoxic $CD8^+$ T cells [39];

(2) shifting the phenotype of $CD4^+$ T cells towards Tregs [40, 41];

(3) transduction of tumor-associated antigen by vesicles and its presentation to cells other than professional APCs or to immature APCs, thus causing T-cell anergy in the absence of costimulatory signaling [42–44];

(4) regulated suppression of the T-cell immune response, which depends on various mechanisms [45];

(5) macrophage re-programming to an M2 phenotype (supporting the tumors) [39, 46, 47]; and

(6) slowing down the proliferation of NK cells [40].

Below, we discuss the pathways through which tumor-derived vesicles can affect $CD4^+$/$CD8^+$ T cells (*Fig. 3)* in more detail.

3. THE NEGATIVE EFFECTS OF THE CROSSTALK BETWEEN EVS AND THE SURFACE RECEPTORS OF TARGET CELLS

3.1. Vesicles induce the apoptosis of $CD8^+$ cytotoxic T cells

The release of EVs carrying apoptosis activation factors by tumor cells is considered to be one of the immunosuppression mechanisms [48, 49]. When incubated with Fas^+ T cells, EVs carrying the highly active membrane protein FasL contribute to cytochrome *c* release into the cytosol, loss of the mitochondrial membrane potential, caspase activation, and DNA fragmentation in T-cell chromatin [48, 50, 51]. The coexpression of FasL and TRAIL on the surface of secreted tumor-derived

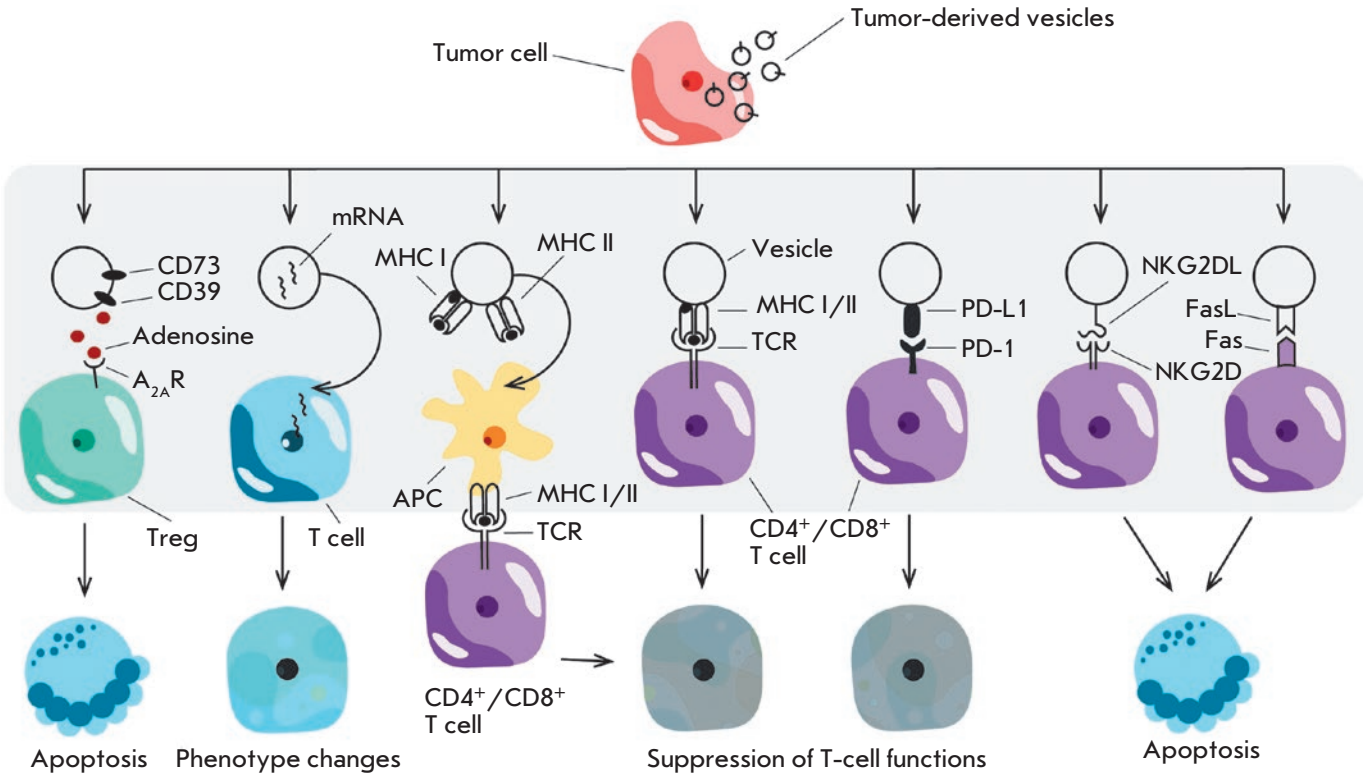

Fig. 3. Effects of tumor-derived vesicle on immune cells. This image shows how tumor-derived vesicles can activate or suppress different populations of immune cells via various mechanisms

vesicles also induces apoptosis in $CD8^+$ T cells [52]. Vesicles released by tumor cells induce apoptosis of Th1 cells via the galectin-9/Tim-3 crosstalk [53]. In turn, vesicles derived from normal cells (fibroblasts or dendritic cells) do not induce the apoptosis of activated cytotoxic $CD8^+$ T cells [54].

It has been experimentally proved that reduced expression of the costimulatory molecule CD3ζ can be observed in T cells in a tumor microenvironment, which results in T-cell anergia and correlates with a decreased release of cytokines such as IL-2, IL-7, and IL-15. Vesicles containing $FasL^+$ can exhibit this capacity: by interacting with Fas^+ lymphocytes, they reduce the number of CD3ζ and JAK3 (Janus kinase 3, tyrosine-specific protein kinase 3) molecules in T cells that have undergone primary activation and facilitate the transition of cells to their apoptotic state [55].

The NKG2D/NKG2DL system also plays an important role in immune cell survival [56, 57]. The NKG2D receptor (Natural Killer Group 2D, a natural killer cell receptor) resides on the membrane of NK cells and $CD8^+$ T cells [58]. MHC class I-like molecules and UL16-binding proteins act as ligands (NKG2DL) of this receptor; they are poorly represented on the surface of normal non-stressed cells. The emergence of these molecules on the membrane is activated by cellular stress (a viral infection or malignant transformation) [59]. Tumor-derived vesicles expressing various NKG2DLs bind NKG2D on the surface of NK and $CD8^+$ T cells, thus blunting the cytotoxic function of T cells [60–62].

3.2. Suppression of T-cell activation via PD-L1/PD-1 crosstalk

The physiological role of the PD-1 (Programmed death-1) immune receptor is to regulate excessive activation of lymphocytes. When interacting with its ligand (PD-L1), the PD-1 receptor transduces a negative signal inside the T cells, which inhibits their proliferation and increases apoptosis. Recent studies have demonstrated that PD-L1 resides on tumor-derived vesicles, allowing them to suppress T-cell activation [47, 63, 64]. In particular, melanoma cells secrete $PD\text{-}L1^+$ EVs in which the PD-L1 level is directly proportional to the level of IFN-γ secreted by lymphocytes [65]. In vivo and in vitro studies showed that hepatocellular carcinoma cells also release $PD\text{-}L1^+$ vesicles, which inhibit $CD4^+$

and $CD8^+$ T cells via the PD-L1/PD-1 crosstalk [66, 67]. When PD-L1-positive vesicles interacted with T cells, the suppression effect was eliminated by pre-incubation with the anti-PD-L1 antibody, which blocked PD-L1 on the vesicles [67].

3.3. Release of immunosupressive adenosine

Adenosine is known to be an immunosuppressive factor [40]. It interacts with one of the isoforms of the adenosine receptor ($A_{2A}R$) expressed on the T-cell surface and increases the cAMP level in $CD4^+$ T cells, thus suppressing their activation [40]. ATP hydrolysis to adenosine is catalyzed by CD39 (an ATP hydrolase converting ATP to 5'-AMP) and CD73 (a 5'-nuclease converting 5'-AMP to adenosine) [68].

Tumor-derived vesicles often carry both of these enzymes (i.e., they are in the $CD73^+CD39^+$ status), which has a negative impact on T cells in a tumor microenvironment [69]. $CD73^+CD39^+$ vesicles induce adenosine secretion; they also activate inosine biosynthesis upon longer contact with cells [70]. Inosine maintains long-term activation of the $A_{2A}R$ receptor on Tregs, which in turn has a strong suppressive effect on $CD4^+$ T cells [71]. It was found that this indirect signal from tumor-derived vesicles is much stronger than that from the cells, as evidenced by the significant contribution of EVs to intercellular communication [72].

4. CHANGES IN CELL BEHAVIOR CAUSED BY ENDOCYTOSIS OF VESICULAR COMPONENTS

4.1. Vesicular RNA modulates T-cell functions

Vesicles contain various types of RNA; mRNA and microRNA being the most abundant and diverse RNA types. 18S and 28S ribosomal RNA and DNA are less abundant. The ExoCarta database based on the results of 286 studies contains approximately 6,000 characterized microRNAs and mRNAs isolated from EVs [73]. The horizontal transfer of mRNA from a vesicle to the target cell may affect the transcription level of some genes which are involved in such processes as suppression/amplification of T-cell functions (in particular, for cells responsible for the production and secretion of proinflammatory cytokines and other biologically active molecules).

The tumor and its microenvironment are involved in the induction of active Tregs and contribute to the conversion of $CD4^+CD25^-$ naïve T cells into $CD4^+CD25^+$ Treg cells. EVs can also induce the conversion of $CD4^+CD25^-$ T cells into the $CD4^+CD25^+Foxp3^+$ phenotype of Treg cells. This conversion of naïve T cells into Tregs requires phosphorylation and coactivation of the transcription factors Smad2/3 (Similar to Mothers Against Decapentaplegic 2/3) and STAT3 (Signal Transducer and Activator of Transcription 3) [74, 75]. The enhanced intensity of Treg formation leads to an imbalance in the proportions of immune cells in a tumor microenvironment, thus inducing the TGFβ-dependent mechanism of apoptosis of effector T cells. In turn, $CD4^+CD25^+Foxp3^+$ Treg cells can also release EVs, which suppress the proliferation of type 1 T helper cells (Th1) and $CD8^+$ T cells and reduce IFN-γ secretion by these cells [74–76].

The verified increase in the number of Tregs in tumor is accompanied by a reduction of the number of differentiated Th1- and Th17-lymphocytes, leading to a Treg/Th imbalance [77, 78]. In the presence of this imbalance, specific microRNAs miR-29a-3p and miR-21-5p of vesicular origin are detected in target cells [79]. As they accumulate in the cells, these microRNAs can affect various signaling pathways associated with the suppression of T-cell activation. Activation of the MAPK1 (mitogen-activated protein kinase) signaling cascade, the STAT3/JAK1 pathway, and other signaling pathways in $CD4^+$ T cells mediated by vesicular microRNA disturbs the cytokine profile of Th and Th17 cells and changes the lymphocyte phenotype of Tregs [80, 81].

The effect of vesicular mRNA on T-cell functions directly depends on whether T cells are naïve or activated. Tumor-derived vesicles were found to significantly increase the expression of genes having a verified immunity-regulating function in activated $CD4^+$ T cells, while in naïve cells, gene expression slightly increased only for FAS1, IL-10, and PTGS2, while decreasing for DPP4, CD40LG, and NT5E [82].

4.2. T-cell activation/suppression by EVs carrying antigen-presenting complexes on their surface

Antigen-presenting cells (APCs) are also capable of releasing vesicles. Moreover, these vesicles carry MHC II (major histocompatibility complex class II) and can indirectly stimulate activated $CD8^+$ T cells but not naïve ones [51]. This process is regulated by the crosstalk between the T-cell receptor (TCR) on the T-cell membrane and the MHC–peptide complex in the presence of additional costimulatory signaling from the CD28/B7 molecules or LFA-1/ICAM-1 adhesion molecules presented on the vesicle surface. The crosstalk between TCR and MHC in the absence of costimulatory signaling is known to cause T-cell anergy (i.e, makes cells unable to divide and secrete cytokines in response to the stimulation of the T-cell receptor) [83, 84].

It has been found that vesicles derived from melanoma cells are also able to transfer MHC I from

tumor cells to APCs, thus changing the expression profile of receptors on the APC surface. EV-derived cytokines and mRNA potentially have an immunosuppressive effect on APCs and decrease the amount of MHC I and CD40 molecules on the cell surface [83]. An APC phenotype shifting toward an immunosuppressive one reduces the probability of stimulation of cytotoxic T cells, which may be the mechanism via which tumor cells "evade" the immune response [83, 84].

5. CONCLUSIONS

To sum up, it is worth mentioning that there is evidence pointing to the fact that tumor-derived extracellular vesicles may be a crucial factor in the formation of an immunesuppressive microenvironment. The negative effect on the immunity can be regulated by receptor-mediated crosstalk between the target cells and EVs, causing T-cell anergy or apoptosis. EVs and their contents can be uptaken by target cells, also leading to transduction of the immunosuppressive signal. The vesicular activity can be one of the reasons behind the treatment resistance and the phenotypic changes in tumor cells induced by chemo- and radiotherapy. Since the effect of EVs on immune cells, and T cells in particular, has been studied insufficiently, a relevant fundamental and practical problem is to characterize EVs and identify the molecular mechanisms underlying their binding and biological effect. ●

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DARPins: Promising Scaffolds for Theranostics

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ABSTRACT **Monoclonal antibodies are the classical basis for targeted therapy, but the development of alternative binding proteins has made it possible to use non-immunoglobulin proteins as targeting modules. The advantages of DARPins, scaffold proteins based on ankyrin repeats, over antibodies are as follows: small size, stability over a wide range of temperatures and pH values, low aggregation tendency, and ease of production in heterologous expression systems. The differences in the structure of the paratope of DARPin and antibodies broaden the spectrum of target molecules, while the ease of creating hybrid fusion proteins allows one to obtain bispecific and multivalent constructs. In this article, we summarize recent data on the development of therapeutic and imaging compounds based on DARPins.**
KEYWORDS **DARPin, targeted therapy, barnase.**
ABBREVIATIONS **DARPin – designed ankyrin repeat protein; scFv – single-chain variable fragment of an antibody; HER2 – human epidermal growth factor receptor 2; EGFR – epidermal growth factor receptor; EpCAM – epidermal growth factor receptor; IgE – immunoglobulin E.**

INTRODUCTION

The hybridoma technology described by Kohler and Milstein in 1975 [1] has enabled the production of monoclonal antibodies, which are used in research and diagnostics, as well as in therapy. Due to their high affinity and specificity, monoclonal antibodies have become the "magic bullet" underlying targeted therapy. The first therapeutic monoclonal antibodies were acquired in 1986. To date, 82 monoclonal antibodies have been approved for clinical use by the Food and Drug Administration (FDA), and the number of approved monoclonal antibodies continues to grow. However, antibodies have some disadvantages: their relatively large size (150 kDa) can limit diffusion in both normal tissues [2] and solid tumors [3]; the Fc region prolongs the time of blood circulation, but it can also cause unwanted effects [4]. In addition, full-length antibodies require complex folding and specific glycosylation and, therefore, have to be produced in mammalian cells, which makes them expensive. Another problem arises from the homology between murine and human proteins, which complicates obtaining antibodies specific to conserved proteins.

Many of the aforementioned problems have been solved by obtaining shortened and single-chain antibodies. The development of recombinant antibody technology has led to the replacement of conventional immunization with fully synthetic libraries free of the restrictions on the autospecificity that is typical of lymphocytes. Later, methods for molecule selection based on its affinity to a ligand were used for other proteins, making antibodies dispensable [5]. In 2018, the importance of these findings was recognized with the Nobel Prize in Chemistry "for the directed evolution of enzymes and binding proteins." Half of the prize was awarded to American bioengineer Frances H. Arnold "for the directed evolution of enzymes," and the other half was awarded to George P. Smith and Sir Gregory P. Winter "for the phage display of peptides and antibodies." With the help of these technologies in the past 20 years, a variety of alternative scaffolds have been developed, including monobodies (derived from fibronectin type III), anticalins (derived from lipocalins), affibodies (derived from immunoglobulin-binding protein A), and DARPins (derived from ankyrin repeats). Like antibodies, these proteins

usually have a "constant" scaffold and "variable" sites in which amino acid substitutions do not alter the protein conformation [6]. Designing alternative scaffolds involves two stages: (1) the design of a library of protein variants by random site-specific mutagenesis and (2) selection of molecules using phage, ribosome, or yeast display, linking genotype (a protein gene sequence) and phenotype (its ability to bind to the target).

The advantages of these alternative binding proteins include their small size, which facilitates tumor penetration; the absence of Fc-avoiding antibody-mediated cytotoxicity and complement-mediated cytotoxicity; in many cases, high thermostability that enables long-term storage of a preparation at room temperature without loss of activity; ease of production in bacteria, and even the possibility of performing direct chemical synthesis.

Many types of alternative scaffolds are based on proteins with repeating motifs: leucine-rich repeats (LRRs), ankyrin repeats (ARs), Armadillo repeats (Arms), and tetratricopeptide repeats (TPRs). Repeat-based proteins are actively used, because they have a long binding surface whose size can be varied and a rigid scaffold formed by the "constant" regions [7]. This paper focuses on DARPins, which are artificial proteins that are based on ankyrin repeats. In eukaryotic cell proteins built from ankyrin, repeats bind to a variety of targets, providing cytoskeletal organization and regulation of enzyme activity [8]. The natural variety of these proteins was used to create a consensus motif containing variable regions and able to combine with neighboring motifs to form a single rigid structure (*Fig. 1A, B*).

USING THE STRUCTURAL FEATURES OF DARPins IN BASIC RESEARCH AND BIOTECHNOLOGY

DARPins can be utilized as binding molecules in most technologies using shortened variants of monoclonal antibodies. However, DARPins have other beneficial properties in addition to their small size. The ease of production in bacteria allows one to create fusion proteins and add sequences for purification and labeling, while the absence of cysteine residues in the DARPin molecule allows one to introduce a unique additional cysteine for precise conjugation.

DARPins consist of tightly packed ankyrin repeats, each forming a β-turn and two antiparallel α-helices. A single repeat typically consists of 33 amino acids, six of which form the binding surface. During recombinant library design, these sites are used to introduce the codons of random amino acids, except for cysteine (to avoid the formation of disulfide bonds), as well as glycine and proline (since some amino acids are part of the α-helix) [6]. DARPins are typically formed by two or three of the binding motifs contained between the N- and C-terminal motifs shielding the hydrophobic regions (*Fig. 1*). DARPins are small proteins (14–18 kDa) that are extremely thermostable (their melting point (T_m) can reach 90°C) and resistant to proteases and denaturing agents. They can be produced in bacteria with a high yield of up to 200 mg of protein from 1 liter of liquid culture [6].

Both ends of the DARPin polypeptide chain form α-helices, facilitating the design of geometrically precise multimers. Thus, the molecular "clamp" wrapping the GFP molecule forming a stable but reversible complex has been created based on two DARPins that recognize different but overlapping epitopes of the

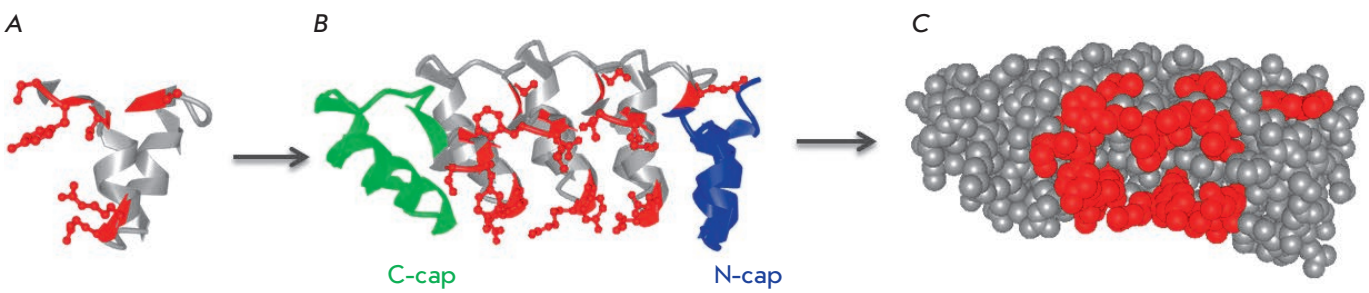

Fig. 1. The structure of DARPins. *A* – the structure of the consensus ankyrin repeat; the constant part is shown in gray; the variable regions are shown in red. *B* – the structure of a DARPin molecule. Two or three binding motifs form the binding surface through variable amino acids (shown in red); the hydrophobic regions are shielded by the C-cap and N-cap. *C* – 3D structure of a DARPin molecule, the variable amino acids are shown in red

green fluorescent protein (GFP) by computer simulations. Such clamps were used for the oriented covering of a sensor chip for surface plasmon resonance with proteins fused to GFP and for chromatographic purification of such proteins on sepharose conjugated to this diDARPin. DiDARPins conjugated to a fluorescent dye amplified the signal from rare GFP-labeled proteins on the cell surface and allowed a more accurate detection of these cells by flow cytometry [9].

A DARPin forming a trimer through the trimerizing motif added to it was created using computer modeling. The obtained DARPin binds to the trimeric protein of adenovirus serotype 5 (Ad5). This protein was shown to be able to almost irreversibly bind to the adenovirus capsid. Adding one more DARPin specific to the target cell receptor enabled efficient infection of cells expressing the corresponding tumor marker (HER2, EGFR or EpCAM) [10].

The rigidity and small size of DARPins made it possible to create dimers that affect signaling of extracellular receptors through fixation of receptors in certain conformations or bringing close molecules that generate competing signals. Utilization of a bivalent DARPin in this way enabled selective suppression of the activity of the mast cells that had bound IgE immune complexes. One of the modules of this dimer recognizes the constant part of IgE in a complex with FcεRI with high affinity; the other module binds to low-affinity FcγRIIB, which exhibits an inhibitory effect on mast cells. This recombinant protein specifically inhibits mast cell degranulation in vivo [11]. A similar approach was applied to create the bispecific diDARPin, which inhibited the proliferation signal from the HER2 receptor and had a cytotoxic effect on HER2-positive cancer cells [12].

Along with multimerization, DARPins can form rigid constructions connected by flexible linkers through introduction of alternative C- and N-terminal motifs sharing a common α-helix. In these di- and trimers, DARPins still retain their ability to simultaneously bind their targets and stabilize them for crystallization [13]. One of such DARPins, which was found to improve the crystallization of its partners, was used to create rigid dimers with DARPin specific to JNK1, which allowed the researchers to obtain crystals of these complexes and reveal the structural features explaining the specificity of DARPins to the kinase isoform and their ability to inhibit its activity [14].

The disadvantages of DARPins as binding modules include their concave binding surface, rigidity, and incomplete randomization of amino acid residues in variable sites, which could potentially limit the range of possible targets. However, these limitations can be overcome: LoopDARPins, a new generation of DARPins, has been created for this purpose. In LoopDARPins, the central β-turn is replaced with a larger convex H3 loop from the immunoglobulin molecule. This insert made it possible to change the geometry of the antigen-binding surface and introduce a flexible motif with a higher amount of variable amino acid residues, as well as improve binding selectivity [15].

However, the concave binding surface of DARPins can also become an advantage. Another DARPin feature (namely, the absence of cysteine residues in the protein that allows introduction of a single cysteine near the surface of interaction with the target and using it for conjugation) makes it possible to take advantage of this drawback. In a study by Kummer et al. [16], DARPin specific to the phosphorylated form of ERK (pERK) was conjugated to an environment-sensitive merocyanine dye: the intensity of its fluorescence increases in a hydrophobic environment; i.e., when DARPin binds to pERK. Hence, a biosensor for detecting ERK phosphorylation was obtained. Since it was shown that DARPin does not itself recognize phosphate but detects changes in the conformation of the activation loop [17], this approach can be used for other proteins that change their conformation during functioning.

Therefore, even the relative disadvantages of DARPins can be used to create unique constructs. However, the advantages of DARPins have made it possible to find many uses for these proteins, primarily in therapy and the diagnosis of cancer.

APPLICATIONS OF DARPins IN CANCER DIAGNOSIS AND THERAPY

The principles for DARPin design were described in 2003 [18]. In 2007, this technology was applied to obtain high-affinity proteins that bind to the HER2 tumor marker [19]. Later, DARPins binding to other molecules involved in carcinogenesis were obtained. The targets included EpCAM [20], EGFR [21], VEGF [22], HGF [22], cathepsin B [23], KRAS [24], etc. However, to date, the majority of targeted agents are based on HER2-binding DARPins. This can be explained by the therapeutic significance of the target. The HER2 (ErbB2) protein is a tyrosine kinase receptor with a low level of expression on the surface of human epithelial cells. HER2 is normally involved in various intracellular signaling pathways but mainly stimulates the HER3/PI3K/Akt pathway and mitogen-activated protein kinase (MAPK) cascade [25], leading to cell proliferation. The HER2 antigen is overexpressed in 20–30% of mammary gland and ovary tumors and bolsters the aggressive properties of the tumor. That is why the standard diagnostic protocols for breast

cancer involve determining the HER2 expression level [26]. *ERBB2* gene amplification can also be observed in gastric and intestinal adenocarcinomas [27], carcinomas of the ovary [28], endometrium [29], prostate gland [30], as well as the salivary glands, vagina, cervix and the bladder [31]. Two murine humanized antibodies are currently used in HER2-positive cancer therapy: trastuzumab (Herceptin, Roche-Genentech) binding to subdomain IV of HER2 and pertuzumab (Perjeta, Roche-Genentech), which binds to subdomain II of the receptor [32]. In addition, trastuzumab conjugated with the microtubule assembly inhibitor (trastuzumab-emtazine, Kadcyla, Roche) [33] and two chemical tyrosine kinase domain inhibitors are used: lapatinib (Tykerb or Tyverb, GlaxoSmithKlein) [34] and neratinib (Nerlynx, Pfizer) [35]. These drugs have been approved for HER2-positive breast cancer, gastric cancer, and gastroesophageal cancer [36]. However, the indications for their use can be expanded in the near future. According to the results of the MY PATHWAY study, a statistically significant response to the trastuzumab and pertuzumab therapy was shown for patients with 9 types of HER2-positive tumors: colorectal cancer (38% of patients), bladder cancer (33%), gallbladder cancer (29%), salivary gland cancer (80%), non-small cell lung cancer (13%), pancreatic cancer (22%), ovarian cancer (13%), prostate cancer, and skin cancer (a single patient in each case) [37]. Therefore, we can conclude that the potential of HER2-specific targeted therapy is not limited to breast cancer and gastric cancer. At the same time, the existing targeted HER2-directed therapy significantly enhances the effectiveness of combination therapy but a complete response or prolongation of patients' survival to more than 5 years are still rare events, which continues to stimulate the search for novel drugs.

Figure 2 summarizes the main ways of using DARPins for developing agents for cancer diagnosis and treatment.

Tumor imaging is important for conducting preclinical trials of new drugs in animals, for validating patient's diagnosis, and evaluating therapy efficacy. In animal models, far-red fluorescent proteins, such as mCherry, can be applied to allow intravital visualization of a tumor [38]. Cherry and HER2-specific DARPin 9_29 were fused to obtain the recombinant protein DARPin-mCherry, which specifically stains HER2-positive cancer cells [39] and is used for the functionalization of nanoparticles [40–43] as described below.

Radionuclides selectively accumulating in the tumor are used for tumor imaging in the human body. Monomeric DARPins can act as binding modules for high-affinity radio immunodiagnostics, in which proteins conjugated to a radionuclide carrier (typically a chelator or quasicovalent technetium complexes) are used [44]. This technology was originally developed for single-chain antibodies, but soon it was applied to other scaffold proteins, since the basic requirements for binding modules for radioimmune diagnostics include high affinity and small size [45, 46]. DARPins have both of these properties and can be successfully utilized for the radioactive imaging of tumors. For example, HER2-specific DARPins G3 and 9_29 were used for obtaining conjugates with the desired pharmacokinetics and reduced accumulation in the liver [47–49]. As for cancer therapy, DARPins can be used both for the delivery of toxic modules and for the inhibition of cell signaling pathways thanks to the specific binding of membrane receptors. A bispecific DARPin dimer with a linker of a certain length was shown to fix the extracellular parts of neighboring HER2 receptors in a nonfunctional conformation that does not allow them to form dimers and transduce mitogenic signals, which had cytostatic and cytotoxic effects on HER2-dependent cancer cells [12]. The dimer was used to design the tetrameric MP0274 drug: it consists of modules recognizing the domains I and IV of the HER2 receptor and two modules that bind to human serum albumin, which increase the circulation time of the protein in the blood. The first phase of clinical trials of this drug was started in 2017 [50].

Clinical trials are underway for MP0250, another multivalent DARPin. One polypeptide chain of this protein contains a module that binds to the vascular endothelial growth factor VEGF-A, a module binding to the hepatocyte growth factor HGF, and two modules binding to human serum albumin [22]. Therefore, the drug inhibits two important cancer cell signaling pathways: VEGF/VEGFR and HFG/cMet; its binding to albumin ensures long-term circulation. MP0250 is the first multimeric DARPin tested in patients [51]. In a phase I clinical trial, this drug was well-tolerated at doses sufficient to suppress VEGF activity. In 2018, phase Ib/II clinical trials to evaluate MP0250 in combination with osimertinib for the treatment of patients with nonsquamous non-small cell lung cancer (NSCLC) with EGFR mutations were started [52]. In 2017, phase II clinical trials of MP0250 in combination with bortezomib and dexamethasone for treating patients with refractory and relapsed multiple myeloma (RRMM) were initiated [53].

Another way to create DARPins with tailored pharmacokinetics is conjugation with polyethylene glycol and topical application of the conjugates. One such conjugate, abicipar specific for VEGF, is used for neovascular age-related macular degeneration (ADE) and diabetic macular edema (DME) [54]. This drug is currently undergoing phase III clinical trials.

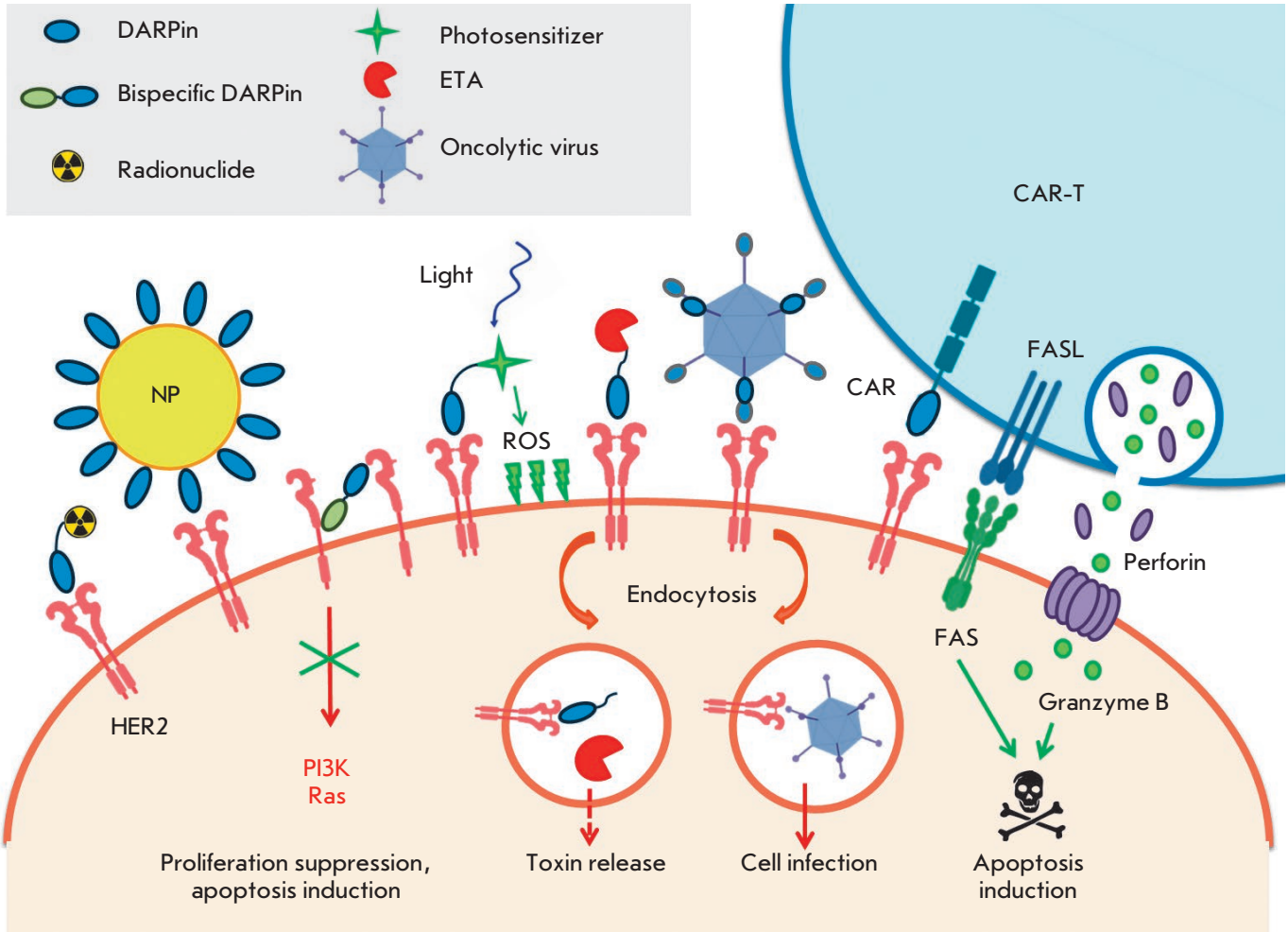

Fig. 2. Application of DARPins in cancer cell visualization and elimination. DARPins can inhibit cell signaling molecules, thus suppressing cell proliferation, or serve as targeting modules for the delivery of various agents: radionuclides, nanoparticles or liposomes, photosensitizers, protein toxins, oncolytic viruses, and lymphocytes with chimeric antigenic receptors. HER2 – human epidermal growth factor receptor 2; NP – nanoparticle; ROS – reactive oxygen species; PI3K – phosphoinositide-3-kinase; Ras – small GTPase Ras; CAR – chimeric antigen receptor; CAR-T – T-lymphocyte expressing the chimeric antigen receptor; FAS – death receptor (CD95, APO-1), an inducer of extrinsic apoptosis pathway; FASL – ligand of the FAS receptor (CD95L, CD178); ETA – truncated *Pseudomonas aeruginosa* exotoxin A

DARPin-BASED TUMOR TARGETING TOXINS

The simplicity of DARPin production in the bacterial expression system has stimulated the development of antitumor agents based on protein toxins. *Pseudomonas aeruginosa* exotoxin A (PE, ETA) is one of the most efficient apoptosis inducers thanks to its own enzymatic activity that inhibits translation. PE consists of three domains. Domain I is specific to the α-2-macroglobulin receptor of animal cells (LRP1, CD91) and provides internalization of the toxin molecule into the cell. Domain II contains furin proteolysis sites and disulfide bonds reduced by protein disulfide isomerases, which are thus involved in the intracellular processing of the toxin molecule. Domain III exhibits intrinsic catalytic activity: it ADP-ribosylates eukaryotic eEF2, thereby blocking protein biosynthesis in the cell, ultimately leading to cell death [55]. The domain structure of the exotoxin allows one to use its truncated variants preserving catalytic activity, while the natural binding domain can be replaced with targeting molecules of desired specificity. In this case, it is sufficient that the agent enters the endosome where the effector module

is cut off by furin protease and the toxin is transported to the endoplasmic reticulum due to the KDEL retrograde transport signal, and subsequently released into the cytosol from it [56].

EpCAM-specific DARPin Ec4 was used to deliver a truncated *P. aeruginosa* exotoxin to colon cancer cells HT29. The resulting DARPin-ETA protein exhibited an antitumor activity both in vitro and in vivo [57]. ETA was also used to suppress the growth of HER2-positive tumors. Since DARPin 9_29 effectively stimulates the internalization of HER2 into a complex with the protein partner [58], this targeting module is well-suited for delivering exotoxin fragments to cancer cells. The DARPin-PE40 targeted toxin was created using the DARPin 9_29 module and a *P. aeruginosa* exotoxin A fragment with a molecular weight of 40 kDa. It successfully induced apoptosis in HER2-overexpressing cells, exhibited selective in vitro toxicity, and effectively suppressed breast cancer cell growth in a xenograft model [59].

One of the problems related to antitumor agents based on the Pseudomonas exotoxin is high immunogenicity. Being a protein of bacterial origin, ETA causes the formation of neutralizing antibodies, which reduce therapy effectiveness and increase the risk of anaphylactic reactions. Various approaches have been developed to solve this problem: mutagenesis of PE, followed by chemical modification (PEGylation); suppression of the patient's immune system; as well as detection and elimination of immunodominant epitopes of B and T cells by mutagenesis. The latter of these approaches is the most universal and compatible with different regimens of tumor therapy [60]. DARPin-LoPE containing an exotoxin fragment with deleted or mutant immunodominant epitopes exhibited selective toxicity with respect to HER2-overexpressing cells in vitro at picomolar concentrations [61] and effectively suppressed the growth of ovarian cancer cells in the xenograft model [62]. Moreover, the nonspecific toxicity and immunogenicity of DARPin-LoPE were lower than those of DARPin-PE40: so, the contribution of DARPin to these side effects was negligible.

APPLICATIONS OF DARPins IN TARGETED PHOTODYNAMIC THERAPY

Photodynamic cancer therapy relies on the use of photosensitizers that convert oxygen into reactive oxygen species (mainly singlet oxygen ($^1O_2$)) at certain wavelengths [63]. The advantage of photodynamic therapy over chemotherapy consists in smaller exposure of healthy tissues, since only part of the body is irradiated. However, such a localized exposure does not completely prevent side effects, such as sensitization of the skin and the retina.

Two approaches are used to solve this problem: increasing selective accumulation of the photosensitizer in the tumor thanks to the physicochemical properties of the molecule per se and covalent binding of the targeting modules to a photosensitizer (targeted photodynamic therapy) [64]. Monoclonal antibodies were the first targeting molecules used to specifically deliver a photosensitizer to a tumor. This approach was developed after the study by Mew D. et al., who showed that hematoporphyrin can be directly conjugated to a monoclonal antibody specific to the myosarcoma antigen and demonstrated the advantages of the resulting immunoconjugate over hematoporphyrin in vivo [65]. Further development of targeted photodynamic therapy has led to the design of conjugates that include other targeting modules selectively accumulating in the tumor due to the biochemistry of malignant cells and their signaling pathways. For example, the application of the conjugates of photosensitizers with folic acid was proposed for tumors dependent on folic acid. Peptide ligands are also currently being developed; these ligands are a tool for delivering chemical photosensitizers to tumor cells carrying specific integrins and hormone receptors on their surface [66].

The conjugates of antibodies and photosensitizers effectively eliminate cancer cells that carry known surface markers in both in vitro studies and in vivo [67]. However, chemical conjugation of photosensitizers and antibodies has a number of drawbacks, such as low reproducibility of conjugate synthesis, aggregation, the presence of an unconjugated photosensitizer in the preparation, loss of antibody affinity to the receptor, and changes in the physical properties of the photosensitizer [68].

A fundamental solution to these problems is to design genetically encoded hybrid molecules containing both phototoxic and targeting components. This eliminates the need for chemical conjugation of components and enables the production of fused recombinant molecules of constant composition, thus ensuring steadily reproducible functionality. It became possible to produce these photosensitizers after phototoxic proteins capable of producing reactive oxygen species when exposed to light at a specific wavelength were discovered. To date, two types of phototoxic proteins are known. These are the KillerRed [69] and KillerOrange proteins [70], the *Aequorea victoria* GFP derivatives, as well as miniSOG [71] and the miniSOG2 [72] protein, the derivatives of the *Arabidopsis thaliana* phototropin.

DARPin 9_29 was used to deliver the phototoxic miniSOG protein (miniSinglet Oxygen Generator) to cancer cells. This protein is obtained from the LOV2 (Light Oxygen Voltage) domain of phototropin

2 (AtPhot2) by site-specific mutagenesis. The LOV domain contains the flavin mononucleotide (FMN) cofactor, which is excited by blue light, after which the energy of the excited state is consumed for the formation of covalent bonds with conserved cysteine 426. Replacing the cysteine 426 participating in this reaction with glycine has altered protein activity. In response to blue light irradiation, all the energy of the excited state of FMN was spent on singlet oxygen formation. After additional mutagenesis, the variant with a quantum yield of singlet oxygen of 0.47 ± 0.05 was selected. The absorption spectrum of miniSOG contains two peaks at 448 and 473 nm; the fluorescence spectrum peaks correspond to 500 and 528 nm [71].

The miniSOG protein was originally designed as a genetically encoded marker for electron microscopy: miniSOG generates singlet oxygen in quantities sufficient for initiating oxidative polymerization of diaminobenzidine (DAB). The polymer obtained by oxidation of DAB interacts with osmium tetroxide; the product of this reaction is used as a label for electron microscopy. In addition, miniSOG can be used as a toxic module for ontogenesis studies, selective inactivation of proteins, and photodynamic therapy [73–75].

A genetically encoded 4D5scFv-miniSOG immunophotosensitizer was based on the anti-HER2 mini antibody and the phototoxic protein miniSOG. 4D5scFv-miniSOG selectively destroys HER2-positive SK-BR-3 breast adenocarcinoma cells under irradiation. The cytotoxic effect of 4D5scF-vminiSOG against this cancer cell line is eightfold stronger than the effect of the chemical conjugate of porphyrin with the same targeting module [76]. However, the overproduction of 4D5scFv-miniSOG in bacteria leads to the aggregation of most of the target protein in inclusion bodies, and its renaturation is ineffective. The replacement of the targeting module with HER2-specific DARPin 9_29 helped to solve the problem related to the production of the target protein in bacteria in soluble form; the yield of the protein was 15 mg from 1 liter of liquid culture. DARPin-miniSOG exhibited selective in vitro toxicity against HER2-overexpressing SK-BR-3 breast adenocarcinoma cells [77]. Notably, the fluorescent properties of DARPin-miniSOG allowed one to estimate the rate of internalization and the recycling of the HER2 molecule [58], as well as compare the internalization rates of 4D4scFv and DARPin 9_29 in a complex with this receptor [78]. Nevertheless, other fluorescent modules or dyes are preferred for the visualization of HER2-positive cancer cells, since miniSOG has a relatively low fluorescence quantum yield and the emission spectrum overlaps with cell autofluorescence [79].

DARPins can also be used to deliver phototoxic nanoparticles, enabling the creation of multifunctional antitumor agents, which will be discussed further.

APPLICATION OF DARPins IN NANOPARTICLE DELIVERY

Nanostructures are increasingly used in basic research, as well as in the diagnosis and therapy of various diseases. Some types of nanoparticles have unique characteristics that make it possible to use them for efficient contrasting of pathogenic foci using X-ray, infrared, and other types of electromagnetic radiation or acoustic waves. Most of the developments have been made in the field of antitumor nanoparticles, primarily due to the fact that imperfect vascularization and disorganization of cell–cell contacts of the tumor make it possible for many types of nanoparticles to penetrate the tumor more efficiently than normal tissue [80, 81]. The advantage of nanoparticles over low-molecular-weight drugs and proteins is that a single agent can have several functions, including particle-targeting to cancer cells using surface modification. Monoclonal antibodies are often used for this purpose. However, the problems related to proper orientation and standardization of the number of antibodies per particle still remain relevant for full-length antibodies [82]. In addition to antibodies and their fragments, other molecules can be used: alternative scaffolds; proteins that are specifically captured by a tumor, such as growth factors and transferrin; aptamers; and low-molecular-weight substances (e.g., folic acid) [56, 57].

Similar to monoclonal antibodies, DARPins can be used to functionalize nanoparticles [83]. DARPin 9_29 was used to deliver upconverting nanoparticles into a tumor during photodynamic therapy. $NaYF_4$: Yb_3^+ Tm_3^+ / $NaYF_4$ particles emitting ultraviolet radiation when exposed to infrared light were coated with the DARPin-mCherry protein [39], which allows visualization of cancer cells thanks to the far-red fluorescent mCherry module [40]. DARPin 9_29 and the DARPin-mCherry protein containing it were also used to coat 5-nm gold nanoparticles [41] and gold nanorods [42]. DARPin was efficiently coupled with the particle surface to form a crown consisting of approximately 35 protein molecules, thus reducing particle aggregation. Notably, DARPin interacted with nanoparticles in a way, leaving its HER2-binding surface free, which ensured selective binding of the resulting nanoparticles to HER2-overexpressing cells [41].

DARPins and DARPin-containing proteins can be successfully coupled with nanoparticles using carbodiimide conjugation. DARPin 9_29 was covalently bound to upconverting radioactive nanoparticles coated with a maleic anhydride and 1-octadecene (PMAO) copolymer. The resulting nanoparticles were used to visualize

breast tumors in a xenograft mouse model and exhibited low side toxicity in vivo [84]. The same conjugation method was applied to functionalize upconverting nanoparticles with the DARPin-mCherry protein [43]. DARPin-PE40 was coupled with upconverting radioactive nanoparticles in the same way, making it possible to visualize tumors in vivo and efficiently eliminate HER2-overexpressing cells both in vitro and in vivo [85]. Insertion of unique cysteine residue allowed one to conjugate HER2-specific DARPin G3 with fluorescein maleimide and then to bind the labeled DARPin to superparamagnetic nanoparticles coated with polylactic acid by activating its C-terminal carboxyl groups with carbodiimide [86]. DARPin was also attached to nanostructures via maleimide conjugation. This method was utilized for DARPin 9_29 conjugation with the surface of ETA-containing liposomes functionalized using Trout's reagent [87].

Hence, the standard methods for immunoglobulin coupling to nanoparticles can be applied to DARPins. However, DARPins can also be embedded into nanostructures in the form of fused proteins that interact with the particle surface. This approach allows one both to achieve the desired orientation of the binding module and to assemble the targeting modules according to the principle of a construction kit. For example, a DARPin-Bn protein consisting of DARPin 9_29, a flexible linker and barnase ribonuclease, was used to create targeted silicon nanoparticles. These nanoparticles are coated with a barstar protein fused to a SiO_2-binding peptide (SBP-Bs), which attaches SBP-Bs to the particle. As barnase and barstar bind to each other with a very high affinity ($K_a = 10^{14}$ M^{-1}), these proteins allowed one to assembly the outer layer of nanoparticles in a solution without using conjugation or to implement the pre-targeting strategy when the targeted protein was delivered to the cells to which the nanoparticles were subsequently added [88]. Fusion of barnase and the peptide binding to the magnetite surface made it possible to utilize the same DARPin-Bn protein to functionalize magnetite nanoparticles and deliver them to cancer cells [89].

To sum up, DARPins can be used, along with antibodies and their fragments, to create targeted nanoparticles. Moreover, their small size and simplicity of production in bacteria (including fusion proteins) provide unique opportunities for maintaining the affinity and specificity of the binding module thanks to the favorable orientation of the molecule.

APPLICATION OF DARPins IN DESIGNING ONCOLYTIC VIRUSES

Molecules derived from viruses and bacteria are widely used to obtain antitumor agents [81], but replicative active viral particles can be utilized for tumor cell destruction [90]. Oncolytic viruses form a new, very peculiar class of therapeutic drugs that largely act in the patient's body on their own. Some viruses have natural tropism to tumor cells, but oncolytic agents are more likely to be based on viruses that can be retargeted by modification of surface proteins (e.g., measles virus, adenovirus, vesicular stomatitis virus, vaccinia virus, and herpes simplex virus) [90]. The natural specificity of the virus can be changed using bispecific adapter proteins, as has been successfully done for adenoviruses using trimerizing DARPins [10]. However, the fusion of targeting modules with envelope proteins is used more often, since in this case all the properties of the virus are encoded by its genome. Like single-chain antibodies, DARPins can be used for such retargeting, and their small size facilitates successful encoding of DARPins sequences in viral vectors.

The measles virus envelope protein was modified by DARPins specific to HER2, EGFR, or EpCAM. The resulting viral particles lost their natural receptors tropism and selectively infected cells, overexpressing the corresponding tumor marker. Viral particles bearing HER2-specific DARPin on the surface caused cell lysis more efficiently than virus functionalized with a HER2-specific single-chain antibody. The use of two DARPin-linked DARPins recognizing HER2 and EpCAM allowed one to create bispecific viral particles that retain the high cytolytic activity of monospecific virions [91, 92].

An adeno-associated virus coated with the DARPin-fused modified envelope protein VP2 was also used to infect HER2-positive cancer cells. The resulting virions specifically infected HER2-positive cells and delivered vectors encoding either the luciferase gene or the herpes simplex virus thymidine kinase gene (HSV-TK) to SK-OV-3 cells *in vivo*. Viral particles containing a gene therapy vector encoding HSV-TK, in combination with ganciclovir, effectively suppressed xenograft tumor growth, without causing hepatotoxicity [93]. Similar viral particles were obtained using EGFR-specific DARPin and affibody, and both agents showed selective toxicity towards EGFR-positive cells *in vitro* [94].

APPLICATION OF DARPins IN THE DESIGN OF CHIMERIC ANTIGEN RECEPTORS

The accumulated knowledge on the functioning of the immune system allowed us to elaborate the technology of targeted cancer therapy based on cytotoxic lymphocytes: T lymphocytes and NK cells. In this case, the lymphocytes are transduced with constructs that encode the chimeric antigen receptor (CAR), which is specific to the tumor antigen and has all the domains necessary for cell activation, including the signal se-

quences of the co-stimulating molecules of the natural receptor [95]. When activated through chimeric receptors, lymphocytes secrete proinflammatory cytokines and induce apoptosis in target cells through the extrinsic FAS receptor pathway and with the help of the granzymes that directly activate effector caspases and the caspase-independent pathways of cell death [96]. T cells with a chimeric antigenic receptor (CAR-T) successfully fought chemotherapy-resistant hematologic tumors to ensure complete cure in a large number of patients [97, 98]. Most of the chimeric receptors developed to date contain single-chain antibodies as an antigen-recognizing domain; however, DARPins can also be used as targeting modules for CAR. Moreover, DARPins have some advantages over single-chain antibodies. Thus, they are more compact, meaning that their coding sequences occupy less space in a lymphocyte transducing virus vector. Furthermore, DARPins are more thermodynamically stable. Finally, their binding surface is formed by a single polypeptide, unlike that of the antibodies whose paratope is formed by two immunoglobulin domains originating from different polypeptides. This means that DARPins can be used to obtain multispecific CARs [99].

CAR-T carrying a receptor based on HER2-specific DARPin G3 had the same level of activation as cells with a chimeric receptor containing single-chain antibody FRP5. The DARPin-containing CAR-T exhibited high toxicity against HER2-positive cancer cells and low toxicity against control cells not expressing HER2 [99]. Similar results were obtained when comparing CAR-T therapy based on 4D5 antibody and CAR-T based on DARPins G3 and 9_29. All the studied cell types specifically recognized HER2 and exhibited high cytotoxicity against HER2-positive cells in vitro. Cells with receptors based on DARPin G3 showed the highest efficacy. In the ovarian cancer xenograft model, the differences between CAR-T based on different DARPins were more pronounced: cells with a receptor based on 4D5scFv and DARPin G3 better infiltrated the tumor and more effectively suppressed its growth [100]. Generally, a conclusion can be drawn that DARPin-based CAR-T therapy does not concede to T lymphocytes that carry artificial receptors containing single-chain antibodies, and the comparative simplicity of obtaining DARPins and their monomeric form facilitates the creation of chimeric receptors for different targets.

Natural killer (NK) cells can also be utilized as agents for tumor recognition using chimeric antigen receptors. Their cytotoxicity is based on the same mechanisms as the activity of $CD8^+$ T cells; the natural activation pathways provide some advantages to CAR-NK over CAR-T. NK cells do not recognize a peptide in complex with MHC I [101], which reduces the risk of graft-versus-host disease (GVHD). This feature has already been used in cancer therapy by transfusion of donor NK cells [102–104] or even cells of the stable NK-92 line [105, 106], which increases therapy effectiveness even without using a chimeric antigen receptor. This makes it possible to design therapy based on stable NK cell lines that does not require cells from the patient [107]. Additional benefits of NK cells include their natural mechanisms of damaged cell recognition, which allows them to remain efficient antitumor agents even if the chimeric antigen receptor gene is lost or mutant. To date, no antitumor CAR-NK therapy based on DARPins has been developed, but it probably will soon be elaborated.

CONCLUSIONS

DARPins were designed as scaffold proteins alternative to antibodies. They are used in most technologies that originally utilize antibodies, except for those technologies where the properties of the constant part of immunoglobulin molecules are needed. The advantages of DARPins, including their small size, independence of animal immunization, and simplicity of production of fusion proteins, make them promising tools for research and efficient components of therapeutic and diagnostic agents. One should refrain from a conclusion that alternative scaffolds can completely replace antibodies; however, they surely have made a substantial contribution to the targeting proteins being utilized and expanded the range of possible targets due to the different paratope structure. Furthermore, they have provided exceptional opportunities for creating bispecific and multivalent constructs. ●

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The Role of Interleukin-37 in the Pathogenesis of Allergic Diseases

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ABSTRACT **Cytokines of the interleukin-1 (IL-1) family play an important role in the realization of the protective functions of innate immunity and are the key mediators involved in the pathogenesis of a wide range of diseases, including various manifestations of allergy. The IL-1 family includes more than 11 members. However, the functions of many of them remain to be elucidated. Recently, new members of the IL-1 family have been discovered. In 2000, several independent research groups reported the discovery of a new interleukin of this family, which was named IL-37, or IL-1F7 (according to the new nomenclature). IL-37 was assigned to the IL-1 family based on its structural similarity with other members of this family. The study of its biological properties showed that its activity changes in inflammatory diseases, such as rheumatoid arthritis, psoriasis, as well as allergic diseases (allergic rhinitis, bronchial asthma, and atopic dermatitis). However, unlike most members of the IL-1 family, IL-37 acts as a negative regulator of inflammation. Activation of IL-37 suppresses inflammation, resulting in the suppression of inflammatory cytokines and chemokines, which in turn prevents infiltration of pro-inflammatory cells, mainly eosinophils and neutrophils. The exact molecular and cellular mechanisms of the anti-inflammatory effect of IL-37 in the development of allergic diseases (AD) have not been fully studied. This review summarizes and analyzes the accumulated experimental data on the role of IL-37 in the pathogenesis of AD, such as allergic rhinitis, bronchial asthma, and atopic dermatitis.**
KEYWORDS **IL-37, bronchial asthma, anti-inflammatory cytokines, pro-inflammatory cytokines, gene expression.**

INTRODUCTION

The development of allergic diseases (ADs), such as bronchial asthma, allergic rhinitis, and atopic dermatitis, is influenced by many factors, including genetic predisposition [1], as well as exposure to allergens, infections, and other negative environmental factors. In certain regions of the world – for instance in EU countries – the incidence of ADs reaches 30%, with predicted growth to 50% expected in the next 15 years [2, 3]. Bronchial asthma (BA) is a heterogeneous disease; it is usually characterized by a chronic inflammation of the respiratory tract [2]. A distinctive feature of allergic BA (aBA), which accounts for about 70–80% of all cases of the disease, is an increased level of allergen-specific IgE antibodies in the serum [4, 5] and high eosinophil count in the blood, mucous membranes of the respiratory tract. and bronchoalveolar lavage (BAL) [6]. Allergic rhinitis (AR), which is characterized by chronic inflammation in the upper respiratory tract [7], can significantly reduce a patient's quality of life by affecting his/her sleep and performance status [2]. Atopic dermatitis (AtD) is a multifactorial inflammatory skin disease which may be partly due to genetically caused impairment of the skin barrier function [8, 9]. According to various studies, AtD developed in childhood persists in adult age in 40–60% of cases [10].

Thus, taking into account the significant prevalence of ADs, the development of new methods of treatment and prevention remains a relevant biomedical issue. However, finding new methods of therapy is impossible without understanding the molecular mechanisms of the disease pathogenesis.

Based on current knowledge [11–13], two stages can be distinguished in the development of AD: the sensitization stage, which is accompanied by the occurrence of hypersensitivity to the allergen, and the effector stage, which is accompanied by inflammation, tissue injury, and remodeling (bronchi in case of BA, skin in AtD and nasal mucosa in AR). During the sensitization stage, an initial encounter with an allergen, which enters the body through the damaged epithelium and is presented by MHC-II molecules on antigen-presenting cells (APCs), occurs. APCs migrate to the regional lymph nodes and trigger polarization of naive Th0 cells to Th2 cells, which produce the cytokines IL-4, IL-5, IL-9, and IL-13, which are responsible for the main manifestations of ADs [14]. This process also promotes the differentiation of B cells into antibody-producing plasma cells. Under the action

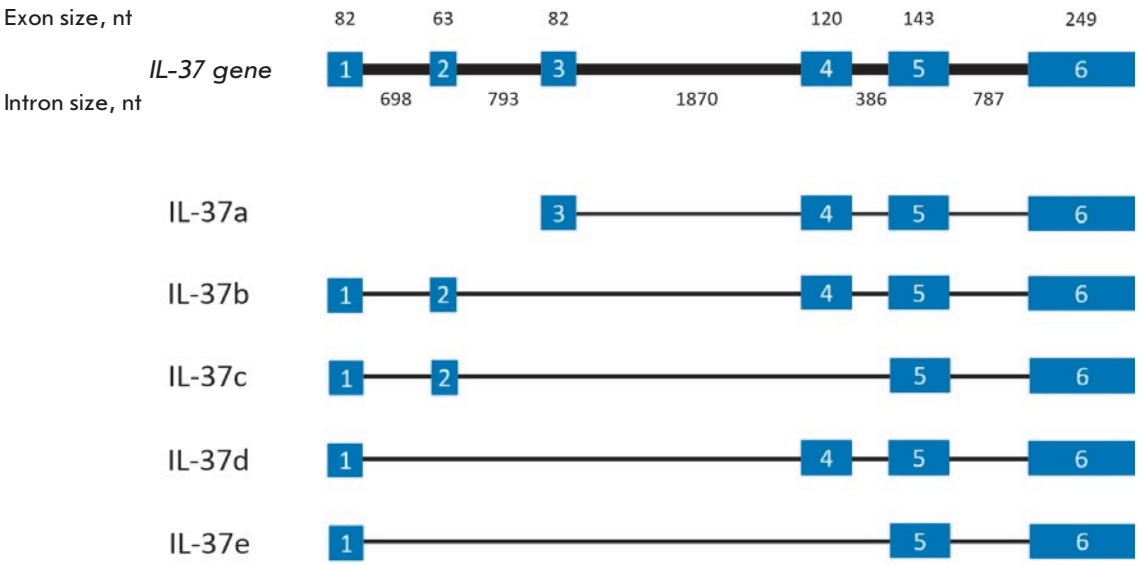

Fig. 1. The structure of the gene *IL-37* and its five alternative transcripts

of IL-4 and IL-13, B cells switch from the synthesis of IgM antibodies to the synthesis of IgE antibodies, which are mainly responsible for the mediation of the subsequent allergic reactions of the body [11, 12].

During the effector stage, IgE-class antibodies interact with mast cells and basophils through the FceRI and FceRII receptors. At a second encounter with an allergen, (the mast cell)??? interacts with surface IgE antibodies, resulting in cell degranulation and the release of pro-inflammatory mediators. The mediators, in turn, recruit pro-inflammatory cells, cause vasodilation, promote microthrombi formation with local tissue damage, and also exert the spasmogenic effect that leads to the contraction of smooth muscle, such as bronchi in BA. In parallel, T2 cells penetrate via chemokine receptors from blood vessels to the inflammation area, where they are activated by the allergen and produce IL-4, IL-5, IL-9, and IL-13. The cytokines IL-4, IL-9, and IL-13 contribute to the overproduction of mucus by bronchial epithelium (in BA) or nasal mucosa (in AR). IL-5 promotes recruitment of eosinophils to the inflammation area. and their activation. Eosinophils, in turn, release the mediators of inflammation during degranulation, which results in damage to surrounding tissues [15–17].

To date, the role of Th2 cells and the cytokines produced by them in the development of ADs has been studied comprehensively (see reviews [18–20]). Meanwhile, there is data on the participation of some recently discovered cytokines in the development of AD. IL-33, a member of the IL-1 family, has been shown to be involved in the development of AD. Secreted by epithelial cells, IL-33 activates innate lymphoid cells 2 (ILC2), which produce significant amounts of IL-5 and IL-13, thereby enhancing the pro-allergic Th2 immune response (see reviews [21–23]). There are reports in scientific literature on the participation of IL-37, another recently discovered representative of the IL-1 family, in the pathogenesis of ADs. The current review is devoted to the role of this IL in the development of ADs.

THE HISTORY OF THE DISCOVERY AND THE MOLECULAR AND GENETIC CHARACTERISTICS OF *IL-37*

Interleukin-37 (IL-37) belongs to the IL-1 family, which includes 10 other cytokines: IL-1α, IL-1β, IL-1Rα, IL-18, IL-36α, IL-36Rα, IL-36β, IL-36γ, IL-38, and IL-33. IL-37 was discovered in 2000, when three research groups independently described five mRNA transcripts of this cytokine using *in silico* methods [24–26]. The study of the biological function of the *IL-37* gene was significantly complicated by the fact that the gene is absent in mice; for this reason, generation of IL-37-defectient mice and subsequent comparison with wild-type mice carrying functional IL-37 was not possible [27]. Unlike in humans, IL-37 is absent in chimpanzees, although the functional cytokine gene has been identified in other primates [28].

IL-37 is located on chromosome 2q12-13, a locus containing the genes of most IL-1 family cytokines [29]. In mice, the *IL-1* gene cluster is also located on chromosome 2 [30–32]. Both loci – human and mouse – are quite similar, with the exception of the region encoding *IL-37*, which is absent in mice [27]. At the same time, in primates, for instance, in gorillas, the *IL-37* gene is located on chromosome 2 [28].

The size of human *IL-37* is 3,617 bp, and its mRNA undergoes alternative splicing, resulting in five different isoforms of IL-37: a–e (*Fig. 1*). The isoforms a, b, and d contain the exons 4, 5, and 6. It would appear that the biological functions of *IL-37* are associated with these exons [33].

IL-37a has a unique N-terminal sequence encoded by exon 3, which is the start exon for this isoform [31]. Axon 3 is absent in four other IL-37 isoforms, and translation of the protein starts at exon 1. Exons 4–6 encode the putative 12 β-folds, which then form the β-trefoil fold, a structure typical of all members of the IL-1 family [34].

The IL-37b isoform is the best-characterized; it consists of 218 amino acid residues. The N-terminal sequence encoded by the first two exons presents a pro-domain, which is cleaved during cytokine maturation. Exons 4–6 play the same role as in isoform IL-37a. Thus, one can assume that both the IL-37b and IL-37a isoforms have biological significance [33].

IL-37c isoform differs from IL-37b: it lacks exon 4, which does not allow IL-37c to form the typical β-trefoil structure during folding. Hence, we can assume that it lacks any biological function. The same is true for the IL-37e isoform, which also does not contain exon 4. Unlike for IL-37b, IL-37d lacks exon 2. Hence, it can form β-trefoils and also serve as a functional cytokine form [33].

Cytokines of the IL-1 family are synthesized as precursor molecules containing a pro-peptide domain. It has been established that caspase-1 is the main enzyme necessary for the processing of precursor molecules into mature cytokine forms and their subsequent secretion [35]. IL-37b is also synthesized as a precursor protein and processed into mature form after cell stimulation (for example, with LPS) [36]. The caspase-1 cleavage site is located in the sequence encoded by exon 1. Therefore, the isoforms b, c, d, e carrying exon 1 also contain a caspase-1 cleavage site. Isoform IL-37a does not contain exon 1 but has a caspase-1 cleavage site, a unique sequence located in exon 3.

Caspase-1 performs the most effective protein cleavage, while caspase-4 acts much slower; and other caspases do not show enzymatic activity against IL-37 [37]. Unlike for IL-33, the secretion of IL-37 is not associated with cell death. Apparently, processing of IL-37 by caspases (and/or other enzymes) is not necessary for its subsequent secretion, since both the processed form of IL-37 and its predecessor were detected in the extracellular space after activation [38]. However, it should be noted that in the case of IL-37, as well as some representatives of the IL-1 family (IL-1β and IL-33), both the processed form and its precursor possess biological activity [37]. Moreover, there is an assumption that unknown proteases can process the secreted mature form of IL-37 in the extracellular space and increase its activity [27]. It has been shown that the recombinant processed protein IL-37 (46–218 aa) lacking 45 amino acid residues at the N-terminus exhibits 20–30 times greater biological activity than the unprocessed protein [39].

Different isoforms of IL-37 were found in various tissues and organs; in some organs, only one isoform is expressed. For instance, only IL-37a is expressed in the brain, the expression of IL-37b is specific to kidneys, and IL-37c is expressed in the heart. Two isoforms, IL-37d and IL-37e, are expressed exclusively in the bone marrow and testes [25, 26]. Mature IL-37 and its proform are secreted by activated macrophages, dendritic cells (DC), and peripheral blood mononuclear cells (PBMCs) [40]. IL-37, which is secreted by these cells, exerts its biological effects via a unique receptor complex.

IL-37 RECEPTOR COMPLEX AND SIGNALING PATHWAYS

The IL-37 receptor complex is similar to the IL-18 receptor, another representative of the IL-1 family. IL-18 is one of the key pro-inflammatory cytokines acting as a pathogenetic factor in a number of diseases [41, 42]. The IL-18 receptor complex consists of two chains: α (IL-18Rα) and β (IL-18Rβ), each of which has a TIR domain [43]. During the formation of the IL-18Rα/IL-18/IL-18Rβ complex, the TIR domains come together, after which the MyD88 factor binds to them and induces the pro-inflammatory effect (*Fig. 2*) [27].

IL-37 can also bind to the IL-18Rα chain; after knockout of this chain, IL-37 could not exert an anti-inflammatory effect [44]. Therefore, a hypothesis has been proposed that IL-37b is a competitive inhibitor of IL-18 and that IL-37b suppresses the inflammatory effect of this cytokine. However, this assumption was not confirmed [37, 45], after which another hypothesis was proposed: in addition to IL-18Rα, IL-37 can also bind to some receptor other than IL-18Rβ, which ultimately results in the activation of the anti-inflammatory pathway. It was soon established that an additional receptor for IL-37 is IL-1R8 (SIGIRR). It was shown that the IL-37–IL-1R8–IL-18Rα complex is assembled on the cell surface, and that its presence is necessary for further triggering of an anti-inflammatory response [46]. The IL-1R8 receptor consists of only one extracellular Ig-like domain with a long "tail" immersed in the cytoplasm and containing a mutant TIR domain [47]. The involvement of IL-1R8 in the anti-inflammatory action of IL-37 has been demonstrated in experiments on mice defective in the gene for this chain. No reduction in inflammation was noted in IL-1R8 knockout mice in response to IL-37 administration (*Fig. 2*) [44, 48]. These facts indicate that IL-1R8 and IL-18Rα are required for the manifestation of the anti-inflammatory activity of IL-37.

In addition, IL-37 is capable of exerting an anti-inflammatory effect via the IL-1R8/IL-18R-independent pathway [36]. Studies of the human lung cancer cell line A549 demonstrated that A549 cells are less sensitive to inflammatory factors when IL-37 is associated

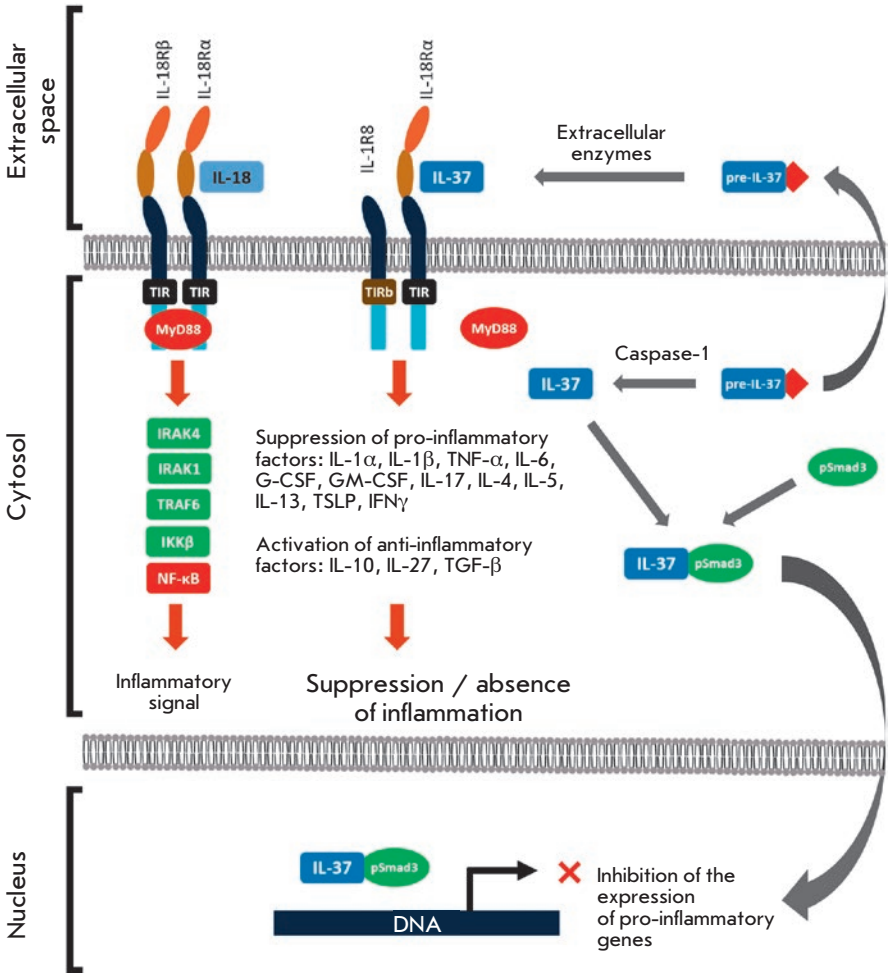

Fig. 2. Mechanisms of the anti-inflammatory effects of IL-37. IL-18 exerts its pro-inflammatory effects through a receptor complex consisting of the IL-18Rα and IL-18Rβ chains. The TIR domains come together and further bind to the MyD88 factor inducing the pro-inflammatory effect. IL-37 is synthesized as a precursor (pre-IL-37) which is capable of secreting into the extracellular space, where it is processed to a mature form through an unidentified mechanism. Mature IL-37 binds to the chains IL-18Rα and IL-1R8 (instead of IL-18Rβ); at the same time, the IL-1R8 chain carries the mutant TIRb domain (instead of functional TIR), which does not allow realization of the MyD88-mediated inflammatory effect [21]. The precursor of IL-37 is also capable of being processed intracellularly into mature form by Caspase-1. In the cytosol, IL-37 binds to the phosphorylated form of the Smad3 factor (pSmad3). Apparently, The IL-37 / Smad3 complex is able to translocate into the nucleus and inhibit the transcription of pro-inflammatory genes (the ability of the complex to bind to DNA has not been confirmed yet)

with Smad3. At the same time, inhibition of Smad3 was shown to increase the production of inflammatory cytokines. *In vivo* experiments performed on IL-37tg mice revealed that the likelihood of LPS-induced pneumonia was increased after Smad3 suppression. However, the exact mechanism of interaction between IL-37 and Smad3 remains unclear. It is believed that the C-terminal domain of IL-37 binds to Smad3, undergoes phosphorylation and then enters the nucleus, where it inhibits the expression of pro-inflammatory genes (*Fig. 2*) [49].

Thus, IL-37 exhibits anti-inflammatory properties in extracellular and intracellular conditions. After intracellular synthesis, a portion of the precursor protein is processed by caspase-1 and then performs negative regulation of pro-inflammatory genes through the Smad3 pathway. Another portion of the IL-37 precursor protein is secreted into the extracellular space, where it is further processed and exerts an anti-inflammatory effect by competitively inhibiting the pro-inflammatory IL-18 and activating the anti-inflammatory signaling pathway via the IL-1R8 and IL-18Rα receptors.

BIOLOGICAL EFFECTS OF IL-37

Most of the research, which has included *in vitro* and *in vivo* experiments, has studied the IL-37b isoform, which is of maximum size among all isoforms (218 amino acid residues). Mice lack IL-37 but have a functional receptor complex capable of binding human IL-37 [27, 39, 48]. For this reason, the biological role of this cytokine was studied not only using a cell culture, but also in laboratory mice.

In *in vitro* studies, recombinant IL-37b reduced the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α by human M1 macrophages after their stimulation with LPS [39]. Moreover, inhibition of IL-37 by monoclonal antibodies has the opposite effect [50]. In addition, IL-37 reduces the activation of pro-inflammatory cells, neutrophils, and prevents their migration along the chemokine gradient [51, 52]. Administration of IL-37 decreases IL-1β expression in mouse alveolar macrophages [48]. Furthermore, introduction of recombinant IL-37 inhibits the proliferation of Th17 cells in mice [53]. Thus, all this data indicates a pronounced anti-inflammatory activity of IL-37 against epithelial cells, macrophages, neutrophils, and blood mononuclear cells.

The biological role of IL-37 was studied *in vivo* in so-called IL-37tg mice with IL-37b gene insertion [49]. LPS was administered to these mice, after which the production of a number of pro-inflammatory and anti-inflammatory factors was evaluated. It was shown that LPS did not increase the level of the pro-inflammatory cytokines IL-6, IL-1β, IL-17, IFNγ,etc. both in wild-type and IL-37tg mice, while the level of anti-inflammatory cytokines, such as IL-10, increased both in transgenic and wild-type mice [36].

The role of IL-37 was also studied in a model of colitis induced by sodium dextran sulfate in mice. The severity of the intestinal inflammation was shown to be significantly lower in IL-37tg mice: infiltration of the colon by all types of leukocytes and production of inflammatory cytokines (IL-1β, IL-17, TNF-α) decreased, while production of anti-inflammatory IL-10 increased. Adoptive transfer of bone marrow cells from IL-37tg mice to wild-type mice led to a significant reduction in signs of experimental colitis. This indicates the anti-inflammatory effect of myeloid cells expressing IL-37 [49] (see review [27]).

The anti-inflammatory role of IL-37 was also confirmed in other models: in an experimental model of ischemic injury [54], acute renal ischemia [55], regional spinal cord injury [56], obesity, and type 2 diabetes in mice [57, 58] (see review [27]).

A number of authors attribute such an anti-inflammatory activity of IL-37 to its ability to attenuate the presentation of antigens and thereby suppress T cell activation. This assumption is supported by the fact that DC isolated from IL-37tg mice had a reduced level of CD40 and MHC class II molecules [59]. In addition, IL-37 increases the level of T regulatory cells, which suppress inflammation via secretion of the anti-inflammatory factor TGF-β [49, 60].

However, it still remains unclear how IL-37 exerts its impact: either via its intracellular form or by binding the extracellular IL-37 to its cell surface receptor. By using antibodies that neutralize extracellular IL-37, it was shown that this cytokine exhibits extracellular activity in some cases, since its neutralization in IL-37tg mice increased the level of pro-inflammatory IL-6 in the serum [38]. In other studies, on the contrary, neutralization of IL-37 in mouse macrophages transfected with the corresponding transgene did not affect their production of IL-6. This fact indicates that, in this type of cells, IL-37 functions more likely via intracellular mechanisms [38]. Injection of mature IL-37b or its precursor into human M1 macrophages suppressed the LPS-induced expression of IL-1β, IL-6, and TNF-α, but the effect was practically absent in M2 macrophages, DCs, and PBMCs [38, 39]. However, introduction of IL-37 into PBMCs of patients with rheumatoid arthritis reduced the expression of pro-inflammatory cytokines [53].

Thus, we can conclude that, in general, IL-37 is a negative regulator of the inflammatory process, apparently due to a decrease in the expression of the main pro-inflammatory cytokines, inhibition of DC maturation and their presentation of antigen, as well as due to the induction of T-regulatory cells and anti-inflammatory cytokines by IL-37.

EXPERIMENTAL DATA ON THE ROLE OF IL-37 IN THE PATHOGENESIS OF ALLERGIC DISEASES

Clinical cases

The first data on the possible involvement of IL-37 in the pathogenesis of ADs appeared after an increase in the expression level of the largest isoform, IL-37b, was detected in the serum of a patient with AtD by using ELISA. Moreover, an increase in IL-37b concentration was directly correlated to the severity of disease symptoms. A study of the local gene expression in skin biopsy material by immunohistochemistry revealed an increase in the IL-37 level in epidermal keratinocytes and some stromal cells of the dermis, but not in lymphocytes infiltrating the skin tissue. Thus, IL-37 was induced both locally and systemically in patients with AtD, which may be due to the activity of another member of the IL-1 family, namely IL-18, in the skin, which is activated through TLR signaling in response to *Staphylococcus aureus* infection, a pathogen that is often present in large quantities on the skin of AtD patients. Considering that IL-18 is a pro-inflammatory cytokine, the simultaneous increase in the expression of anti-inflammatory IL-37 can be explained by a compensatory response of the body to excessive inflammation in AtD (*Table 1*) [61].

On the contrary, a significant decrease in IL-37 activity in AR was found in a study published by Liu et al. [62]. In particular, a significant decrease in IL-37 concentration was observed both in the nasal lavage and systemic circulation of AR patients. Ten out of the 40 children with AR included in the study were diagnosed with BA. At the same time, no changes were observed in the systemic and local levels of IL-37. Despite the fact that AR is associated with dysregulation of the Th1/Th2 cytokine balance, a decrease in IL-37 activity in AR patients occurs due to the activation of Th2 cytokines ((IL-4, -5 and -13) and suppression of Th1 cytokines (IL-12 and IFNg). The severity of such manifestations of AR, as well as the level of specific IgE in the serum and eosinophilia negatively correlated with IL-37 activity. In a more detailed *in vitro* study [62], stimulation of PBMCs, which were obtained from AR patients, with recombinant IL-37 (rIL-37) suppressed the production of Th2 cytokines but had no effect on the production of Th1 cytokines and IL-10. In

Table 1. The role of IL-37 in allergic diseases. Clinical cases

Pathology	Study design	Detection method	Result	Reference
AtD	55 adult patients with moderate and severe AtD.	Evaluation of IL-37b in sera samples using ELISA, evaluation of local expression in skin biopsy material by immunohistochemistry.	Serum IL-37 levels are significantly higher in patients with AtD. IL-37 level positively correlated with the severity of AtD symptoms.	[61]
AR	40 children with AR (among them 10 with BA).	Evaluation of IL-37b in sera samples and nasal lavage using ELISA.	The level of IL-37b was decreased in serum and nasal lavage in AR. The level of nasal Th2 cytokine was negatively correlated with local expression of IL-37b. Blood levels of ECP, IgE, and eosinophilia were negatively correlated with the level of serum IL-37b. Intranasal administration of a glucocorticosteroid drug led to the induction of IL-37b and a decrease in AR symptoms.	[62]
aBA	21 children with aBA.	Evaluation of IL-37 in the supernatants of stimulated PBMCs using ELISA.	Production of IL-37 by PBMC-stimulated cells is significantly reduced in children with aBA.	[50]
aBA and nBA	92 children, among them 74 with aBA and 18 with nBA.	The expression level of IL-37 in stimulated PBMCs was evaluated by RT-PCR.	Patients with nBA have an increased level of IL-37, increased number of neutrophils in the blood, and increased level of pro-inflammatory cytokines IL-1β and IL-17.	[64]
BA	40 children with mild and moderate BA.	The expression level of IL-37 in serum and sputum was evaluated using ELISA and RT-PCR.	The expression of IL-37 mRNA in sputum and its serum level are significantly decreased in BA patients. Suppressed production of pro-inflammatory cytokines TNFα, IL-1β and IL-6 was observed in sputum cells cultured with rIL-37 after cell stimulation with LPS. Stimulation of $CD4^+$ T cells of the sputum in the presence of IL-37 decreased the production of IL-17.	[65]
BA	40 children with mild to moderate BA.	Incubation of sputum cells in the presence of IL-37.	The production of TSLP by sputum epithelial cells was significantly decreased in the presence of IL-37.	[66]
AR	32 adults with AR.	Analysis of $CD4^+$ and T cells isolated from PBMCs and cultured with rIL-37.	After incubation with IL-37, the production of IL-17 and IL-4 by $CD4^+$ T cells was significantly decreased in patients with AR.	[63]

Note: AtD – atopic dermatitis; AR – allergic rhinitis; ELISA – enzyme-linked immunosorbent assay; ECP – eosinophil cationic protein; PBMCs – peripheral blood mononuclear cells; BA – bronchial asthma; aBA – allergic bronchial asthma; nBA – non-allergic bronchial asthma; RT-PCR – real-time polymerase chain reaction; rIL-37 – recombinant interleukin-37.

contrast, the production of IL-37 by PBMCs decreased significantly after stimulation with recombinant Th2 cytokines, did not change after stimulation with Th1 cytokines, and was activated in response to IL-10 [62]. Similar results were obtained by Li et al. [63]. They confirmed that the production of both IL-4 and IL-17 is suppressed in the presence of recombinant IL-37 in the cultures of T cells isolated from the PBMCs of AR patients. However, IL-37 did not affect the production of such cytokines as IL-1b, IL-6, and IL-10 by dendritic cells isolated from the PMBCs of the same volunteers and did not alter the expression of the co-stimulatory molecules CD80, CD40, HLA-DR, and CD86 on their surface. Moreover, the presence of IL-37 in the culture medium did not affect the ability of DC to activate the production of IL-4 and IL-17 by T cells. This suggests that IL-37 acts as a regulator of innate rather than adaptive immunity (*Table 1*) [63]. It is known that Eosinophils secreting the eosinophil cationic protein (ECP) and other pro-inflammatory factors are known to be actively involved in the damage to the respiratory tract epithelium. A dose-dependent decrease in ECP was observed in eosinophils isolated from the peripheral blood of children with AR and then treated with IL-37, which confirms the anti-inflammatory role of this cytokine. The use of nasal steroid agents is one of the most common approaches in the treatment of this disease. In this study, a four-week course of corticosteroids resulted in a twofold decrease in the severity of AR symptoms and a subsequent significant increase in IL-37. Thus, Liu et al. showed that the development of AR symptoms is associated with a decreased IL-37 activity, while restoration of IL-37 expression reduces the disease symptoms (*Table 1*) [62].

Changes in the expression of IL-37 were also studied in aBA, and a significant decrease in IL-37 production by stimulated PBMCs harvested from children with aBA was shown in comparison with healthy volunteers [50]. A reduced expression level of a number of genes of the innate immune system, including the gene encoding for IL-37, was also demonstrated in children with aBA [64]. This effect is ascribed to the activity of Treg cells, whose blood count is increased in children with aBA. Moreover, these cells were able to suppress IL-5, IL-13, and IFNg in experiments *in vitro*. In children with non-allergic BA (nBA), despite the increased number of Treg cells, a significant increase in the expression of IL-37, as well as the pro-inflammatory cytokines IL-1b and IL-17, is observed, which is associated with a different functional state of Treg cells in children with nBA. Unlike for Treg cells isolated from children with aBA, the Treg cells of children with nBA were unable to suppress the expression of pro-inflammatory cytokines in *in vitro* experiments (*Table 1*) [64].

More evidence of a decrease in IL-37 activity in children with BA has been obtained. A significant decrease in IL-37 expression was found both at the level of mRNA and protein in the serum, as well as in the sputum of children with controlled BA (40 children in total, about 70% of whom had allergic asthma) compared with healthy volunteers. In addition, cells isolated from the sputum of children with asthma and cultured in the presence of rIL-37 exhibited a decrease in the production of the pro-inflammatory cytokines IL-1b, IL-6, and TNF-α, which confirms the anti-inflammatory properties of this cytokine. Similarly, the production of IL-17 was decreased in the $CD4^+$ T cells of the sputum in the presence of IL-37, which indicates the ability of IL-37 to exert an anti-inflammatory effect via its direct effect on cells (*Table 1*). This data indicates that IL-37 deficiency in BA contributes to inflammation in this pathology [65]. In a similar study, rIL-37 inhibited the production of another pro-inflammatory factor, TSLP, by epithelial cells isolated from the sputum of children with BA (*Table 1*) [66].

Summarizing the results of studies of the role of IL-37 in the pathogenesis of AD, we can state that this cytokine has pronounced anti-inflammatory properties, which are realized via its direct action on eosinophils, T cells, and epithelial cells. Most studies have demonstrated a decrease in both the systemic and local activities of IL-37 in such ADs as aBA and AR. Apparently, the low activity of IL-37 contributes to a more severe course of the Th2-mediated pathology. However, different results were obtained in a number of studies: for example, an increase in the systemic and local expressions of IL-37 was shown in AtD. A possible explanation for this difference might be related to the specificity of the AtD pathogenesis, in which Th2 cells play a crucial role at the early stage of the disease, while Th1 cells are involved in the late stage [67]. In addition, an increase in the systemic and local activities of IL-37 was also shown in nBA. Bronchial asthma is a heterogeneous disease; it can develop not only via the pro-allergic Th2-dependent pathway, which is associated with the infiltration of eosinophils in the lung tissue, but also via the Th17-dependent pathway, when other pro-inflammatory cells, namely neutrophils, are detected in the lungs. Considering the heterogeneity of the BA pathogenesis, it was suggested that IL-37 might play a different role in different BA endotypes. This assumption was confirmed by Raedler et al. [64], who observed a decrease in IL-37 production in children with aBA, while IL-37 expression was increased in nBA.

Such contradictory data on the changes in IL-37 activity indicate the heterogeneity of the molecular mechanisms of ADs. It is possible that the inclusion of patients with a more accurate phenotyping in such studies will allow us to better understand the biological role played by this interleukin.

Animal studies

Studies on laboratory animals allow a more detailed evaluation of the biological role of a factor, since there is a wider range of instrumental methods of molecular biology that are not available in clinical practice. Such methodological tools include the use of neutralizing monoclonal antibodies, generation of knockout mice, and the use of rIL-37.

It is known that mice lack the gene encoding for IL-37. However, a receptor complex capable of activating the intracellular signal upon interaction with human IL-37 is localized on their cell surface. In one of the first such studies [48], the effect of rIL-37 obtained in *E. coli* cells was studied using a model of pulmonary aspergillosis in mice. Mice were subjected to sensitization with *Aspergillus fumigatus* fungus parenterally with further intranasal administration of the same pathogen. A few hours prior to intranasal provocation, mice were injected intraperitoneally with rIL-37 in a wide range of doses, from 1000 to 1 ng/mouse. IL-37 at doses of 1000 and 100 ng/mouse prevented lung tissue damage, which manifested itself in suppressed lung infiltration by neutrophils, Th2 and Th17 cells, and in reduced bronchial remodeling, such as peribronchial collagen deposition and metaplasia of bronchial epithelium. IL-37 was shown to reduce the level of the pro-inflammatory cytokines IL-1β, IL-6, and IL-17A in lung tissue and to activate IL-10 [48]. Intranasal administration of recombinant human IL-37 at a dose of 1 μg/mouse decreased the level of the pro-inflammatory cytokines IL-6, IL-12, IL-4, IL-5, and IL-13 in the

Table 2. The role of IL-37 in allergic diseases. Animal studies

Animal model/species	Experimental protocol	Result	Reference
Pulmonary aspergillosis. C57BL/6 mice.	Intraperitoneal administration of rIL-37 at doses of 1000, 100, 10 and 1 ng/mouse prior to infection.	Decrease in the number of neutrophils in the BAL. Suppression of NLRP inflammasome in the lungs. Decreased IL-1β, IL-6, and IL-17A in the lung tissue. Activation of IL-10 in the lung tissue. Reduced signs of bronchial remodeling (collagenosis of lung tissue and metaplasia of bronchial epithelium). Suppression of lung infiltration with Th2/Th17 cells.	[48]
OVA-induced BA. C57BL/6 mice.	Intranasal administration of rIL-37 at a dose of 1 μg/mouse 1 day prior to aerosol administration of OVA.	Decreased eosinophil count in BAL and lung tissue. Suppression of bronchial epithelium hyperplasia, mucus production and bronchial hyperreactivity. Suppression of pro-inflammatory cytokines in BAL: IL-6, IL-12, IL-4, IL-5, and IL-13.	[50]
HDM-induced AR. BALB/c mice.	Intranasal administration of rIL-37 at a dose of 1 μg/mouse in combination with nasal provocation with HDM.	Threefold reduction in nasal hyperreactivity. Decreased levels of allergen-specific antibodies of the IgE class. Suppression of eosinophilic infiltration to the mucosa of the nasal cavity. Suppression of the pro-inflammatory cytokines IL-4, IL-5, IL-13 and IL-17 in the nasal mucosa and activation of regulatory IL-10.	[70]
OVA-induced BA. BALB/c mice.	Intranasal administration of rIL-37 at a dose of 1 μg/mouse in combination with allergen provocation.	Decreased bronchial hyperreactivity and lung infiltration by pro-inflammatory cells (lymphocytes, neutrophils, and eosinophils). Inhibition of IL-4, IL-6, and IL-13 in lung tissue. Decrease in proliferation and migration of respiratory smooth muscle cells and epithelial-mesenchymal transition.	[68]
HDM-induced BA. BALB/c mice.	Intranasal administration of rIL-37 at a dose of 0.2 μg/mouse combined with sensitization or nasal provocation with HDM.	Decrease in the number of eosinophils in BAL and lung tissue, decreased bronchial hyperreactivity. IL-37 suppressed IL-4/13-induced production of CCL11 fibroblasts and respiratory smooth muscle cells.	[69]

Note: rIL-37 – recombinant interleukin-37; BAL – bronchoalveolar lavage; BA – bronchial asthma; OVA – ovalbumin allergen; HDM – house dust mite allergen.

BAL of OVA-induced asthmatic mice. Furthermore, a decrease in the level of these interleukins resulted in attenuated symptoms of experimental BA: there was a significant reduction in lungs eosinophilia, signs of bronchial remodeling, as well as bronchial hyperreactivity. The biological effect of IL-37 in this BA model could be due to its ability to competitively bind the IL-18Rα receptor for the pro-inflammatory IL-18. However, further experiments on animals with gene knockout of receptors IL-18Rα and SIGIRR demonstrated a loss of the positive effects of IL-37 upon inactivation of the abovementioned receptor chains. This suggests that IL-37 does not only act as a competitive inhibitor of IL-18 but also activates its own anti-inflammatory signals [50]. Similar results were obtained in a similar OVA-induced BA model in mice [68]. Intranasal administration of IL-37 at a dose of 1 μg significantly attenuated the manifestations of BA in mice. In particular, there was a decrease in bronchial hyperreactivity and pneumonia, which is ascribed to a suppression of the pro-inflammatory Th2 cytokines IL-4, IL-6, and IL-13 and activation of the Th1 cytokine IFNg (*Table 2*) [68].

A more detailed study of the molecular and cellular mechanisms of the anti-inflammatory effect of IL-37 was conducted by Lv J. et al. [69] in a mouse model of BA induced by a house dust mite (HDM) allergen. Unlike in the case of OVA-induced BA, the HDM model is closer to the clinical case in humans, since a clinically significant allergen is used. Aerosol exposure without intraperitoneal sensitization was used for the administration of HDM to mice at the stages of sensitization and provocation. Intranasal administration of rIL-37 at a dose of 0.2 μg/mouse did not suppress BA symptoms, while administration of IL-37 at the provocation stage significantly attenuated disease manifestations, such as eosinophilic pneumonia and bronchial hyperreactivity. It is noted that, unlike in the study by Lunding et al. [50], IL-37 did not affect the differentiation of Th2 cells in the lungs and did not suppress the production of IL-4, IL-5, IL-13, and IL-17A. Moreover, no IL-37 effect on the production of IgE antibodies was detected. These results indicate that IL-37 is incapable of inhibiting T cell activation. However, despite high levels of Th2 cytokines, a significant suppression of the CCL11

chemokine was detected after intranasal administration of IL-37. In BA, this chemokine is responsible for the recruiting of eosinophils to the site of the inflammation, the lungs, which explains the ability of IL-37 to suppress eosinophilia in the lungs. The main sources of CCL11 in the lungs are fibroblasts and respiratory smooth muscle cells. It is noteworthy that, in contrast to the studies *in vivo*, in *in vitro* experiments, IL-37 did not inhibit CCL11 production by these cells. This is due to the fact that the receptor for IL-37 is poorly presented on these cells; therefore, they are insensitive to IL-37-mediated signals. Apparently, IL-37 exerts an indirect effect on CCL11 production by fibroblasts and respiratory smooth muscle cells. The largest number of the receptor copies was detected on the cells of the tracheobronchial epithelium. In order to detect such indirect effects *in vitro*, the cells of the tracheobronchial epithelium were treated with IL-37 and then cultured together with fibroblasts or smooth muscle cells in the presence of CCL11 inducers (IL-4 and IL-13). This experiment allowed researchers to establish a strong inhibition of CCL11 production by fibroblasts and smooth muscle cells in response to the treatment of tracheobronchial epithelial cells with IL-37. This indicates the indirect effect of IL-37 on IL-4/IL-13-mediated production of CCL11, which requires intercellular contact between these types of cells. Thus, using a mouse model of aBA, Lv J. et al. [69] confirmed the positive effects of IL-37 and shed light on the molecular and cellular mechanisms of its anti-inflammatory activity. Exogenous IL-37 activates tracheobronchial epithelial cells, which, upon contact with fibroblasts and respiratory smooth muscle cells, inhibit the production of CCL11 chemokine by these cells, which ultimately leads to a decrease in eosinophilic pneumonia and restoration of the respiratory function of the lungs [69].

A similar anti-inflammatory effect of IL-37 was revealed in a mouse model of AR. Kim et al. [70] induced AR in mice by intraperitoneal injection of the HDM allergen, followed by intranasal provocation with the same allergen. As a result, the animals developed AR signs: an increased level of IgE, eosinophil infiltration of the nasal mucosa, and nasal hyperreactivity, which manifested itself in an increased frequency of sneezing. Intranasal administration of rIL-37 (1 μg/mouse) in combination with allergen provocation resulted in mitigated symptoms of the pathology. Apparently, the attenuation of AR manifestations in mice is associated with the ability of IL-37 to suppress the activity of the pro-inflammatory cytokines IL-4, IL-5, IL-13, and IL-17 and activate regulatory IL-10 (*Table 2*) [70].

CONCLUSION

Almost 20 years have passed since the discovery of IL-37. During this time, a large body experimental evidence of the anti-inflammatory properties of this cytokine has been accumulated. Analysis of the data on the role of IL-37 in ADs confirmed the unique function of IL-37 as an anti-inflammatory agent, which is atypical of other representatives of the IL-1 family. The results achieved in studies using both clinical material obtained from AD patients and mouse AD models showed that activation of IL-37 leads to the suppression of pro-inflammatory Th2 cytokines (IL-4, IL-5 and IL-13), Th17 cytokine (IL-17A), chemokines, and transcription factors (CLL11, STAT3, NF-κB, etc.). Suppression of these cytokines and factors ultimately leads to an attenuated inflammation, which manifests itself as a decrease in the degree of infiltration of the target organs (nasal mucosa in AR and lung tissue in BA) by pro-inflammatory cells (neutrophils and eosinophils) and a decrease in pulmonary hyperreactivity. The extracellular form of IL-37 exerts its biological effect through the receptor complex consisting of IL-18Rα and IL-1R8 chains; the intracellular form of IL-37 is able to translocate into the nucleus and inhibit the expression of pro-inflammatory genes. The revealed positive effects of IL-37 allow us to offer it for consideration as a potential anti-inflammatory agent for cytokine therapy of ADs. ●

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The Role of Heritable Tumors in Evolution of Development: a New Theory of *Carcino-evo-devo*

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ABSTRACT **The hypothesis of evolution by tumor neofunctionalization (the "main hypothesis") describes the possible role of hereditary tumors in evolution. The present article examines the relationship of the main hypothesis to other biological theories. As shown in this paper, the main hypothesis does not contradict to the existing biological theories, but fills the lacunas between them and explains some unexplained (or not completely understood) questions. Common features of embryonic development and tumorigenesis are described by several recognized theories. Similarities between normal development and tumorigenesis suggest that tumors could participate in the evolution of ontogenesis and in the origin of new cell types, tissues and organs. A wide spectrum of non-trivial explanations and non-trivial predictions in different fields of biology, suggested by the main hypothesis, is an indication of its fundamental nature and the potential to become a new biological theory, a theory of the role of tumors in evolution of development, or *carcino-evo-devo*.**
KEYWORDS **heritable tumors, embryonic development,** *evo-devo, carcino-evo-devo*.
ABBREVIATIONS **AIS – adaptive immune system; CRC – Core Regulatory Complex; C/T antigens – cancer/testis antigens; RAG – recombination-activating genes; TCR genes – T-cell receptor genes; TEBs – Terminal end buds; WGDs – whole-genome duplications.**

INTRODUCTION

Multicellular organisms needed a continuous source of additional cell masses with high biosynthetic and morphogenetic potential as a material for progressive evolution, especially in the line Deuterostomia – Chordata – Vertebrata. The problem of the origin of such cell masses has not been resolved. It is clear that stem cells should participate in this process, but adult and embryonic stem cells are regulated by functional feedback loops and cannot provide considerable amounts of excessive cells. Physiological proliferative processes existing in normal organisms could not provide sizeable extra cell masses because such proliferative processes are functional and are regulated with feedback loops.

On the other hand, tumors and tumor stem cells are not (or less) regulated and potentially could provide the evolving multicellular organisms with unlimited amounts of extra cells with high biosynthetic and morphogenetic potential.

The hypothesis of evolution by tumor neofunctionalization (below I will call it "the main hypothesis") suggests that the possible role of hereditary tumors in evolution might consist in providing extra cell masses for the expression of evolutionarily novel genes and gene combinations, and for the origin of new cell types, tissues and organs [1]. The main hypothesis formulated several non-trivial predictions; some of them have already received experimental confirmation [1–3]. In the present article, I will examine the relationship of the main hypothesis to other biological theories.

NON-TRIVIAL EXPLANATIONS OF THE MAIN HYPOTHESIS AND ITS RELATIONSHIP TO OTHER BIOLOGICAL THEORIES

The main hypothesis does not contradict the existing biological theories but fills the lacunas between them and explains some unexplained (or not completely understood) questions (*Fig. 1*). Explanation of the phenomena unexplained on not completely explained by the pre-existing theories, together with non-trivial predictions, is the fundamental demand to the new scientific theory.

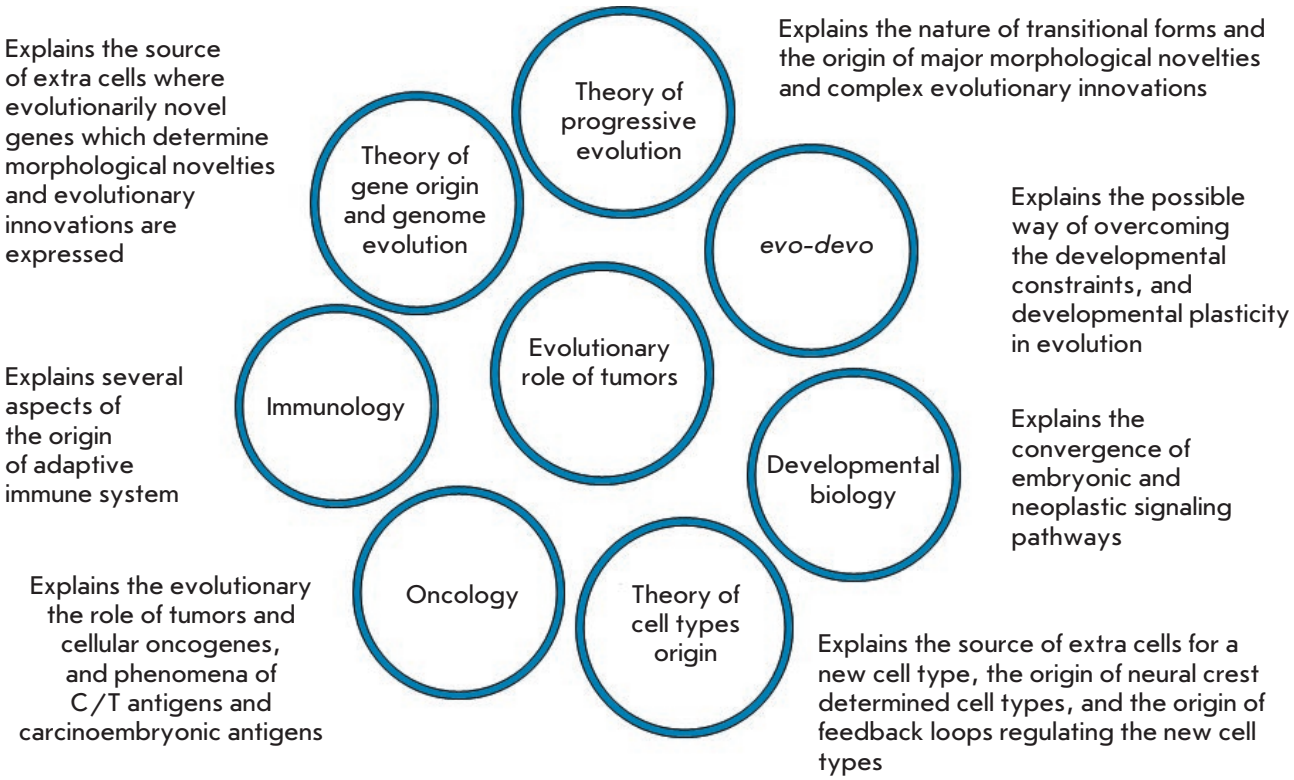

Fig.1. Non-trivial explanations of the main hypothesis and its relationships to other biological theories

In theory of progressive evolution, the main hypothesis explains the nature of transitional forms, and the origins of complexity. It explains the possible mechanism of the origin of major morphological novelties such as evolutionarily new organs and complex evolutionary innovations such as the adaptive immune system.

In *evo-devo*, the main hypothesis explicates the possible way to overcome developmental constraints, and the mechanism of developmental plasticity in progressive evolution. It also suggests the neoplastic mode of evolution of ontogenesis.

In developmental biology, this hypothesis offers an explanation for the convergence of embryonic and neoplastic signaling pathways.

In the theory of cell types origin, it explains the source of extra cells for a new cell type, the origin of neural crest determined cell types, and the origin of feedback loops regulating the new cell types. The role of oncogenes, tumor suppressor genes, and novel genes and gene combinations in the origin of new cell types is also explained.

In the theory of gene origin and genome evolution, it offers an explanation for the source of extra cells where the evolutionarily novel genes determining the morphological novelties and evolutionary innovations are expressed.

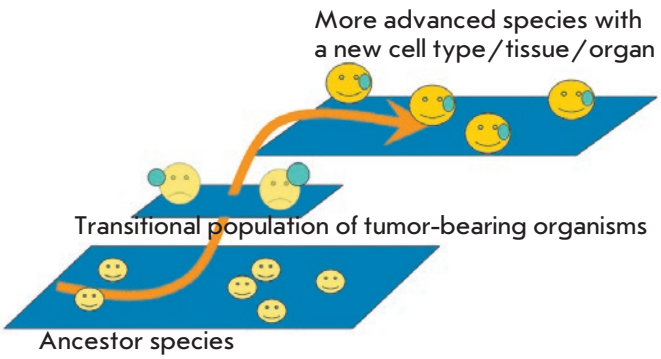

Fig.2. Population of tumor-bearing organisms with heritable tumors as transition between established species of organisms at different levels of complexity. Modified from [1], with permission

In oncology, it construes the evolutionary role of tumors and cellular oncogenes, phenomena of cancer/testis antigens and carcinoembryonic antigens, etc.

In immunology, the main hypothesis explains several aspects of the origin of the adaptive immune system.

Non-trivial explanations offered by the main hypothesis were well accepted by representatives of

corresponding branches of biological science during a number of my presentations to different audiences.

The explanations being most important for the present paper are those of the problem of transitional forms in progressive evolution, the mechanisms of overpassing the developmental constraints, and the origins of complexity and major evolutionary innovations and morphological novelties. I will now examine them in more detail.

TUMOR-BEARING ORGANISMS AS TRANSITIONAL FORMS IN PROGRESSIVE EVOLUTION

According to the main hypothesis, tumor-bearing organisms with hereditary tumors could represent relatively unstable transitional forms that linked phyla with different levels of complexity (*Fig. 2*). Their stabilization was achieved through the expression of novel genes and gene combinations, and the origin of new functions and functional regulatory feedbacks. As we know from physics, the unstable elementary particles (or some unstable transuranium elements) are difficult to observe. In chemistry, the unstable highly reactive transitionary molecules are difficult to observe as well. Similarly, it is difficult to find tumor-like transitionary structures in paleontological records. A.N. Severtsov has already pointed out that this is because periods of complexity growth were rare and of short-duration [4]. I would add that transitional populations of tumor-bearing organisms could be small, and tumors were soft and not well preserved.

The examples of transitional populations of tumor-bearers are tumor-bearing voles and Xiphophorus fishes with melanomas which were discussed in my book [1]. During certain periods of phylogenesis, differentiation of tumor cells in different organisms of these populations could be frequent enough to result in populations of organisms with a new cell type. The organisms with the new cell type would then be selected for their fitness and competitive abilities. Examples of such selection were discussed in the book [1]. New cell types could participate in the formation of new tissues and organs.

TUMORS AS THE GENERAL MECHANISM TO OVERCOME DEVELOPMENTAL CONSTRAINTS

Developmental constraints are defined as limitations on phenotypic variability caused by structural and other features of the developmental system [5]. Restraints on variant phenotype production include physical, morphological, genetic and phyletic constraints [6, 7]. Developmental constraints seriously restrict evolutionary changes in animals [8]. The body plan at certain stages is so embedded in the organism's development that any modification may be lethal [9].

But despite the existence of developmental constraints, morphological novelties have been realized in progressive evolution. The mechanisms through which such transitions happen are not completely understood. The existing hypotheses, e.g. the hypothesis of facilitated variation [10], do not explain how it happened.

My main hypothesis explains that tumors may represent a general way to overcome the developmental constraints in evolutionary perspective, although tumors are connected with present-day pathological conditions. The concept of tumors as engines that search for all possible molecular combinations and innovations by cancellation of major restraints and incompatibilities, formulated in my book [1], helps to understand the possible mechanisms of overpassing the developmental constraints.

Tumors as search engines work in the space of possibilities that have not realized themselves yet. The concept of possibility space is being developed in scientific literature [11–13]. The concepts of morphological, phenotypic and genotype space were also used [5, 7]. The "tumors as a search engine" idea gravitates towards the chaos theory and the complexity theory, which looks for the source of complexity in evolution [14, 15].

The molecular basis of search engine is the global hypomethylation of DNA (discussed in the book), increased global transcription activity [16], and dysregulated transcriptional programs ("transcriptional addiction" [17]) in tumor cells. Gene competition and antagonistic relations between the genes [18, 19] may change significantly in tumor cells due to additional space and resources there. As a result, many unusual genes not expressed in normal cells, including evolutionarily novel genes, are expressed in tumors [2]. Thus, developmental constraints and the compatibility/incompatibility issues are completely or partially abandoned, and unrealized developmental potential is fulfilled.

For morphological innovations, not only novel genes and gene combinations are necessary, but also additional cell masses. According to the main hypothesis, valuable coincidences of unusual gene expression and cell proliferation, which may incidentally happen in tumors, are frozen by natural selection ("frozen accidents" discussed in the book [1]), and lead to the origin of morphological novelties.

Thus, tumors may represent a general mechanism of evolvability of complex organisms and/or developmental plasticity in evolution (see [10, 20] for evolvability and [21, 22] for developmental plasticity). In particular, tumors may facilitate new combinations of "core components" of J. Gerhart and M. Kirschner [10], and/or core regulatory genes of G. Wagner [23], as well as expression of evolutionarily novel genes. On the contra-

ry, anti-cancer selection may be the source of developmental and evolutionary constraints [24].

TUMORS AND THE ORIGIN OF NEW CELL TYPES

The number of cell types in Metazoa increased during evolution and may be a measure of their complexity [19, 25]. That is why scientists were looking for the mechanisms of the origin and evolution of new cell types.

The main hypothesis suggests that evolutionarily novel genes and gene combinations are expressed in tumor cells and give rise to a new function and a new regulatory feedback loop. The new function is selected for its enhancement, which also enhances the regulatory feedback. This leads to differentiation of tumor cells in the novel direction and the origin of a new cell type. The new cell type is inherited due to the mechanisms similar to those in preexisting cell type (see discussion in the book [1]). The evolutionary role of cellular oncogenes might consist in sustaining a definite level of autonomous proliferative processes in evolving populations of multicellular organisms and in promoting the expression of evolutionarily new genes. After the origin of a new cell type, the corresponding oncogene should have turned into a cell type-specific regulator of cell division and gene expression [26, 27]. Non-trivial predictions that follow from such a scenario were confirmed in my lab and discussed in the previous paper [3].

The "sister-cell-type model" suggests that novel cell types arise as pairs (sister cell types) from an ancestral cell type by sub-specialization at the last stages of differentiation [28]. This hypothesis works best in the case of terminally differentiated cells but has difficulties in explaining developmental cell types like neural crest-derived cells [23]. In later publication, the authors formulated the "serial sister cell type" hypothesis: "It is now well established that early animal evolution involved the repeated subdivision of the animal body into distinct regions. We propose that these regionalization events also led to the duplication and subsequent diversification of at least one of the cell types that populated that region. This process produced an iterated series of topographically separate sister cell types that we refer to as serial sister cell types. It is plausible that these cell type duplication events also led to the evolution of serial sister stem cells, as virtually all animal cell types co-occurring in one region develop from asymmetrically dividing, multipotent stem cell-like cells" [29]. This hypothesis may also be called "evolution by cell type duplication". It does not contradict my main hypothesis but even converges with it. The pre-existing cell type is under control of natural selection. It is also under regulatory control in the organism. The duplication of a cell type means the origin of extra cells, which escape selection and regulatory control, like in case of gene duplication. The uncontrolled extra cell mass is a neoplasm by definition, which brings the serial sister cell type hypothesis close to the hypothesis of evolution by tumor neofunctionalization.

In tumors, the combination of genes expressed in unrelated or distantly related cell types may be transcribed. In this case, the new cell type will not be in hierarchical relationships predicted by sister-cell-type model. The origin of many cell types from neural crest may be explained in this way. Evolutionarily novel genes expressed in tumors may become targets for core regulatory genes (see Core Regulatory Complex, CRC, [23]) of pre-existing cell types. In such cases, the hierarchical relationship may be conserved.

TUMORS AND THE ORIGIN OF MAJOR EVOLUTIONARY MORPHOLOGICAL NOVELTIES AND COMPLEX EVOLUTIONARY INNOVATIONS

In my book [1], I presented examples when expression of evolutionarily novel genes in tumors was connected with the origin of new organs (placenta in Mammalia and root nodules in Legumes) and new cell types (macromelanophores in Xiphophorus fishes). The mammary gland and the adaptive immune system are new examples of the possible connection with tumors during the origin of new organs and complex evolutionary innovations.

The mammary gland, an evolutionarily novel organ, may represent a neomorphic hybrid, a mosaic organ whose evolution involved the incorporation of characteristics already encoded in the genome but expressed differently by separate populations of skin glands [30]. The mammary gland coopted signaling pathways and genes for secretory products from earlier integumentary structures [31, 32]. The ancestral tumor could be a mechanism for expression of evolutionarily novel gene combinations in breast tissue, as discussed above. A recent study of evolutionarily novel genes in placental mammals also discovered several novel genes expressed in breast tissue [33].

The adaptive immune system (AIS) originated in jawed fishes and represents a major innovation in evolution of complexity [34]. Two macroevolutionary events – the invasion of the RAG transposon and two whole-genome duplications (WGDs) – are believed to determine the relatively rapid ("big bang") emergence of the AIS in jawed vertebrates [35]. But the origin of clonal expansion and clonal selection of lymphocytes, as well as of different immune cell types and organs, is hard to imagine with only the RAG transposon and WGDs hypotheses. The AIS requires large populations of cells for clonal selection and clonal expansion, and these populations of cells could be provided by an-

cestral tumors. The computer-like search in ancestral tumors for all possible combinations of molecular and cellular events – the search engine – could be a mechanism of the origin of such complicated evolutionary innovation as AIS, with its combinatorial joining of V, D and J elements.

The number of potential Ig/TCR V region is huge, far exceeding the number of available lymphocytes. The expressed repertoire was studied by variety of methods. The conclusion is that the antibody diversity in non-mammalian vertebrates is low, as opposed to mammals, which make the most of this potential [36, 37].

In frogs, the organization and usage of Ig gene loci is similar to that in mammals, but the diversity of antibodies is much smaller, several orders of magnitude less than in mammals. This is due to major difference in cell number and lymphoid organ architecture. There are few cells in the differentiating immune system of frogs, not enough to realize the potential diversity of the V_H locus. Tadpoles have less efficient immune response, i.e. skin graft rejection, and lower Ig and TCR diversity. Simpler organization of the lymphoid frog organs, without lymph nodes or germinal centers, results in poor affinity maturation [36–39].

Thus, cell number limitation represented a serious restriction for the evolution of AIS. Coevolution of lymphoid cell compartment with Ig gene loci might involve tumors. Tumors might provide not only combinatorial possibilities, but also the additional cells necessary for clonal expansion and selection, and for building the structure of lymphoid organs. Indeed, true lymphoid tumors have been discovered in frogs [40, 41].

Without tumors, the origin of such combinatorial innovations as mammary gland and AIS is not possible, because of developmental constraints in established organs and ontogenies.

According to the main hypothesis, the origin of a major evolutionary morphological novelty or complex evolutionary innovation cannot happen by saltatory manner, because it needs the coincidence of too many independent events at different levels of organization. The mechanism for saltatory origin of complex structures does not exist. That is why the unstable transitionary state with search engine capabilities – the tumor – is necessary.

TUMOR-LIKE PROPERTIES OF EVOLUTIONARILY NEW ORGANS AS AN INDICATION OF THEIR ORIGIN FROM TUMORS

Parallels between the normal and neoplastic development result in solid tumors with many features of normal organs (atypical tumor organs, [42]), on one side, and some normal organs with features of tumors, on the other side.

Normal organs that have features of tumors may be called tumor-like organs. In my book [1], I examined such tumor-like organ, the placenta. Many tumor-like features of placenta were reviewed, and relation of its origin to recurrent germline retrovirus infection was analyzed. The conclusion was drawn that the placenta may be considered a regulated tumor-like organ. After publication of the book, several reviews have been published that basically confirm this point of view [43–45]. Thus, the placenta is a tumor-like organ, first identified in the literature as such.

The developing mammary gland demonstrates many of the properties associated with tumors, e.g. invasion. Terminal end buds (TEBs), a rapidly proliferating mass of epithelial cells, invades into stromal tissue much like a solid tumor [46]. The mammary gland is an evolutionarily young organ. The evolutionary novelty of the mammary gland may be a reason for higher incidence of breast cancer as compared to cancer incidences in evolutionarily older organs [47].

Like the mammary gland, the prostate gland demonstrates correlation of evolutionary novelty with the highest incidence of cancer [47]. Genes differentially expressed in prostate cancer progression overlap with the genes expressed at the earliest stages of prostate development [48]. This indicates the tumor-like nature of the prostate gland.

The common features of tumor-like organs (placenta, mammary gland and prostate) is the presence of the regulated invasion stage in their organogenesis, and the young evolutionary age of these organs. The mammary gland and prostate also demonstrate the highest incidence of cancer. The main hypothesis suggests that atypical tumor organs can give rise to normal organs in evolution, with tumor-like organs as transitional phase.

TUMORS AND THE GROWTH OF COMPLEXITY

According to the main hypothesis, tumors may be not a consequence, but a prerequisite of the growth of complexity, by providing the building material – extra cells – for expression of evolutionarily novel genes and gene combinations. As it is evident from the above discussion of the origin and evolution of the adaptive immune system (AIS), the access to additional cells necessary for this evolution was not a trivial problem, e.g. for amphibians with their available cell types and stem cells. This problem was resolved in the line Amphibia – Mammalia, with the help of hereditary tumors and tumor stem cells, as suggested by the main hypothesis. With the origin of new functions, atypical tumor-like organs could be stabilized by functional feedbacks, accumulate larger proportion of cells differentiated in new directions and become new organs. The origin of complex organs such as the mammary gland and com-

plex systems such as the AIS may be explained with the help of the "tumors as a search engine" concept discussed above: tumors search for unrealized possibilities in the gene expression possibility space and in the morphological possibility space. Thus, the "tumors as a search engine" concept suggests that chaotic neoplastic development may be a source of complexity in evolution, similarly to suggestion of the dynamical systems theory [14, 15].

TUMORS AND EMBRYONIC DEVELOPMENT

Normal embryonic development and tumorigenesis have many common features, e.g. invasiveness and cell migration, expression of certain genes and signaling pathways, epithelial-mesenchymal transition, etc.

These commonalities are usually explained by re-activation or deregulation of embryonic signaling pathways in tumors [48–52]. On the other hand, many signaling pathways connected with normal development were first discovered as protooncogenes and tumor-suppressor genes. The terminology of "convergence" of embryonic and tumor signaling pathways is also used (e.g. [53]).

Common features of embryonic development and tumorigenesis are described by several recognized theories. The "embryonal rest" or "embryonic remnants" theory of cancer, formulated over a hundred years ago, suggested that tumors may originate from embryonic cells [54, 55]. This theory was finally proved last year by the results of single-cell transcriptome analysis: the transcriptomes of childhood Wilms tumor cells matched to those of specific fetal cell types [56].

The loss of differentiated functions (e.g. due to mutations) causes tumors. On the other hand, tumor cells can differentiate with the loss of malignancy. This and similar evidence constituted the basis of the differentiation theory of cancer. The more recent stem cell theory of cancer interconnects cancer, cell differentiation, and embryonic development.

The similarities between normal development and tumorigenesis suggest that tumors could participate in the evolution of ontogenesis and in the origin of new cell types, tissues and organs. If true, it explains all the above similarities.

TUMORS AND *EVO-DEVO*

A.N. Severtsov defined the following major ways of the evolution of ontogenesis, or modes of phylembryogenesis, as he called them: archallaxis (the change in original anlages), when changes were introduced at the earliest stages of organ embryonic development, or *de novo* formation of evolutionarily new organ occurred; deviation, when the changes were introduced in the intermediary stages of organ embryogenesis; and anaboly, when changes were added at terminal stages of organ ontogenesis, i.e. addition of final stages of morphogenesis [57–59] (see also [60] for review].

From the discussion above it is evident that evolutionarily novel tumor-like organs (placenta, mammary gland, and prostate) represent examples of true archallaxis. The neural crest with its tumor-like cells, recapitulating those of prototype tumor-like formations in early vertebrates [1], may be another example of archallaxis (some researchers consider the neural crest to be a fourth germ layer [61]). Thus, tumors may be a mechanism of the origin of phylogenetically new formations. A.N. Severtsov wrote that unregulated embryonic changes at the earliest stages of organ development produce material for archallaxis, and archallaxis is the most rapid mode of evolution of development [59]. This agrees with the main hypothesis. It is interesting that A.N. Severtsov used the term "new formations," like oncologists did, and claimed that phylogenetic new formations originated by the archallaxis mode.

The origin of the neocortex in humans, related to tumor-like processes as discussed in my book [1], may be connected to deviation and/or anaboly modes. An interesting example of deviation was discussed by A.N. Severtsov in his classical "Morphological Laws of Evolution" [59]: the evolution of nasal pits in Osteichthyes. In *Belone acus*, there is a serious deviation in development of its olfactory pit, which consists in formation of the large mushroom-like outgrowth at the bottom of the pit. The development of this outgrowth resembles tumor growth.

Embryonic, fetal, infantile, and adult tumors, the possible candidates for playing a role in evolution, could participate in evolution of ontogenesis at its different stages. This assumption predicts recapitulations

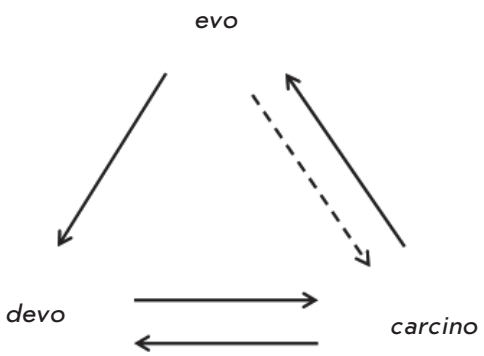

Fig. 3. *Carcino-evo-devo* diagram: *devo* – normal ontogenies, *carcino* – ontogenies with neoplastic development, *evo* – progressive evolution of ontogenies. Arrows indicate participation in the corresponding process, or essential connections

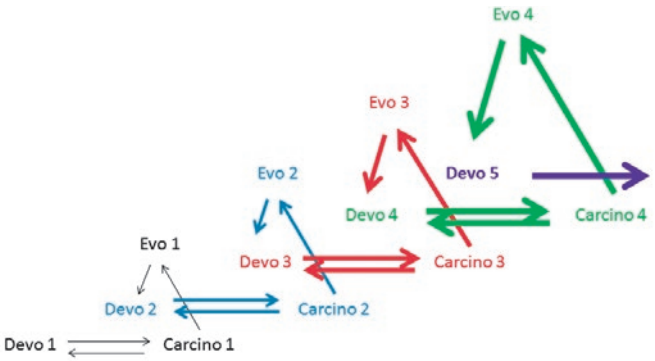

Fig. 4. *Carcino-evo-devo* diagrams showing four successive steps in progressive evolution of ontogenesis with tumor participation. Devo 2, Devo 3, Devo 4 and Devo 5 – ontogenies with evolutionarily new progressive traits

of some tumor features in the most recently evolved organs. Indeed, evolutionarily young organs (placenta, mammary gland, and prostate) recapitulate features of tumors such as invasiveness, the capability of indefinite growth (prostate), the high rates of cancer incidence (mammary gland and prostate), etc.

Thus, tumors may participate in evolution of ontogenesis. Participation of hereditary tumors in evolution of ontogenesis and in the origin of major evolutionary morphological novelties, or phylogenetic new formations, may become an integral part of evolutionary developmental biology, and may be called *carcino-evo-devo*.

CARCINO-EVO-DEVO, A NEW THEORY OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY

A broad spectrum of non-trivial explanations and non-trivial predictions in different fields of biology, suggested by the main hypothesis, is an indication of its fundamental nature and the potential to become a new biological theory, a theory of the role of hereditary tumors in evolution of development, or *carcino-evo-devo*. Evidently, this abbreviation stems from two other abbreviations – *carcinoembryonic* and *evo-devo* – related to two big areas or research that have brought to formulation of the main hypothesis.

The interrelationships between the processes of progressive evolution, normal and neoplastic development may be presented as a diagram (*Fig. 3*). This diagram represents the relationships between normal ontogenesis and neoplastic development (*devo* ↔ *carcino*); participation of hereditary tumors in progressive evolution (*carcino* → *evo*); and generation of more complex ontogenies in the course of progressive evolution (*evo* → *devo*). This diagram shows that normal ontogenies do not directly participate in progressive evolution (i.e., the lack of *devo* → *evo* arrow), and evolution can influence neoplastic development (e.g. anti-cancer selection, dashed arrow between *evo* and *carcino*).

According to the *carcino-evo-devo* theory, tumor-bearing organisms participate in progressive evolution that generates new more complex ontogenies. In *Fig. 4*, four *carcino-evo-devo* diagrams show successive steps in progressive evolution of ontogenesis leading to the origin of different morphological novelties and complex evolutionary innovations, with participation of tumors.

The *carcino-evo-devo* diagram reminds the central dogma of molecular biology not only in its outward appearance. Like the central dogma, it contains a fundamental prohibition: a prohibition of saltatory origin of complex evolutionary innovations and morphological novelties directly from normal ontogenies. As I wrote above, the mechanisms of saltatory origin of complex structures do not exist. The *carcino-evo-devo* theory demands the necessity of transitionary intermediates with search engine capabilities, which I think are tumor-bearing organisms (*carcino*). I hope that the *carcino-evo-devo* diagram will cause discussion on what the transitionary intermediates should be, and on the number of arrows and their possible directions.

Thus, a new theory of the possible role of hereditary tumors in evolution – *carcino-evo-devo* – is being developed. This theory possesses a predictive power, explains many previously unexplained biological phenomena, accommodates a large amount of data, and has a potential of unifying several existing biological theories. It may become a new theory of evolutionary developmental biology. ●

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Surface Charge Mapping on Virions and Virus-Like Particles of Helical Plant Viruses

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ABSTRACT **Currently, the assembly of helical plant viruses is poorly understood. The viral assembly and infection may be affected by the charge distribution on the virion surface. However, only the total virion charge (isoelectric point) has been determined for most plant viruses. Here, we report on the first application of positively charged magnetic nanoparticles for mapping the surface charge distribution of helical plant viruses. The charge was demonstrated to be unevenly distributed on the surface of viruses belonging to different taxonomic groups, with the negative charge being predominantly located at one end of the virions. This charge distribution is mainly controlled by viral RNA.**
KEYWORDS **plant viruses, magnetic nanoparticles, surface charge mapping.**
ABBREVIATIONS **TMV – tobacco mosaic virus; PVX – potato virus X; AltMV – alternanthera mosaic virus; CP – coat protein; VLP – virus-like particle; vRNP – viral ribonucleoprotein; MN – micrococcal nuclease; MNP – magnetic nanoparticle; TEM – transmission electron microscopy.**

INTRODUCTION

Investigation of the physico-chemical characteristics of plant virus virions, including the charge distribution on their surface, may lead to a better understanding of the molecular mechanisms of infection development in the primary infected cell or during the movement of a transport form of RNA viruses (virions/vRNPs) to neighboring uninfected cells. To date, studies exist that describe the features of surface charge formation in icosahedral viruses [1–3]; isoelectric points of virions with various symmetry types have been determined [4, 5]. According to published data, the isoelectric points of most plant viruses fall within the range of 3.6 to 6.3. At neutral pH values, these viruses have total negative surface charges [4, 5]. However, the surface charge distribution of viral particles with a helical capsid remains largely unexplored. According to our preliminary data, the surface of some virions may be charged unevenly. The infection-induced translational activation of the RNA-containing plant virus genomic nucleic acid may also be associated with the surface charge distribution on the viral particle.

In this work, we propose a method for surface charge mapping in helical virions using fluidMAG-DEAE magnetic nanoparticles (MNPs). We studied the surface charge distribution in helical plant viruses from different taxonomic groups (tobacco mosaic virus (TMV) of the *Tobamovirus* genus and potato virus X (PVX) and alternanthera mosaic virus (AltMV) of the *Potexvirus* genus), as well as in virus-like particles (VLPs) and viral ribonucleoproteins (vRNPs) derived from virion components.

A suggestion of a relationship between either the surface charge distribution in plant viruses or the surface charge heterogeneity throughout the entire virion and the accessibility of encapsidated RNA for interaction with ribosomes and RNA packaging in the coat protein (CP) has not yet been discussed.

EXPERIMENTAL

Isolation of viruses, viral RNA, and coat proteins

PVX and TMV samples were isolated according to [6] and [7], respectively; AltMV was isolated according to [8]. RNA was isolated by a modified phenolic method [9]. PVX and AltMV coat proteins were obtained by salt deproteinization [10]. The TMV coat protein was obtained by the acetate method [11].

Preparation of TMV and AltMV CP (VLP) repolymers and PVX vRNPs

TMV and AltMV CP repolymers were prepared using the techniques described in [11, 12], respectively; PVX vRNPs were obtained according to [13].

Treatment of virions and virus-like particles with RNase A and micrococcal nuclease

Virions and vRNPs with a final concentration of 0.05 mg/mL were treated with RNase A at the ratio 1 μg of enzyme per 4 μg of the virus. Incubation was carried out for 30 min; the reaction was stopped by placing the samples in ice. Micrococcal nuclease (MN) (50 units of active enzyme per 1 μg of RNA) was added to the samples pre-treated with 100 mM $CaCl_2$. The reaction was stopped by adding 250 mM EGTA. tRNA was used as a co-precipitator during RNA isolation from nuclease-treated virions.

Ultrasonic treatment of TMV virions

TMV particles were sonicated using an Ultrasonic Processor homogenizer. The treatment was performed at a TMV concentration of 0.05 mg/mL, in ice for 60 s.

Preparation of virion/virus-like particle (VLP)–magnetic nanoparticle complexes

Virions/VLPs were incubated with fluidMAG-DEAE magnetic particles (Chemicell, Germany) in an aqueous solution at a final virion/VLP concentration of 0.05 mg/mL for 20 min.

Transmission electron microscopy and nanoparticle tracking analysis

Samples were adsorbed on copper grids and contrasted according to the procedure described in [14]. The analysis was performed using the JEOL JEM-1011 and JEOL JEM-1400 electron microscopes (JEOL, Japan) at 80 kV.

Samples in liquid were studied by nanoparticle tracking analysis using a NanoSight NS500 instrument and NanoSight NTA 2.3 software (NanoSight, UK). The particles' Brownian motion was recorded and processed using the following settings: 10 video recordings 60 seconds long each at a camera level of 14 and a detection threshold of 5. The mean hydrodynamic diameter and particle concentration are presented as a 95% confidence interval.

RESULTS AND DISCUSSION

Virion surface charges were mapped using fluidMAG-DEAE magnetic nanoparticles (MNPs) (Chemicell, Germany) with a specified hydrodynamic diameter of 50 nm. These MNPs consist of an iron oxide magnetic core and a shell composed of starch functionalized with diethylaminoethyl groups. Due to the positive charge of these groups, the MNPs can be used to map a negative charge on the surface of biological structures. The magnetite core enables detection of the MNP position in a complex with viral particles by transmission electron microscopy (TEM).

The mean MNP hydrodynamic diameter measured by nanoparticle tracking analysis was 72 ± 3 nm. According to the TEM data, individual MNPs were assembled into aggregates of 2 to 20 nanoparticles.

The assembly of TMV virion–MNP complexes was performed at a total negative charge of viral particles. The interaction between MNPs and TMV in liquid was studied by nanoparticle tracking analysis. The mean equivalent hydrodynamic diameter of TMV was 115 ± 3 nm. Addition of MNPs to the virus led to an increase of the mean diameter to 134 ± 8 nm at a constant particle concentration of $(1.6 \pm 0.1) \times 10^{14}$ and $(1.6 \pm 0.2) \times 10^{14}$ particles/ml, respectively, which indicated complex formation.

Analysis of TMV–MNP complexes by TEM showed that the magnetic particles effectively formed complexes with TMV bounding to only one end of the virion (*Fig. 1A*). The so-called "spider" complexes were also detected, which are a group of virions simultaneously interacting by one end with magnetic nanoparticles (*Fig. 1B*). No complexes of MNPs interacting simultaneously with two opposite TMV ends were detected.

This fact (MNPs interacting with only one end of TMV particles) attracted our attention. In further studies, we used a TMV preparation of 300 nm native particles "broken" by ultrasound (TMV^{US}). The mean length of these particles was 149 ± 83 nm.

TEM revealed that MNPs were bound strictly to one end of TMV^{US} fragments (*Fig. 2A*), like in the case of native virions (*Fig. 1A*). However, a certain amount of TMV^{US} particles did not interact with MNPs (*Fig. 2B*). If MNPs had interacted with the "broken" end of fragmented virions, TMV^{US}s, both ends of which are associated with MNPs, would statistically have occurred in the solution. However, this type of complexes was not detected.

Each TMV CP subunit is known to contain an RNA binding site that interacts with three nucleotides of the viral RNA. This CP–RNA interaction is observed when a guanine residue occurs in the third position of the binding site [15, 16]. Guanine residues are absent among the 69 first nucleotides of TMV RNA [17]. Therefore, the CP–RNA interaction is weak throughout the first 50–60 nucleotides, which may affect the surface charge distribution of the TMV virion.

To elucidate the role of individual virion components in the formation of an increased negative charge area at one end of the TMV virus particle, we prepared

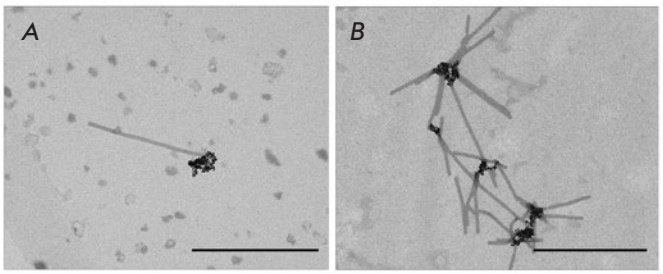

Fig. 1. MNPs bind to one end of the native TMV virion. Scale bars are 500 nm

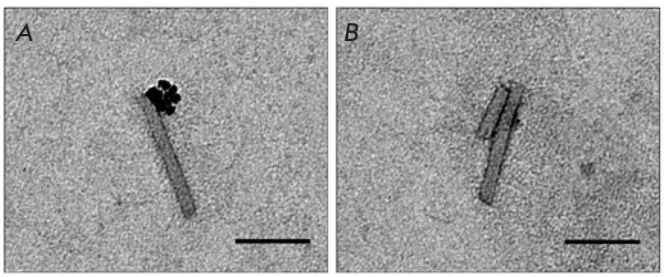

Fig. 2. MNPs form complexes with one end of ultrasound-treated TMV (TMV^{US}). Scale bars are 100 nm

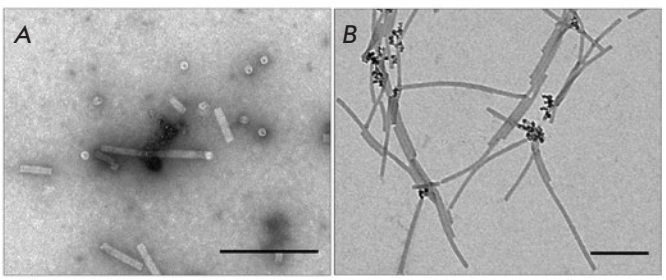

Fig. 3. MNPs lack specific affinity to the ends of TMV CP repolymers (*A*). Formation of native TMV–MNP complexes during preparation of TMV CP repolymers, control (*B*). Scale bars are 200 nm

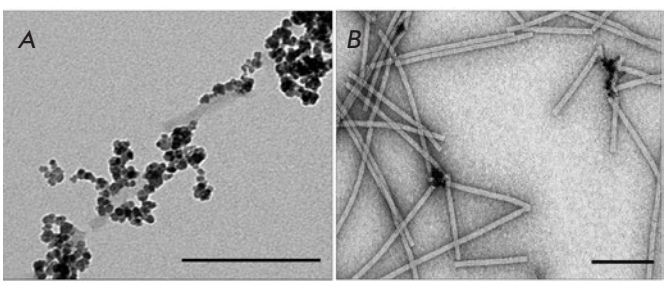

Fig. 4. Analysis of the interaction between MNPs and TMV virions treated with RNase A (*A*) and micrococcal nuclease (*B*). Scale bars are 200 nm

TMV CP repolymers (virus-like particles, VLPs) with a helical structure similar to that of the virion, but lacking RNA [18]. An analysis of the complexes produced upon incubation of TMV repolymers with MNPs showed that MNPs either did not interact with TMV VLPs or bound to the entire surface of the repolymers (*Fig. 3A*). No complexes between MNPs and TMV VLP ends were observed. Because the binding of repolymers to MNPs occurred at pH 5.6 (the condition for TMV repolymer assembly), the TMV–MNP complexes obtained under the same conditions were used as controls. At pH 5.6, MNPs were found to interact also with only one end of the TMV virions (*Fig. 3B*).

Therefore, the negative charge is uniformly distributed on the surface of VLPs obtained by polymerization of the TMV CP in the absence of RNA and is not localized at one of the ends, in contrast to the virion. RNA is likely to contribute significantly to the formation of an increased negative charge density area at one end of the native TMV.

To assess the contribution of viral RNA to the surface charge formation, TMV virions were treated with two nucleases: RNase A and micrococcal nuclease.

The ability of RNases to affect the TMV virion RNA was analyzed in preliminary experiments. Virions were treated with the nucleases. Then, RNA was isolated and analyzed by electrophoresis in 1% agarose gel. RNase A hydrolyzed the nucleic acid to fragments with an electrophoretic mobility comparable to that of the tRNA used as a co-precipitator or less (data not shown).

Micrococcal nuclease, as shown previously [19], does not hydrolyze RNA in the virion. However, it cannot be ruled out that at one of the virion ends, where the increased negative charge density area is located, the CP packaging is less dense, or the interaction between CPs and RNA is weaker throughout the first 50–60 nucleotides [17, 20], and small TMV RNA fragments within this area may be cleaved out. The analysis method used did not allow us to detect these changes.

There was no predominant affinity of magnetic nanoparticles to the ends of the virions treated with RNase A; the interaction occurred over the entire surface of the viral particles (*Fig. 4A*). The surface charge of TMV virions containing degraded RNA was evenly distributed, as in TMV VLPs (*Fig. 3A*). However, an unusual pattern was observed upon the incubation of MNPs with MN-treated virions. Magnetic nanoparticles were not only located at the virion ends but also interacted with the entire surface of virions (*Fig. 4B*). One may assume MN to be able to partially hydrolyze an RNA fragment that forms an increased negative charge density area. This is consistent with a model of the cotranslational TMV disassembly mechanism [21].

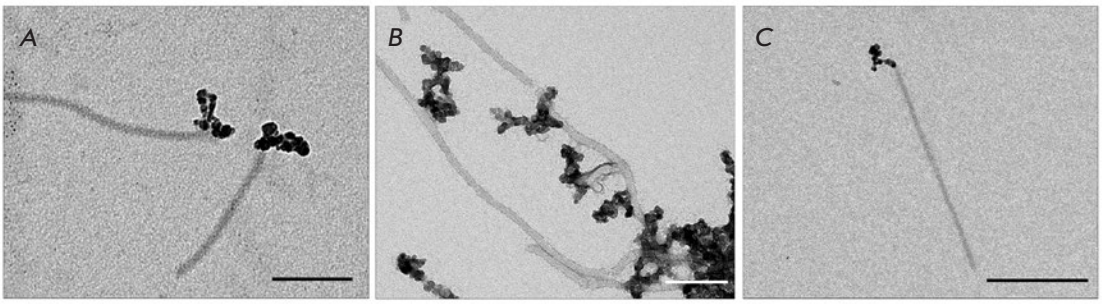

Fig. 5. Complexes of MNPs with AltMV virions (*A*), AltMV VLPs (*B*), and PVX virions (*C*). Scale bars are 100 (*A*, *B*) and 200 (*C*) nm

Most likely, the TMV virion end interacting with the MNP contains the 5'-end of TMV RNA.

Further investigation of the virion surface charge distribution was carried out on viruses with a flexible filamentous virion, which belong to the *Potexvirus* genus: potato virus X (PVX) and alternanthera mosaic virus (AltMV). We did not find any published data on the charge distribution on the AltMV or PVX surface; only the PVX isoelectric point is known (pI of 4.4).

Like the TMV CP, the AltMV CP is able (in the absence of RNA) to form *in vitro* stable extended particles similar in length and morphology to AltMV virions – AltMV virus-like particles (AltMV VLPs) [12].

As seen from *Figure 5*, MNPs, as in the case of TMV, form complexes with AltMV, binding to one end of the native virion (*Fig. 5A*).

An analysis of MNP–AltMV VLP complexes by electron microscopy showed that MNPs were distributed over the VLP surface. However, MNPs had no affinity to the VLP ends (*Fig. 5B*). Therefore, as in the case of TMV, the charge is unevenly distributed over the AltMV surface and the increased negative charge density area is also located at one end of the virion and is due to the presence of RNA. Similar results were obtained for PVX virions (*Fig. 5C*).

Earlier, we demonstrated that encapsulated PVX and AltMV RNAs, unlike TMV RNA, cannot be translated *in vitro* [22, 23].

Starting this study, we assumed that the previously discovered translational properties of RNA in TMV and some potexviruses [22, 24] would correlate with the charge density distribution on the surface of viral particles and that cotranslational TMV disassembly might be associated with an uneven distribution of the negative charge on the particle surface and with localization of the charge at the end comprising the 5'-end of the TMV RNA [20, 25]. At the same time, in potexviruses, whose viral RNA is not accessible to the ribosomes in the virion until specific translational activation, we thought we would observe a uniform charge distribution on the virion surface. Our data did not confirm this assumption.

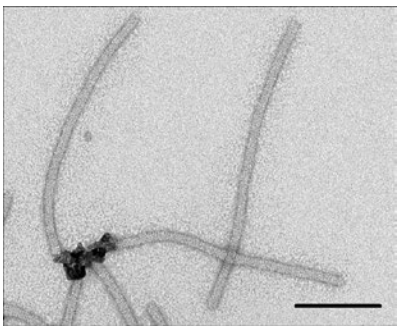

Fig. 6. Complexes of MNPs with RNase A-treated PVX virions. The scale bar is 100 nm

The unexpected result was obtained for RNase A-treated PVX virions. Previously, treatment of PVX with RNases (A and T1) was shown to lead to RNA degradation into short segments (5 to 6 nucleotides) [26]. In this case, degraded RNA fragments remained in virions that were morphologically similar to filamentous PVX particles. In contrast to TMV, the interaction between MNPs and the ends of the PVX virions was detected (*Fig. 6*). Therefore, the surface charge of the PVX virions containing degraded RNA is unevenly distributed, like in native virions (*Fig. 5C*). It may be assumed that an RNA fragment located at the viral particle end remains bound to the CP and forms an increased negative charge density area. This result is consistent with the differences in the translational properties of encapsulated RNA in PVX and TMV virions.

Unlike TMV, the PVX CP is incapable of polymerization in the absence of RNA [27]. However, upon incubation with RNA *in vitro*, the PVX CP is able to form RNA-containing vRNPs and PVX CPs that have a helical "head," whose structure is identical to that of the protein helix of PVX virions, and a "tail"–CP-free RNA (one-tailed particles) [28].

Investigation of the interaction between magnetic nanoparticles and PVX vRNPs revealed binding of MNPs to CP-free RNA (vRNP tails) (*Fig. 7A*). The lack of interaction between MNPs and the vRNP head surface may be explained by the competitive binding of all

Table. Mapping of the surface negative charge of helical plant viruses

Object/Treatment	TMV	AltMV	PVX
Virions			
Ultrasound treatment			
VLP			
RNase A			
MN			
vRNP			
vRNP + MN			

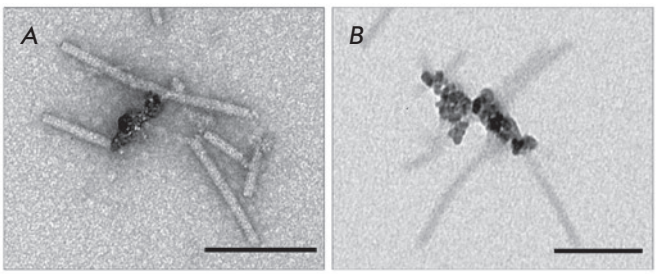

Fig. 7. Complexes of MNPs with PVX vRNPs (*A*) and MN-treated vRNPs (*B*). Scale bars are 100 nm

available MNPs to free RNA. To prevent this, vRNPs were treated with MN. The PVX CP is known to cap the 5'-end of RNA upon vRNP treatment, and the 5'-terminal CP-encapsulated RNA segments in vRNPs retain their integrity and translational properties during treatment with MN [13]. An analysis of these complexes revealed that most MNPs, upon removal of free RNA, interact with the ends of MN-treated vRNPs (*Fig. 7B*), as in the case of native PVX (*Fig. 5C*).

Most likely, the end of viral particles, which interacts with MNPs, contains the 5′-end of RNA.

The experimental results are summarized in the *Table*.

CONCLUSION

In this study, the surface charge of TMV (Tobamovirus genus) and PVX and AltMV (Potexvirus genus) was mapped.

During the TMV assembly, the CP in the form of 20S discs is known to interact with an RNA site located at a distance of about 1,000 nucleotides from the 3'-end of the molecule (assembly origin) [18]. At the same time, the assembly of PVX virions begins directly at the 5'-end of the molecule; RNA interacts with CP monomers and dimers [27]. There is little data on the assembly of AltMV, but the assembly apparently occurs in the same way as in PVX [29]. It should be noted that in TMV, PVX, and AltMV, despite the fact that the assembly of virions occurs in different scenarios, the surface charge is distributed unevenly and an increased negative charge density area is located at one end of the virion. The key role in the formation of this area is apparently played by the 5'-end of viral RNA. Most likely, this may be explained by a less dense packing of the 5'-end of RNA in CP, which is required to initiate translation of RNA-dependent RNA polymerase at the early stages of infection of RNA-containing viruses with a positive genome. ●

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"Shielding" of Cytokine Induction by the Periodontal Microbiome in Patients with Periodontitis Associated with Type 2 Diabetes Mellitus

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ABSTRACT **Periodontal diseases, especially those with polymicrobial etiology, are often associated with type 2 diabetes mellitus, proceeding more severely and affecting the course of diabetes mellitus. Recently, this feature has been associated with the ability of periodontopathogen microflora to cause not only a local infectious process in the oral cavity, but also to interact with the human immune system and induce various systemic effects. We investigated changes in the salivary cytokine profile of patients with chronic periodontitis, associated and not associated with type 2 diabetes mellitus. We observed a statistically significant decrease of MCP-1/CCL2, GM-CSF, IL-5, IL-6, and IFN-γ in the saliva of patients with chronic periodontitis associated with type 2 diabetes mellitus in comparison with patients with chronic periodontitis only. All of these cytokines are associated with macrophage activation. These data are an important contribution to the elucidation of the mechanism of periodontopathogens involvement in the manifestation of the systemic effects of type 2 diabetes.**

KEYWORDS **chronic periodontitis, type 2 diabetes mellitus, interleukins, chemokines, bioplex, IL, periodontal pathogens, salivary cytokine profile.**

ABBREVIATIONS **IL – interleukin; TNFα – tumor necrosis factor-α; IFNγ – interferon gamma; MCP-1 – monocyte chemoattractant protein-1; MIP-1β – macrophage inflammatory protein-1β; G-CSF – granulocyte-colony stimulating factor; GM-CSF – granulocyte-macrophage colony-stimulating factor; AUC – area under the ROC curve; ROC curve – receiver operating characteristic.**

INTRODUCTION

Almost half of the world population is affected by oral diseases. Periodontal diseases initiated by bacterial species are especially significant, since they occur in people of all ages and ethnicities residing in any region [1, 2]. From 50 to 90% of all oral diseases are those that affect the periodontium [1, 3, 4]; approximately 7% of the world population has some severe form of chronic periodontitis [5].

A special feature of chronic periodontitis is that it has a polymicrobial etiology, as a certain stable bacterial community (periodontal pathogens) that has pronounced invasive properties is in a symbiotic relationship, and is capable of suppressing the immune response and promoting chronic inflammation [6, 7] (which becomes systemic with time [8, 9]) predominates in the periodontal biofilm.

Because of the systemic effects of periodontal pathogens that derive from their ability to persist in the human body within macrophages [10, 11], these pathogens are widely disseminated and are involved in the development of various systemic conditions [6,

12], such as infective endocarditis [13], atherosclerosis [14] and other cardiovascular diseases [15], bacterial pneumonia [16], obesity [17], diabetes mellitus [18], pregnancy outcomes [19], rheumatoid arthritis [20, 21], Alzheimer's disease [22], inflammatory bowel disease [23], colon cancer [24], etc. Diabetes mellitus holds a prominent place among these disorders [18, 25].

Diabetes mellitus is one of the most common metabolic disorders [26]. As estimated by the International Diabetes Federation (IDF), the number of patients with diabetes mellitus will steadily increase to reach more than 500 million by 2030 [27]. Diabetes develops either because pancreatic β cells are unable to produce insulin or because peripheral tissues become insulin-resistant [28]. Therefore, two types of this disease have been singled out. Type 1 (insulin-resistant) diabetes is diagnosed in approximately 10% of all patients with diabetes mellitus and is associated with autoimmune destruction of pancreatic β cells, resulting in the body's inability to produce insulin. Insulin-independent type 2 diabetes mellitus (90% of all diabetic patients) manifests itself in relative hyperinsulinemia caused by insulin resistance in cells [29]. Obesity and systemic inflammation are considered the shared risk factors for type 2 diabetes mellitus [30].

There are four major mechanisms for the pathogenesis of type 2 diabetes mellitus: hyperglycemia, insulin resistance, hyperlipidemia, and immune dysfunction [31]. The disorders caused by these mechanisms are tightly interrelated in the pathogenesis of obesity, inflammation, and diabetes mellitus. Chronic periodontitis fits well into this combination of pathological processes, since a high prevalence of periodontitis among all age groups is typical of patients with disorders of carbohydrate and lipid metabolism [29]. The key markers of type 2 diabetes mellitus are related very closely to the level of inflammatory cytokines and the severity of periodontal lesions in patients with chronic periodontitis [32, 33]. The severity of the inflammation in patients with different diseases can be assessed according to the changes in the cytokine profiles in the blood [34, 35], cerebrospinal fluid [36, 37], or saliva [38].

In patients with type 2 diabetes mellitus, local changes in the periodontium are characterized by increased production of reactive oxygen species and proinflammatory cytokines (IL-1, IL-6, and TNFα), as glycation products accumulate and become engaged in vigorous interaction with receptors. The increased levels of proinflammatory cytokines induce oxidative stress and subsequent periodontal tissue degradation [39].

There is ambiguity in the recent data regarding which of these diseases (type 2 diabetes mellitus or periodontitis) should be considered the underlying one and which one has a stronger impact on its comorbidity [40]. Thus, diabetic nephropathy and cardiovascular complications were reported to occur significantly more often in patients with type 2 diabetes mellitus associated with chronic periodontitis than in those without chronic periodontitis [41], while effective treatment of one of these comorbidities has a favorable impact on the course of the other one [42, 43]. This conception has also been proved in other studies showing that the systemic inflammatory status caused by pathogenic periodontal bacteria in patients with chronic periodontitis favors the development of type 2 diabetes [44]. It has been proved that proinflammatory cytokines play a considerable role in the appearance of insulin resistance [45] and are involved in the development of hyperlipidemia, one of the key pathogenetic signs of diabetes mellitus [46].

The objective of this study was to identify the characteristic features of the cytokine profile of the oral fluid of patients with comorbid chronic periodontitis and type 2 diabetes mellitus, using the clinical model of association between these pathological processes.

Both chronic periodontitis and type 2 diabetes mellitus are multifactorial disorders with rather diverse pathogenetic mechanisms, which make the development of sufficiently efficient experimental animal models a challenge.

Since periodontal disorders occur exceptionally rarely in animals, they are simulated by applying ligatures or using other traumatizing techniques. However, despite the large body of data that have been collected in animal experiments, in some cases it is extremely difficult to evaluate whether the results are applicable to humans, since today there is no simple, reproducible model that would actually mimic the pathogenesis of periodontal disorders in humans [47, 48].

The same can be said of the attempts to elaborate a robust experimental animal model of diabetes mellitus. For example, chemically and surgically induced models or genetically modified animals are used. However, it is believed that only separate aspects of the pathogenesis of diabetes mellitus can be studied using these models. Furthermore, most of the existing experimental models have failed to differentiate between types 1 and 2 diabetes mellitus [49, 50].

Because of the aforementioned problems, we used the clinical model which is based on the significant constraints imposed on the possible effect of individual symptoms of the diseases. Therefore, the study group involved patients older than 45 years who had moderate chronic periodontitis and type 2 diabetes mellitus complicated by stage 2 hypertension and had grade

1 or 2 obesity, since type 2 diabetes mellitus is almost always accompanied by complications.

EXPERIMENTAL

Patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus and healthy subjects (controls)

The clinical model used in this study involved three sex-matched groups of patients aged 45–60 years: (1) the study group consisting of 11 patients with chronic periodontitis associated with type 2 diabetes mellitus, (2) the comparison group consisting of 9 patients with chronic periodontitis, and (3) the control group consisting of 12 healthy donors. Patients with chronic periodontitis had no congenital maxillofacial anomalies; the papillary marginal and attached gingival (PMA) index was ≤ 60%, and the periodontal pocket depth was 3–5 mm (tooth mobility grade 1–2). The patients did not undergo any therapeutic interventions for a period of 6 months preceding the enrollment. The duration of type 2 diabetes mellitus was 3–10 years. All the patients were on oral antihyperglycemic therapy. The patients were monitored by controlling the blood level of glycated hemoglobin (HbA1c), the key criterion used to assess the quality of carbohydrate metabolism compensation in patients with diabetes mellitus [51]. The glycated hemoglobin (HbA1c) level during the observation period was 6.5–11.3%.

The study protocol was approved by the Ethics Committee of the A.I. Yevdokimov Moscow State University of Medicine and Dentistry (Ministry of Health of the Russian Federation). All the patients signed a written informed consent for participation in the research.

Analysis of the cytokine profile in the oral fluid

Whole unstimulated saliva samples (1 ml) were collected into sterile tubes. The saliva samples were stored at −80°C until further analysis. The unfrozen saliva samples were centrifuged at 16 100 *g* at 4°C for 10 min. The supernatant was diluted 2.5-fold with phosphate buffered saline (PBS) supplemented with 0.5% Tween 20.

The cytokine levels in saliva samples were determined by multiplex magnetic fluorescent immunoassay using a Human Cytokine 17-plex Assay kit on a Bio-Plex 200 system (Bio-Rad, USA) in accordance with the manufacturer's recommendations. The cytokine levels were measured for a combination of 17 different cytokines, including (1) chemokines: interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1β (MIP-1β); (2) growth factors: granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 7 (IL-7); (3) proinflammatory cytokines: interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-α (TNFα), interleukin 17A (IL-17A); (4) cytokines related to humoral immunity: interleukins 4, 5, 13 (IL-4, IL-5, IL-13); (5) cytokines related to cell-mediated immunity: interleukins 2, 12 (IL-2, IL-12p70), interferon-γ (IFNγ); and (6) immunosuppressive cytokines: interleukin 10 (IL-10).

Statistical analysis

Data was analyzed using the SPSS (version 21) and Sigma-Plot 12.5 statistical software. The difference between patients and healthy subjects (controls) was compared by using the nonparametric Mann–Whitney U test. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The levels of various cytokines in saliva samples were analyzed by comparing the 95% confidence intervals for the measured values and plotting the ROC curves, which show the ratio between the sensitivity and specificity for each test presented as the area under the curve (AUC) (see the diagrams).

Figure 1 and *Table* show the results obtained in a comparative analysis of salivary chemokine levels and the ROC curves, indicating their diagnostic significance in all study groups. Comparison of 95% confidence intervals demonstrated that the salivary levels of such chemokines as IL-8 and the MIP-1β protein tended to increase in both groups of patients with chronic periodontitis; however, only in patients without type 2 diabetes mellitus is this increase statistically significant compared to that in the control group (the diagnostic significance of the test being rather high: AUC = 0.8–0.96). Meanwhile, no significant differences in elevation of the IL-8 level were revealed in the groups of patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus (AUC = 0.574), while the MIP-1β levels could be assessed as being moderately high (AUC = 0.725). The salivary level of monocyte chemoattractant protein 1 (MCP1) was significantly lower in patients with chronic periodontitis associated with type 2 diabetes mellitus. In this case, the diagnostic significance of the test can be considered high (AUC = 0.775).

Anbalagan et al. [52] also reported that MCP-1 chemokine in the oral cavity has a special diagnostic significance in patients with type 2 diabetes mellitus. In particular, they emphasized that it is directly associated with the bacterial load in the oral cavity, since a reduction in the bacterial load due to therapeutic and

Table. The salivary cytokine profile of patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus and healthy donors

Cytokines	Chronic periodontitis		Chronic periodontitis associated with type 2 diabetes mellitus		Healthy donors	
	median	IQR	median	IQR	median	IQR
IL-1β	▪591	(82–867)	▪226	(53–399)	50	(40–128)
IL-2	▪19	(11–25)	7.4	(4.9–14.4)	2.3	(2.0–7.7)
IL-4	▪2.5	(1.2–2.6)	1.2	(0.6–2.1)	0.6	(0.5–1.2)
IL-5	13.7	(4.9–14.0)	2.2	(1.5–10.7)	7.5	(7.4–8.7)
IL-6	▪278	(14–303)	20	(5–52)	8.0	(6.4–74.7)
IL-7	5.5	(2.9–10.6)	▪9.9	(2.8–16.6)	1.5	(1.5–1.5)
IL-8	▪2554	(760–2859)	1245	(522–2964)	381	(362–588)
IL-10	▪10.3	(4.2–12.0)	4. 8	(1.0–17)	3.0	(1.0–4.8)
IL-12(p70)	1.5	(0.5–4.0)	▪1.2	(0.8–1.5)	0.6	(0.5–0.6)
IL-13	▪1.1	(0.4–1.3)	0.4	(0.4–0.9)	0.4	(0.4–0.4)
IL-17A	▪23	(14–43)	9.1	(5.8–19.5)	4.3	(4.0–11.0)
G-CSF	▪296	(141–305)	161	(33–279)	82	(71–85)
GM-CSF	▪6.5	(5.9–8.7)	2.4	(1.0–5.4)	1.0	(1.0–1.7)
IFNγ	18	(17–26)	7.3	(4.6–9.5)	7.1	(3.6–24.9)
MCP-1	302	(251–415)	151	(126–269)	87	(65–260)
MIP-1β	▪27	(26–41)	17	(11–36)	10.2	(9.2–14.6)
TNFα	▪115	(45–268)	▪43	(26–92)	12	(11–23)

Note. The results are presented as the median value and the interquartile range (IQR). Cytokine concentrations significantly differing from those in healthy donors ($p < 0.05$) are marked with the symbol "▪" and shown in red color. Gray color denotes cytokine concentrations that differ significantly between the groups of patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus ($p < 0.05$).

prophylactic measures is also accompanied by a decline in the salivary MCP-1 level [52]. However, paradoxical results were obtained in our study: all other conditions being equal and no treatment measures being performed, the MCP-1 level in the oral cavity of patients with chronic periodontitis associated with type 2 diabetes mellitus was lower than that of patients with chronic periodontitis without the somatic comorbidity. One might expect that pathogenic periodontal bacteria would play a considerably greater role in this case [53], but alternative results have also been reported [54].

Figure 2 and *Table* show the data obtained for a similar analysis of the salivary levels of several growth factors in patients and healthy subjects. The salivary G-CSF level in patients with chronic periodontitis tends to increase; however, this trend becomes a statistically significant deviation only in patients without type 2 diabetes mellitus. In all patients with chronic periodontitis, the salivary level of GM-CSF was higher than that in the control group. The IL-7 level was significantly high only in patients with chronic periodontitis associated with type 2 diabetes mellitus. Among these deviations, special attention should be paid to the differences between the GM-CSF levels in patients with chronic periodontitis associated and not associated with type 2 diabetes mellitus: they are higher in the latter group of patients.

Miranda et al. [55] demonstrated that in patients with chronic periodontitis associated with type 2 diabetes mellitus, the GM-CSF level (albeit in serum, not in saliva) is considered to be one of the pathogenetically important cytokines. Indirect evidence of the potential deficiency of this cytokine in patients with

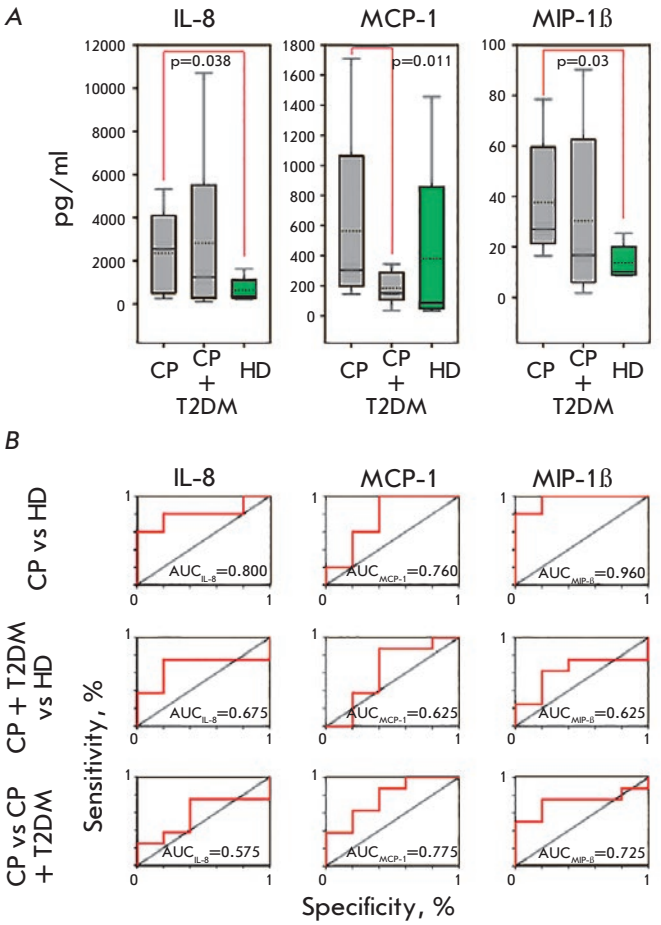

Fig. 1. Levels of chemokines in the saliva (A) of patients with chronic periodontitis (CP), chronic periodontitis associated with type 2 diabetes (CP+T2DM), healthy donors (HD), and the corresponding ROC-curves (B). The interquartile range is shown by boxes. The median in each group is shown by the bold line. Bars represent the 95% confidence interval. Statistically significant differences with their respective *p* values are indicated; AUC – area under the ROC curve

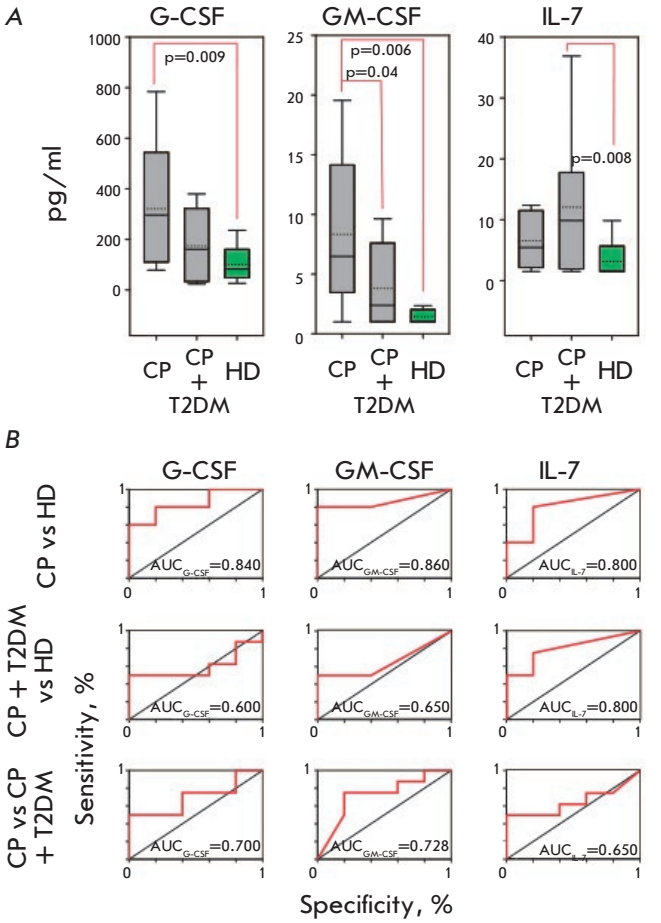

Fig. 2. Levels of growth factors in the saliva (A) of patients with chronic periodontitis (CP), chronic periodontitis associated with type 2 diabetes (CP+T2DM) and healthy donors (HD), and the corresponding ROC-curves (B). The interquartile range is shown by boxes. The median in each group is shown by the bold line. Bars represent the 95% confidence interval. Statistically significant differences with their respective *p* values are indicated; AUC – area under the ROC curve

the comorbidities under study has been obtained, since exogenous administration of GM-CSF increases the survival rate of experimental animals [56].

Next, the profile of four proinflammatory cytokines was analyzed: three of these cytokines are secreted mainly by innate immune cells (primarily by the macrophages IL-1β, IL-6, and TNFα), while IL-17A is a secretion product of Th17, one of the subtypes of T-helper cells (*Fig. 3*, *Table*).

Figure 3 demonstrates that the salivary levels of IL-1β and TNFα were statistically significantly elevated in both groups of patients with chronic periodontitis regardless of whether or not they had comorbid type 2 diabetes. The IL-17A level was significantly increased in patients with chronic periodontitis, while only tending to increase in patients with both comorbidities.

The groups with chronic periodontitis associated and not associated with type 2 diabetes mellitus differed only in terms of the salivary level of IL-6, which was significantly elevated only in patients with chronic periodontitis with no comorbidity.

IL-6 is considered one of the key predictors of type 2 diabetes mellitus and its vascular complications. Assumptions have been made that the mechanism through which this cytokine is involved in the pathogenesis of atherosclerosis is systemic (via the activation

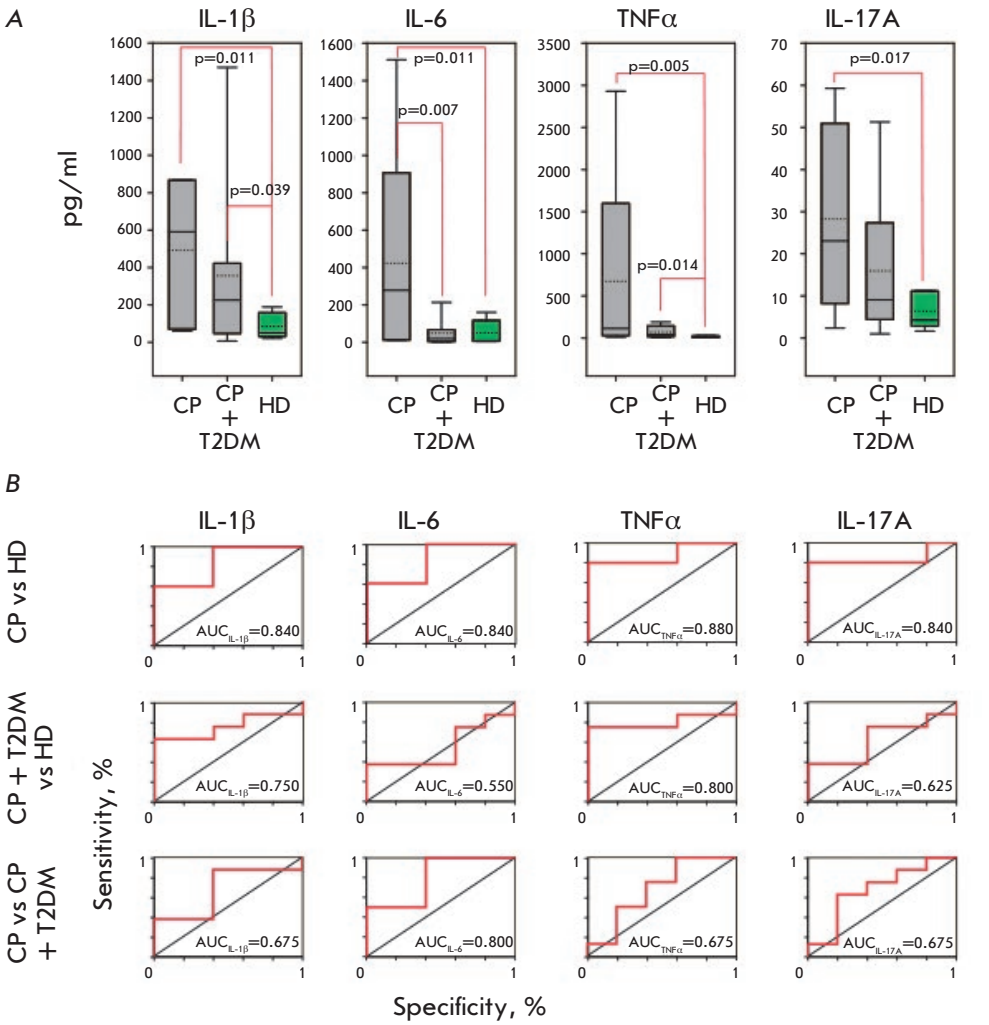

Fig. 3. Levels of proinflammatory cytokines in the saliva (A) of patients with chronic periodontitis (CP), chronic periodontitis associated with type 2 diabetes (CP+T2DM) and healthy donors (HD), and the corresponding ROC-curves (B). The interquartile range is shown by boxes. The median in each group is shown by the bold line. Bars represent the 95% confidence interval. Statistically significant differences with their respective *p* values are indicated; AUC – area under the ROC curve

of endothelial cells, due to the increasing role played by the thrombogenic function of platelets, via stimulation of proliferation of vascular smooth muscle cells, and due to the increased lipid accumulation in the macrophages) [57, 58]. We showed that the local salivary level of IL-6 is reduced in patients with chronic periodontitis associated with type 2 diabetes mellitus.

The levels of the cytokines that are secreted mainly by type 2 T-helper cells (Th2) and are to a certain extent associated with eliciting the humoral immune response were also determined (*Fig. 4*, *Table*).

Similar to other cytokines, the salivary levels of Th2-secreted IL-4, IL-13, and IL-10 in patients with chronic periodontitis associated with type 2 diabetes mellitus was higher than those in the control group, but lower than those in patients with chronic periodontitis without the comorbidity. However, these differences had a low diagnostic significance (AUC < 0.65). Only the levels of IL-5, which is secreted not only by Th2, but also by type 2 innate lymphocytes [59], differed in two groups of patients with chronic periodontitis (AUC = 0.75).

IL-5 is a growth factor that promotes eosin proliferation in adipose tissue, including in patients with type 2 diabetes mellitus [60]. Hypereosinophilia contributes to the transition of activated macrophages to the M2 phenotype, followed by suppression of inflammatory responses [60, 61]. As one of the components of this system (such as IL-5 in our study) is eliminated, the adipose tissue starts triggering insulin resistance (a typical feature of type 2 diabetes mellitus) and aggravating the inflammation [60, 62].

The measured levels of cytokines secreted by dendritic cells, macrophages, type 1 T-helper cells, and cytotoxic T cells are shown in *Figure 5* and *Table*. All of them (the active fraction of interleukin-12 (IL-12(p70)), IL-2, and interferon-γ (IFNγ)) are related to the eliciting of the cell-mediated immune response.

Significantly greater elevation of the salivary levels of IL-12 and IL-2 was observed in patients with chronic periodontitis (both associated and not associated with type 2 diabetes mellitus) compared to the control group; however, no differences between these groups

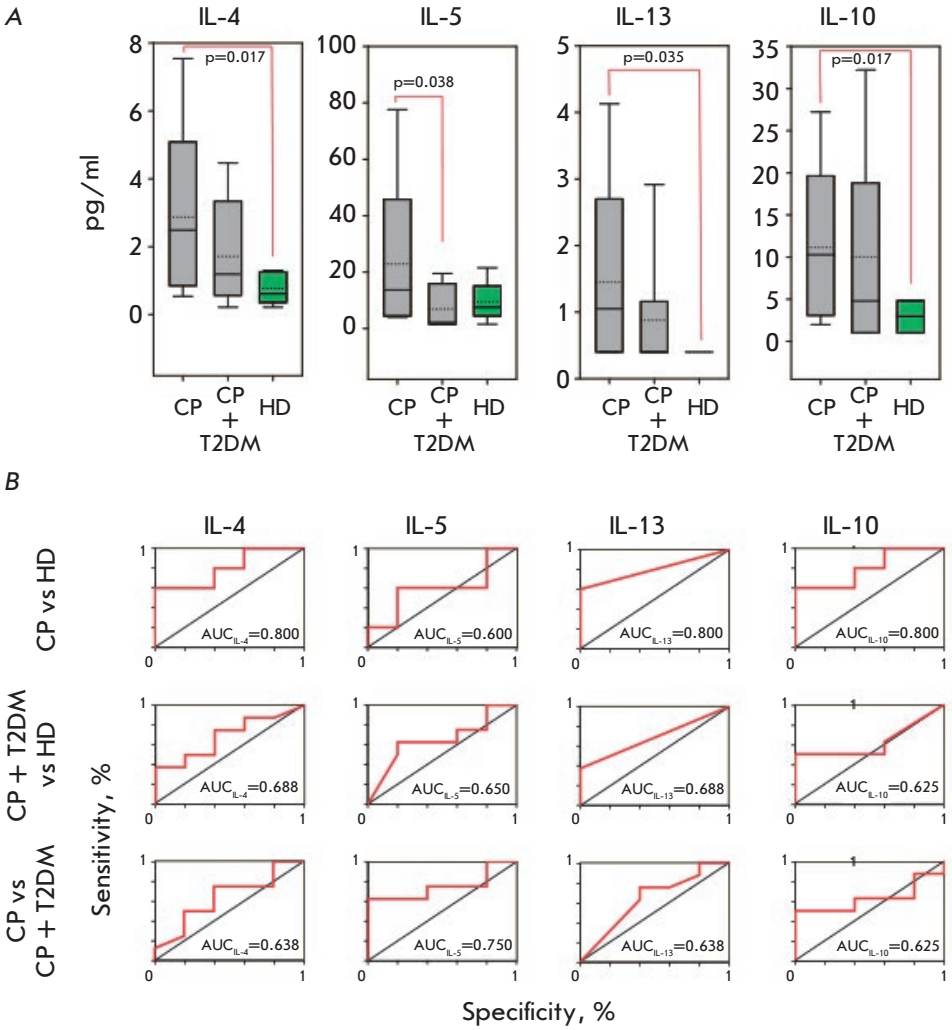

Fig. 4. Levels of Th2 cytokines in the saliva (A) of patients with chronic periodontitis (CP), chronic periodontitis associated with type 2 diabetes (CP+T2DM) and healthy donors (HD), and the corresponding ROC-curves (B). The interquartile range is shown by boxes. The median in each group is shown by the bold line. Bars represent the 95% confidence interval. Statistically significant differences with their respective *p* values are indicated; AUC – area under the ROC curve

were revealed. The salivary level of IFNγ suggests that IFNγ secretion is reduced in patients with chronic periodontitis; this reduction is greater when chronic periodontitis is associated with type 2 diabetes mellitus.

It has been reported that the serum IFNγ levels are reduced in patients with type 2 diabetes mellitus, especially during treatment [63]. Meanwhile, induction of the M1 macrophage phenotype is one of the functions of IFNγ [64].

Table summarizes the salivary cytokine levels in patients with chronic periodontitis (both associated and not associated with type 2 diabetes mellitus). It is obvious that the levels of 12 out of the 17 cytokines are significantly higher in patients with periodontitis compared to those in the control group. The levels differed for only five cytokines. Unambiguous differences between the groups of patients with chronic periodontitis associated or not associated with type 2 diabetes mellitus were established for the levels of only five cytokines.

An international research group with Russian participation has put forward an interesting hypothesis [65]. According to this hypothesis, hyperglycemia is the key factor in the pathogenesis of diabetes mellitus. Among all immune cells, an important role is played by macrophages whose activation is accompanied by a polarization of their functions, giving rise to two phenotypes: the classically activated M1 macrophages and the alternatively activated M2 macrophages. Due to the production of different cytokines, both of these phenotypes play a crucial role in the development of the inflammation and vascular complications associated with diabetes. Hyperglycemia per se (without allowance for additional effects) induces the mixed M1/M2 cytokine profile, which is responsible for the specific ratio between the inflammatory and vascular responses.

The observed features of the cytokine profile in patients with chronic periodontitis associated with moderate type 2 diabetes mellitus are apparently caused by

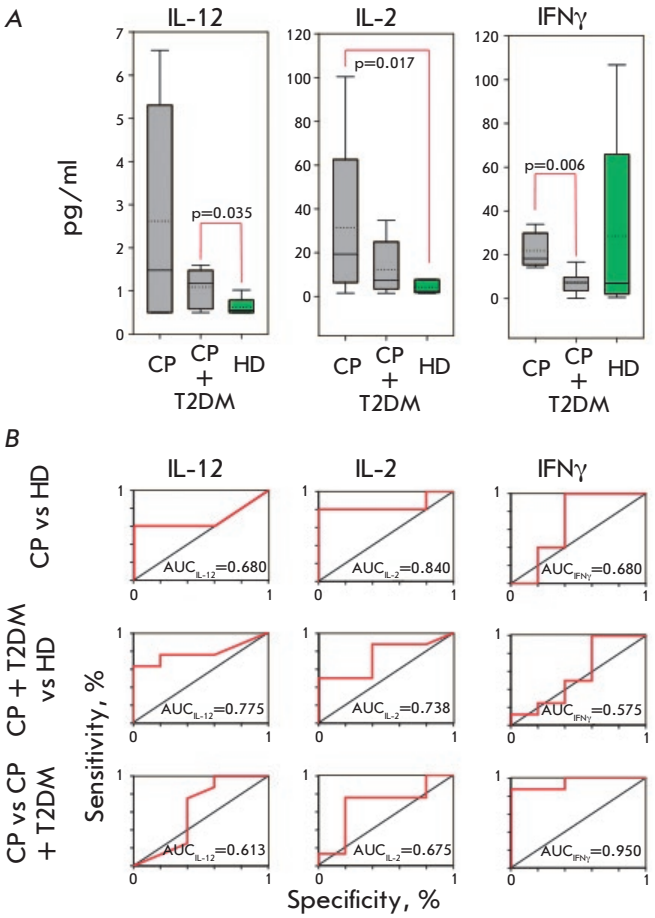

Fig. 5. Levels of cell-mediated immunity cytokines in the saliva (A) of patients with chronic periodontitis (CP), chronic periodontitis associated with type 2 diabetes (CP+T2DM) and healthy donors (HD), and the corresponding ROC-curves (B). The interquartile range is shown by boxes. The median in each group is shown by the bold line. Bars represent the 95% confidence interval. Statistically significant differences with their respective *p* values are indicated; AUC – area under the ROC curve.

an additional factor; chronic periodontitis etiologically related to the community of pathogenic periodontal bacteria that persist.

CONCLUSIONS

The features of the local salivary cytokine profile typically observed in patients with chronic periodontitis associated with type 2 diabetes mellitus have been identified. These features were not observed in patients with chronic periodontitis not associated with diabetes and include statistically significant changes in the levels of MCP-1, GM-CSF, IL- 6, IL-5, and IFN-γ.

The key feature of the changes in the cytokine profile is the reduced secretion of the aforementioned cytokines, which is ground for assuming that the factor inducing cytokine secretion is "shielded" in patients with comorbid chronic periodontitis and type 2 diabetes mellitus. Pathogenic periodontal microflora etiologically related to chronic periodontitis can be such a factor.

Another important feature of the changes in the cytokine profile is the potential association between these deviations, the macrophage system, and the conditions required for macrophage activation. The combination of these features suggests that the selective effect of periodontal pathogens on the salivary cytokine profile is "shielded" as they switch to intracellular parasitism of macrophages, which subsequently elicits systemic effects. ●

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Calcitonin Gene-Related Peptide and Substance P As Predictors of Venous Pelvic Pain

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ABSTRACT **The purpose of this work was to study the contents of calcitonin gene-related peptide (CGRP) and substance P (SP) in the blood plasma of patients with pelvic varicose veins. Thirty women with pelvic varicosities and a reflux blood flow were investigated using duplex ultrasonography. Group 1 included 18 patients with clinical signs of the pelvic congestion syndrome (PCS), including venous pelvic pain (VPP). Group 2 consisted of 12 patients with pelvic varicosities with no clinical signs of PCS.** *Group 1.* **The score of VPP intensity ranged from 4 to 8; the mean score being 4.84 ± 0.43. The CGRP level in the studied group ranged from 0.39 to 1.01 ng/mL; the SP level ranged from 0.005 to 1.33 ng/mL.** *Group 2.* **The CGRP values were 0.15–0.32 ng/mL, and the SP range was 0.003–0.3 ng/mL. In this group, the levels of the studied peptides were 3–5 times lower than those for the patients with VPP.** *Group 3.* **The mean CGRP values were 0.06 ± 0.003 ng/mL, and the mean SP values were 0.03 ± 0.001 ng/mL. These values were considered as the reference parameters; a statistical analysis was performed for them. The correlation analysis revealed a strong relationship between the CGRP and VPP levels (r = 0.82) and a medium correlation between the SP level and pelvic pain in Group 1. The CGRP and SP levels in blood plasma highly correlate with the presence of pelvic venous pain.**
KEYWORDS **venous pelvic pain, calcitonin gene-related peptide, substance P.**
ABBREVIATIONS **VPP – venous pelvic pain; PCS – pelvic congestion syndrome; CGRP – calcitonin gene-related peptide; SP – substance P.**

INTRODUCTION

Chronic pelvic pain (CPP) is a highly relevant and challenging problem of modern medicine [1, 2]. According to the World Health Organization (WHO), the prevalence of CPP ranges from 2.4 to 24% of the population, with women of reproductive age being the most predominant group affected [5]. Other data indicate that 3.8% of women suffer from CPP, and that the annual cost of treatment of the disease in Europe amounts to 3.8 billion euro [3, 4]. The pelvic congestion syndrome (PCS) is a cause of CCP in 10–30% of patients with PCS, whereas 10% of the entire female population has pelvic varicose veins and a reflux blood flow and PCS appears in 60% of them [6, 7, 8, 9]. Hansrani et al. (2016) have convincingly proved that there is a relationship between CPP and PCS in women with pelvic vein incompetence [10]. Thus, pelvic venous insufficiency is a serious factor behind the development of CPP. The reasons behind the emergence of venous pelvic pain (VPP) remain unclear, and the available hemodynamic and inflammatory hypotheses cannot fully explain what causes the pain syndrome in some patients and why other patients with identical morphofunctional changes in pelvic veins do not have it [11, 12, 13]. As proved by earlier studies, there is no obvious relationship between the diameter of pelvic veins and the severity of VPP [14, 15]. Meanwhile, the findings obtained by several authors indicate that there might be a relationship between neurogenic inflammation, hyperproduction, and increased activity of vasoactive neuropeptides and the emergence of VPP formation [16, 17, 18, 19].

The objective of this work was to study the levels of calcitonin gene-related peptide (CGRP) and substance P (SP) in the blood plasma of patients with pelvic varicose veins and to determine the degree of correlation between the levels of these algogens and VPP.

EXPERIMENTAL

Patients

Thirty women aged 22–42 years with pelvic varicosities and a pathological reflux blood flow along those veins were enrolled in the study using the results of transabdominal and transvaginal duplex ultrasonography (DUS) of pelvic veins. The study was approved by the Local Ethics Committee of the N.I. Pirogov Russian National Research Medical University and registered at clinicaltrials.gov (NCT03921788). All patients signed a consent form to take part in the study. Group 1 consisted of 18 patients with clinical signs of the pelvic congestion syndrome (PCS), including venous pelvic pain (VPP). The severity of VPP was evaluated using the Visual Analog Scale (VAS). In this group of patients, this parameter ranged from score 4 to 8. Patients in group 2 (12 patients) had pelvic varicose veins but showed no clinical signs of PCS. The inclusion criteria were as follows: women of reproductive age; pelvic vein dilatation and reflux blood flow along parametrial, uterine, and gonadal veins higher than 0.5 s according to the DUS data; the absence of any pathology accompanied by CPP; and signed informed consent form obtained from the patient. The exclusion criteria were the absence of dilated pelvic veins and a reflux blood flow along them during DUS; diseases whose clinical course assumes that patients have CPP and other varieties of the chronic pain syndrome, including migraine. For this purpose, all the patients consulted a gynecologist, an urologist, and a neurologist; they also underwent ultrasonography of internal genitalia and the urinary system.

In addition, 10 healthy subjects without any acute or chronic diseases accompanied by the pain syndrome took part in the study. These subjects had no varicose veins of the pelvis or lower extremities as assessed both visually and according to the DUS data. These patients composed the third (control) group (Group 3).

The results of the clinical and ultrasonography examination are summarized in *Table 1*.

ELISA (enzyme-linked immunosorbent assay) procedure

Venous blood was taken from the cubital vein at the same time (8:00–8:30 a.m.) on an empty stomach, in sitting position, and seven days after the end of the last menstruation. The blood was sampled into 4.0 mL vacuum tubes containing K_2-EDTA. The blood samples were then centrifuged for 10 min at 3000 rpm. The obtained blood plasma was divided into 1.0 mL aliquots and placed into two Eppendorf tubes. The biological material was immediately frozen and stored at −80°C for subsequent analysis. The levels of calcitonin gene-related peptide (CGRP) and substance P (SP) were determined by competitive enzyme-linked immunosorbent assay (ELISA) using commercial kits (Peninsula laboratories, LLC, Bachem Group, USA). The reference and test samples were analyzed in doublets. Protocol no. 5, recommended by the manufacturer (incubation at 4°C for 14–16 h (overnight)), was used. The absorbance was measured on a Stat Fax 2100 immunoenzymatic analyzer (microplate photometer, Awareness Technology Inc., USA) in standard 96-well plates at a wavelength of 450 nm. Concentrations of neuropeptides were calculated using the Cobas EIA recalibration software (F. Hoffmann – La Roche Ltd, Switzerland).

Statistical analysis

The statistical analysis was performed using the Microsoft Excel and Statistica 6.0 software and the med-

Table 1. Clinical and ultrasonography data (n = 30)

Parameter		Group 1 (n = 18)	Group 2 (n = 12)	Group 3 (n = 10)
Age, years		30.2 ± 2.4	31.6 ± 1.9*	21.3 ± 0.8**
Body mass index (BMI)		23.4 ± 0.8	22 ± 0.6*	20.4 ± 0.3**
Childbearing, n		1–3	1–3	0
Duration of the disease/observation of varicose pelvic veins, years		4.9 ± 1.3	3.3 ± 1.1*	0
Venous pelvic pain, n/%		18/100	0	0
Chronic pain of any other localization, %		0	0	0
Valvular dysfunction	Parametrial veins, n/%	30/100	30/100	0
	Uterine veins, n/%	9/50	5/41.6	0
	Gonadal veins, n/%	4/22.2	3/25	0

*$p > 0.05$; **$p < 0.05$

statistic.ru statistical online calculator. The arithmetic mean (M) and standard deviation (σ) were calculated. The data are presented as absolute and relative values. The differences were considered statistically significant at $p < 0.05$. Correlation regression analysis (r) and calculation of the relative risk (RR) were used to evaluate the relationships between the clinical and laboratory parameters.

RESULTS AND DISCUSSION

Duplex ultrasonography data

The transabdominal and transvaginal DUS data indicated that there were no significant distinctions in the incidence rate of valvular insufficiency of pelvic veins in the two groups of patients. No symptoms of pelvic congestion syndrome (PCS) were observed in Group 2 patients in spite of the pathological reflux blood flow along the gonadal (25%) and uterine (41.6%) veins. The diameter of intrapelvic veins was ignored, because there was no significant correlation with the presence and severity of VPP as confirmed by previous studies [14,15]. Statistically significant intergroup differences were observed for the laboratory results.

ELISA DATA

Group 1

Among the patients in this group, the severity of VPP ranged from score 4 to 8; the mean score was 4.84 ± 0.43. The CGRP level in the studied group ranged from 0.39 to 1.01 ng/mL (mean, 0.71 ± 0.11 ng/mL); the SP level ranged from 0.005 to 1.33 ng/mL (mean, 0.42 ± 0.18 ng/mL). The CGRP levels lay in the range of 0.69–1.01 ng/mL, the SP level, from 0.006 to 1.45 ng/mL. In two patients with maximum pain severity (score 8), a combination of increased levels of neuropeptides was revealed: in one patient, the CGRP and SP levels were 0.69 and 1.33 ng/mL, respectively; in another patient, these values were 1.01 and 1.45 ng/mL, respectively. The simultaneous increase in the production of these proteins probably contributes to the aggravation of the pain syndrome. In six patients, pelvic pain with a severity score = 4 was accompanied by a less significant increase in the levels of CGRP (0.39–0.51 ng/mL) and SP (0.005–0.38 ng/mL). *Figure* shows the clinical and laboratory parallels between the severity of VPP and the levels of neurotransmitters under study.

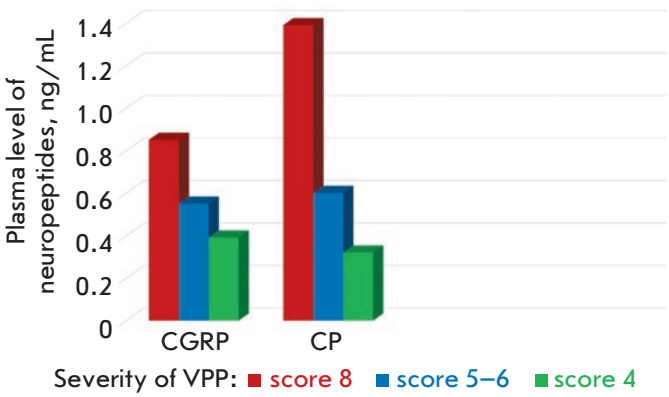

Fig. The CGRP and SP levels and severity of venous pelvic pain in group 1 patients

In contrast to the CGRP level, the plasma levels of SP varied widely, from normal values to a significant increase of up to 1.45 ng/mL. The cause of this phenomenon will be investigated in further studies.

Group 2

No VPP was observed in Group 2 patients. The CGRP levels were 0.15–0.32 ng/mL (mean, 0.26 ± 0.02 ng/mL); the SP levels were 0.003–0.3 ng/mL (mean, 0.15 ± 0.06). In this group, the levels of the studied neuropeptides were 3–5 times lower than those in patients with VPP. No correlations between the GGRP and SR levels were revealed in patients without pelvic pain.

Group 3

No signs of chronic pain syndrome of any localization were observed in healthy subjects. The mean CGRP and SP levels were 0.06 ± 0.003 and 0.03 ± 0.001 ng/mL, respectively. These levels were considered as the reference values and were used for the statistical analysis.

The correlation analysis showed a strong relationship between the CGRP and VPP levels (r = 0.82) and a medium relationship between the SP level and the pelvic pain severity in Group 1 patients. The calculated relative risk (RR) of developing VPP with increasing CGRP level in Group 1 is 19-fold higher than that in Group 2 (*RR* = 19.19; 95% CI: 2.78–132.35) and indicates that there is a direct relationship between VPP severity and the CGRP level. No such evident correlations were revealed for Group 2.

Table 2 lists the VPP severity and the CGRP and SP levels in the studied groups.

Significant differences in the plasma levels of CGRP were revealed for Groups 1 and 2. The differences in the plasma level of SP for these two groups are statistically insignificant, but this parameter apparently tends to increase in patients with VPP. The CGRP and SP levels in Group 3 are statistically significantly lower than those in Group 2, which probably indicates that the mere existence of varicose veins can be accompanied by an increase in the levels of these neuropeptides

Table 2. Severity of VPP and plasma levels of CGRP and SP in the study groups

Parameter	Group 1 (n = 18)	Group 2 (n = 12)	p^*	Group 3 (n = 10)	p^{**}
VPP, score	4.84 ± 0.43	0	-	0	-
CGRP level, ng/mL	0.71 ± 0.11	0.26 ± 0.02	0.0004	0.06 ± 0.003	0.0001
SP level, ng/mL	0.42 ± 0.18	0.15 ± 0.06	0.166	0.03 ± 0.001	0.05

*Groups 1 and 2 were compared; **Groups 2 and 3 were compared.

regardless of whether or not patients display the pain syndrome.

Back in 1985, J.A. Fisher and W. Born observed pronounced cardiovascular effects for CGRP injected intravenously (vasodilatation, hypotension, positive chronotropic and inotropic effects on the heart) [20]. The maximum efficacy of CGRP was observed at the microcirculation level (its vasodilatory activity was tenfold higher than that of prostaglandins). CGRP is abundant in the peripheral and central nervous system; its receptors are expressed in the pain pathways and usually colocalize with other neuropeptides, including substance P [21]. Receptors to CGRP and SP were also observed in the pelvic veins of women [17, 22]. Stones et al. (1995) detected SP in endothelial cells of the ovarian vein and proved that it is involved in the regulation of the vascular tone of this vessel [22]. They suggested that the disruption of venous outflow in women with PCS increases the elimination of CP, and that the hypersensitivity of receptors to this neuropeptide causes the pain syndrome. The synergistic effect of substance P and CGRP on the venous tone may play a significant role in the occurrence of venous pelvic pain. The number and sensitivity of receptors to these neurotransmitters probably determine whether or not patients with PCS will develop venous pelvic pain. Stones et al. found that intravenous injection of CGRP to patients with PCS leads to a high SP level in endothelial cells of ovarian veins and aggravation of pelvic pain. This proved a compelling argument for studying the influence of these neurotransmitters on the development of VPP in patients with PCS.

The reported results of the study of the plasma levels of CGRP and SP in groups of patients with pelvic varicosities accompanied by a reflux blood flow indicate that there is a tight correlation between the level of these neuropeptides and pelvic pain. To a certain extent, this fact indirectly confirms the theory of a vein-specific inflammation that emerges during varicose vein transformation and is accompanied by vein wall hypoxia, which should be regarded as a damaging factor contributing to neurogenic inflammation in the vein wall, enhanced synthesis of neuropeptide algogens, and development of the pain syndrome.

Today, the reference CGRP and SP levels in healthy people are unknown. The available data is contradictory: some of the data indicate that the plasma of healthy people does not contain these substances. Meanwhile, other data strongly indicate that the normal CGRP level ranges from 2 to 36 pmol/L and that of SP does not exceed 0.1–0.19 ng/mL [23, 24, 25]. Our study demonstrates that the CGRP and SP levels in healthy female subjects do not exceed 0.06 ± 0.003 and 0.03 ± 0.001 ng/mL, respectively. However, the distinctions in the test systems used by independent authors should be taken into account. In our work, we report on the preliminary results of a study that will be continued until the necessary power and representativeness are achieved. Meanwhile, the obtained data indicate that the chosen scientific research is quite promising.

It should be noted that CGRP and SP are only two vasoactive neuropeptides whose levels were studied in patients with venous pelvic pain. However, the development of pain in patients with PCS involves the activation of the entire range of neurotransmitters and algogens (neurokinin A, endothelin, prostaglandins, nitric oxide, interleukin-1, tumor necrosis factor-α, etc.). In particular, Agu et al. (2002) and Yang et al. (2008) showed that decreased expression of endothelin-1 (ET-1), in combination with a decreased number of endothelin-B receptors, is a factor responsible for a reduction of the vasoconstrictor activity of veins and their varicose transformation [26, 27]. Pietrzycka et al. (2015) found that therapy with a micronized purified flavonoid fraction in female patients with a chronic venous disease (CVD) is accompanied by an increase in ET-1 levels, while the level of tumor necrosis factor-α (TNF-α) decreases, which indirectly indicates that ET-1 participates in the regulation of the venous tone in patients with CVD [28]. These data suggest that further research into the neurobiological aspects of venous pelvic pain is needed, which could allow one to evaluate the effect of other protein derivatives on the pathological processes taking place in the vein wall.

CONCLUSION

The plasma levels of CGRP and SP strongly correlate with pelvic venous pain. These neuropeptides probably play a substantial role in the development of the pain syndrome in patients with the pelvic venous congestion syndrome. The high levels of CGRP and SP in patients with VPP resistant to conventional phlebotropic therapy can be an indication towards administering medications that block these neurotransmitters to treat such patients. ●

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Genetic Variability of the AcrAB-TolC Multidrug Efflux Pump Underlies SkQ1 Resistance in Gram-Negative Bacteria

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ABSTRACT **SkQ1, a novel antibiotic targeting bacterial bioenergetics, is highly effective against both gram-positive and gram-negative bacteria. However, some gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, are highly resistant to it. In different gram-negative bacteria, this resistance is associated with the identity of their AcrB transporter protein sequence with the sequence of the AcrB protein from *E. coli*. SkQ1 is expelled from *E. coli* cells by the AcrAB-TolC multidrug efflux pump. In this study, we demonstrate that SkQ1 resistance in *E. coli*, in contrast to chloramphenicol resistance, does not depend on the presence of the multidrug efflux pump accessory protein AcrZ.**
KEYWORDS **SkQ1, AcrZ, AcrAB-TolC efflux pump, multidrug resistance.**

INTRODUCTION

SkQ1, decyl triphenylphosphonium-conjugated plastoquinone, is a member of a new class of antibiotics that directly affect bacterial bioenergetics. The SkQ1 ability to inhibit growth of a variety of gram-negative and gram-positive bacteria may be used in medicine and agriculture; therefore, it is important to study its effect on microbial ecosystems and the development of resistance to it. We have demonstrated [1, 2] that SkQ1 resistance in *E. coli* is due to the presence of a specific multidrug resistance (MDR) pump AcrAB-TolC *(Fig. 1)* that underlies resistance to a wide range of antibiotics, surfactants, bile salts, pigments, and small organic molecules [3]. However, our study [1] did not analyze all TolC-dependent pumps, namely the putative TolC-dependent pump EmrKY-TolC, EntS, and the protein AcrZ. The small accessory protein AcrZ (also known as YbhT) of 49 amino acid residues is known to bind to the AcrAB-TolC complex, which comprises the AcrA, AcrB, and TolC proteins, and enhance the pump ability to remove certain classes of substrates from the cell: e.g., tetracycline, puromycin, and chloramphenicol [4].

Bacteria have genetic plasticity, which allows them to respond to a wide range of environmental threats, such as antibiotics. Bacteria use two main genetic survival strategies: (1) acquisition of resistance determinants through horizontal gene transfer and (2) mutations associated with antibiotic targets [5]. The amino acid sequences of the AcrA, AcrB, and TolC proteins are identical in laboratory *E. coli* B and K-12 sub-strains [6]. Previously, we demonstrated that removal of any of the AcrA, AcrB, or TolC proteins led to a complete loss of SkQ1 resistance [1]. The distance between the TolC and AcrB operons in the *E. coli* chromosome is about 175 kbp [7]; therefore, the likelihood of acquiring AcrAB-TolC pump-mediated resistance through interspecific horizontal gene transfer is very negligible.

To date, MDR pump-mediated resistance is the only known mechanism of SkQ1 resistance, and AcrAB-TolC is the only known pump that removes SkQ1 from the cell. Based on the data on the ability of the small protein AcrZ to regulate resistance to antibiotics, such as tetracycline, puromycin, and chloramphenicol [4], it may be supposed that SkQ1 resistance is also modu-

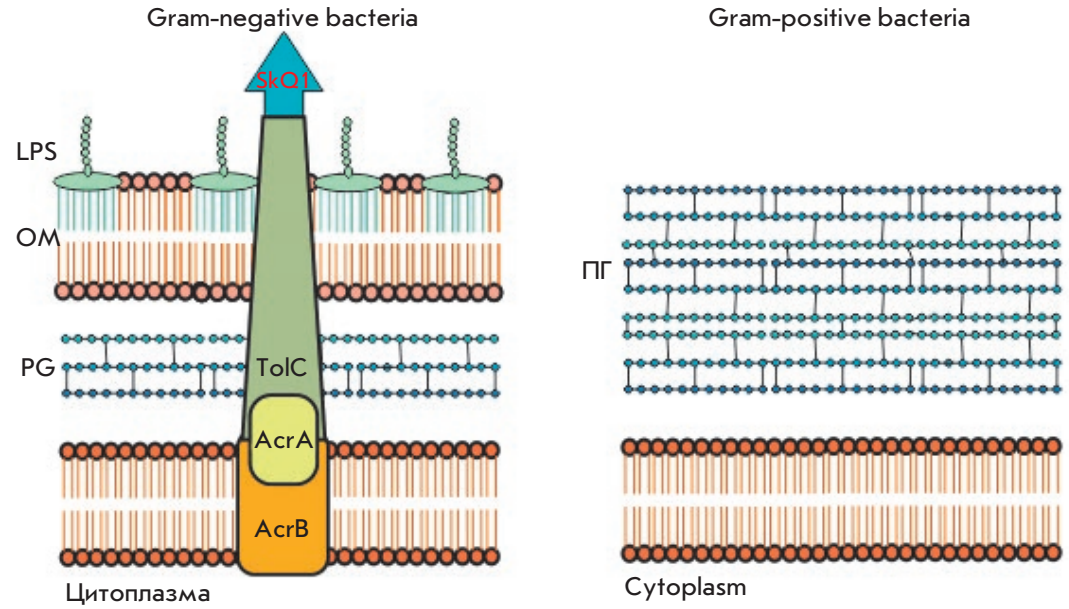

Fig. 1. Schematic of the bacterial cell wall (LPS – lipopolysaccharides, OM – outer membrane, PG – peptidoglycan layer) and the antibacterial effect of SkQ1 against gram-positive and gram-negative bacteria. The sensitivity of gram-negative bacteria to SkQ1 depends on the structure of the protein components of the AcrAB-TolC pump

	MIC, μg/mL
Escherichia coli	19
Rhodobacter sphaeroides	0.6–1.2
Photobacterium phosphoreum	0.6–1.2

	MIC, μg/mL
Bacillus subtilis	0.6–1.2
Staphylococcus aureus	0.6–1.2

lated by AcrZ. On the other hand, SkQ1 resistance might be modulated by local and global transcriptional regulators, as well as through post-transcriptional and post-translational regulation [8].

EXPERIMENTAL

The standard laboratory *E. coli* strains MG1655 and W3110 (F-lambda-IN (rrnD-rrE) 1 rph-1) were used in the study. The *E. coli* strains MC1061, DH5α, and BL21 (DE3) were provided by S.S. Sokolov (Belozersky Institute of Physico-Chemical Biology, Moscow State University); the *E. coli* strain JM109 was provided by L.A. Novikova (Belozersky Institute of Physico-Chemical Biology, Moscow State University); the *E. coli* strain GR70N was received from Yu.V. Bertsova (Belozersky Institute of Physico-Chemical Biology, Moscow State University); and the *E. coli* strain XL1-Blue was purchased from Eurogen company (Moscow, Russia).

The *E. coli* deletion strains ECK0751 (devoid of the *acrZ* gene), ECK0584 (devoid of the *entS* gene), ECK2363 (devoid of the *emrY* gene), ECK2364 (devoid of the *emrK* gene) were kindly provided by H. Niki (National Institute of Genetics, Japan) [9].

Staphylococcus aureus was received from the microorganisms collection of Lomonosov Moscow State University (No. 144). *Photobacterium phosphoreum* was provided by A.D. Ismailov (Belozersky Institute of Physico-Chemical Biology, Moscow State University). *Rhodobacter sphaeroides* was provided by G. Klug (Institute for Microbiology and Molecular Biology at Justus-Liebig-University of Giessen, Germany).

Bacterial cells were grown at 37°C in LB or a Mueller–Hinton medium at a shaking rate of 140 rpm as described in [1].

SkQ1 resistance was studied by double dilutions in a liquid nutrient medium using home-made panels according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Mueller–Hinton broth (HIMEDIA, Mumbai, India) was used in the study. A dilutions panel was prepared in a 96-well microtiter plate in a volume of 200 μL per well. A bacterial suspension (50 μL) in Mueller–Hinton broth was added to each well to a final suspension volume of 250 μL (5×10^5 CFU/mL). The resulting suspension was incubated at 37°C for 20 h [1].

The minimum inhibitory concentration (MIC) was determined as the lowest concentration completely inhibiting bacterial growth. Bacterial growth was observed visually, along with OD_{620} measurements [1].

Table 1. Bacterial susceptibility to SkQ1: measurements of the minimum inhibitory concentration (MIC). Comparison of SkQ1 activity against *Staphylococcus aureus* with that of various antibiotics under identical conditions

Bacterium	Antibiotic	MIC, μg/mL	Reference
E. coli strain			
W3110	SkQ1	19	[1]
MG1655	SkQ1	19	Present study
JM109	SkQ1	19	«
BL21(DE3)	SkQ1	19	«
XL1-Blue	SkQ1	19	«
DH5α	SkQ1	19	«
MC1061	SkQ1	19	«
GR70N	SkQ1	19	«
Deletion *E. coli* **MG1655** strains			
AcrD, AcrE, AcrF, MacA, MacB, MdtA, MdtB, MdtC, MdtE, MdtF, EmrA, EmrB	SkQ1	19	[1]
AcrZ, EmrK, EmrY, EntS	SkQ1	19	Present study
AcrA, AcrB, TolC	SkQ1	0.6–1.2	[1]
R. sphaeroides	SkQ1	0.6–1.2	Present study
P. phosphoreum	SkQ1	0.6–1.2	«
K. pneumoniae	SkQ1	>19	«
S. aureus	SkQ1	0.6–1.2	Present study, [1]
	Kanamycin	2.5	Present study
	«	3.1	[10]
	Chloramphenicol	5	Present study
	«	3.1	[10]
	Ampicillin	2.5	Present study
	«	1.6	[10]
	Streptomycin	6.3	[10]
	Polymyxin B	100	[10]

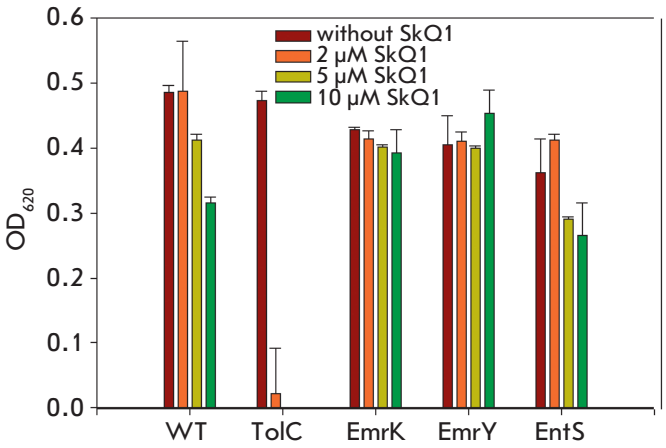

Fig. 2. Toxicity of SkQ1 against the *E. coli* MG1655 strain and its deletion mutants ΔTolC (positive control), ΔEmrK, ΔEmrY, and ΔEntS. SkQ1 (2–10 μM) was added to bacterial cultures (1–5 × 10^5 cells/mL) placed in 96-well plates. Cell density was determined by absorption at 620 nm. After that, bacteria were allowed to grow at 37°C for 20 h and the cell density was again measured. Data are presented as a mean value ± standard deviation for at least three experiments

For bioinformatics analysis, we used the BLASTp search tool (NCBI, https://blast.ncbi.nlm.nih.gov), STRING v.10.5 database (EMBL, http: //string.embl.de/), and BioCyc database from the Pathway/Genome Database Collection (PGDBs, https://biocyc.org/).

RESULTS AND DISCUSSION

We compared the resistance of various *E. coli* laboratory strains and found that all these strains were resistant to SkQ1 (*Table 1*). This is apparently explained by the identity of the primary structure of the AcrA, AcrB, and TolC proteins in all the studied strains [6].

Earlier [1], we showed that the gram-negative bacteria *P. phosphoreum* and *R. sphaeroides*, unlike *E. coli* strains, were not resistant to SkQ1. According to the data given in *Table 2*, the amino acid sequence of the proteins, annotated as AcrB, from these bacteria is quite different from the sequence of the AcrB protein from *E. coli*. The levels of their identity with the *E. coli* AcrB protein are 65 and 33%, respectively, which apparently manifests itself in a rather high sensitivity of these bacteria to SkQ1. Of note, the AcrD protein, removal of which does not affect SkQ1 sensitivity, is 66% identical to the AcrB protein sequence, which is comparable to the AcrB proteins from *P. phospho-*

Table 2. Comparison of the *acrB* gene sequences from different strains of gram-negative bacteria with the *acrB* sequence from the *E. coli* strain

Bacterium	Identification number	Overlap, %	Identity, %	Resistance to SkQ1
E. coli MG1655	NP_414995.1	100	100	YES
E. coli W3110	BAE76241.1	100	100	YES
E. coli AcrB*	NP_416965.1*	99	66	NO
E. coli BL21(DE3)	CAQ30935.1	100	100	YES
E. coli DH5α	KGA88788.1	100	100	YES
R. sphaeroides	ANS33442.1	97	33	NO
P. phosphoreum	CEO37741.1	98	65	NO
K. pneumoniae	CDO13174.1	99	91.5	YES

Note. In the case of the *E. coli* AcrB deletion mutant MG1655, denoted by an asterisk, comparison was performed with the AcrD protein sequence. The amino acid sequence identity was defined as the percentage of identical amino acid residues at the corresponding positions in aligned sequences. Overlap was defined as the percentage of aligned AcrB protein sequences. The absence (NO) and presence (YES) of resistance to SkQ1 was determined with respect to *E. coli*, where MIC of SkQ1 comparable to MIC of SkQ1 for *E. coli* was a criterion for resistance.

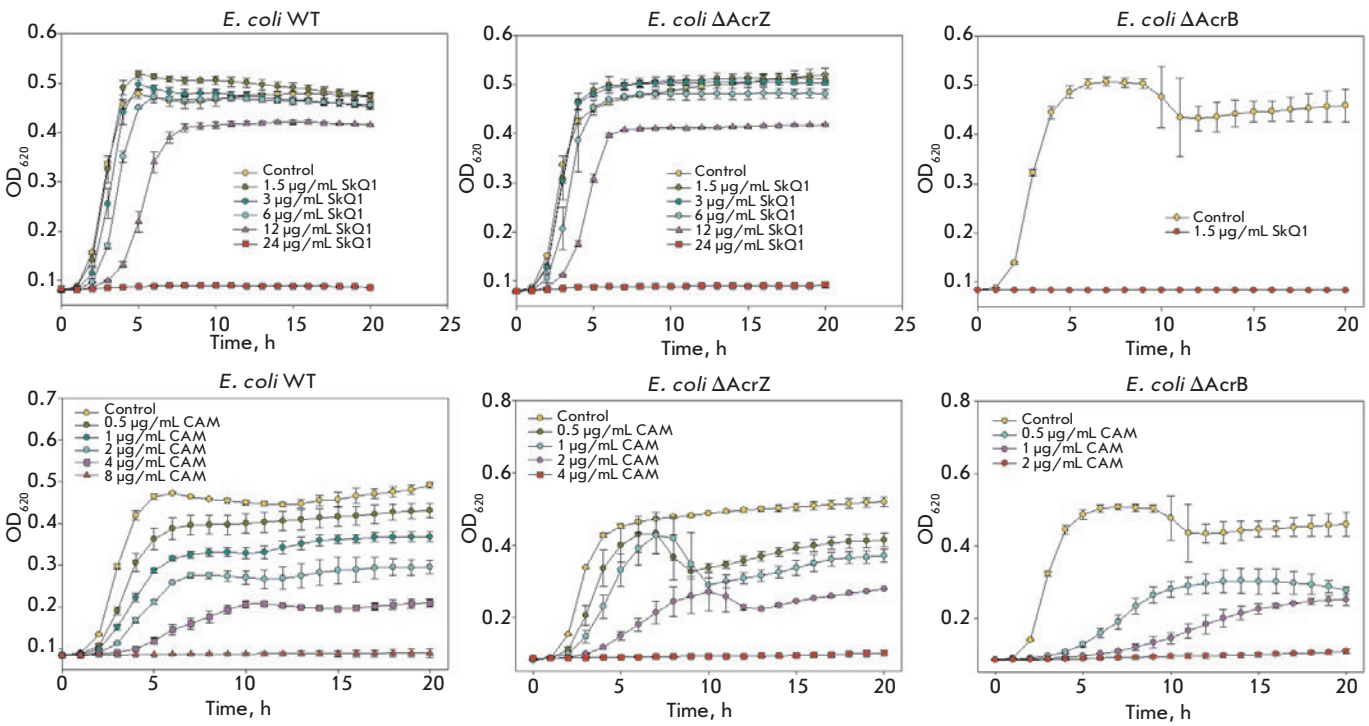

Fig. 3. Effect of SkQ1 (upper panel) and chloramphenicol (CAM) (lower panel) on growth of *E. coli* bacteria (WT, ΔAcrB, and ΔAcrZ). SkQ1 (2.5–40 μM) or chloramphenicol (0.5–8 μg/mL) was added to the bacterial cultures (5×10^5 cells/mL) placed in 96-well plates. Growth was assessed by hourly measured absorbance at 620 nm on a Multiskan FC plate reader (Thermo Fisher Scientific) during incubation. Bacteria were incubated at 37°C for 20 h. Data points are mean value ± standard deviations for at least three experiments

reum and *R. sphaeroides*. Therefore, SkQ1 resistance in bacteria requires a higher similarity of the amino acid sequence of their AcrB protein to the *E. coli* AcrB protein sequence. To examine this conclusion, we determined the primary structure of the AcrB protein from another gram-negative bacterium, *Klebsiella pneumoniae*, which was found to be 91.5% identical to the *E. coli* AcrB protein structure. This suggested the presence of SkQ1 resistance in *K. pneumoniae*, which was confirmed experimentally (*Tables 1* and *2*).

An analysis of the SkQ1 antibacterial activity in *E. coli* mutants with deletions of the EmrK, EmrY, and EntS proteins (*Fig. 2*) revealed that the minimum inhibitory concentrations of SkQ1 were the same as those determined for the wild-type *E. coli* strain (*Table 1*).

To elucidate the role of the AcrZ protein in *E. coli* resistance to SkQ1, we compared the resistance of the wild-type *E. coli* MG1655 strain and that of strains with deletions of the AcrZ and AcrB proteins. If the AcrZ protein is involved in the AcrAB-TolC MDR pump functioning with formation of the AcrABZ–TolC complex, then removal of SkQ1 requires that the stability of the AcrZ protein deletion mutant be higher than that of the AcrB protein deletion mutant but lower than that of the wild-type protein. If the AcrZ protein is not involved in the AcrAB-TolC pump functioning, then the resistance of the AcrZ protein deletion mutant should be the in the wild-type strain and higher than in the case of an AcrB protein deletion. As a positive control in these experiments, we used chloramphenicol [10], removal of which from the cell is enhanced by the AcrZ protein [4]. The AcrZ protein impact on resistance to SkQ1 and chloramphenicol was determined simultaneously to exclude the impact of experimental conditions on the obtained result.

In our experiments, the AcrZ protein deletion mutant exhibited SkQ1 resistance similar to that in wild-type *E. coli* strains (*Fig. 3*), while three *E. coli* strains (WT, ΔAcrZ, and ΔAcrB) demonstrated different levels of resistance to chloramphenicol (*Fig. 3*), as described previously [4]. According to [4], binding of AcrZ to AcrB may cause conformational changes in its periplasmic domain, which affects recognition and capture of low hydrophobic substrates. Because SkQ1 is a highly hydrophobic compound (logP = 4.11) [11, 12], its recognition by the pump may not be regulated by the binding of AcrZ to AcrB.

An analysis of deletion mutants revealed that the EmrKY-TolC pump is not involved in the expelling of SkQ1 from the bacterial cell. Removal of the *entS* gene also had no effect on the expelling of SkQ1 from the bacterial cell. Thus, our conclusion that AcrAB-TolC was the only pump expelling SkQ1 was confirmed.

Another possible modulator of resistance to SkQ1 may be 6S RNA, a regulator of sigma-70-dependent gene transcription [13]. Our preliminary studies did not reveal differences in SkQ1 resistance between an *E. coli* SsrS protein deletion mutant and the wild-type *E. coli* strain. It cannot be ruled out that resistance to antibiotics targeting bacterial bioenergetics, such as SkQ1, may be enhanced by a trivial increase in the expression level. Expression of pleiotropic drug resistance pumps in *Saccharomyses cerevisiae* yeast was recently shown [13] to be induced by dodecyl triphenylphosphonium, another member of this class of antibiotics: i.e., dodecyl triphenylphosphonium can act as both an activator and an inhibitor of drug resistance [14, 15]. However, there is no direct correlation between temporal activation of expression and a constant increase in pleiotropic resistance to these compounds.

CONCLUSION

Therefore, these findings indicate that SkQ1 is an effective antibiotic; SkQ1 resistance in *E. coli* bacteria is associated only with the AcrAB-TolC pump. An essential factor underlying SkQ1 resistance in other gram-negative bacteria is the identity of their AcrB proteins to AcrB from *E. coli*. The AcrZ protein is not involved in the development of SkQ1 resistance; in other words, the routine way to regulate resistance by affecting the AcrAB-TolC MDR pump through the AcrZ protein is ineffective in the case of SkQ1. ●

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Tear Fluid Catecholamines As Biomarkers of the Parkinson's Disease: A Clinical and Experimental Study

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ABSTRACT **An important approach to an early diagnosis of Parkinson's disease (PD) is screening for peripheral biomarkers in patients at the early clinical stage. In this study, we evaluated catecholamine concentration changes in the tear fluid of untreated PD patients as biomarkers. Norepinephrine and dopamine concentrations in the tear fluid of patients were found to increase compared to those in age controls, which was especially pronounced on the side where motor symptoms appeared. On the contrary, the epinephrine concentration in the tear fluid of patients was reduced bilaterally. Since there was no reason to consider the markers found in the clinical stage of PD as markers of the preclinical stage, we additionally studied the tear fluid composition in mouse neurotoxic models of PD preclinical and clinical stages. The norepinephrine concentration in the tear fluid of mice from the clinical stage model was found to be higher than that in controls; in the preclinical stage model, the norepinephrine concentration had a tendency to increase. Therefore, both PD patients and mice from PD preclinical and clinical stage models manifest unidirectional changes in their tear fluid compositions, which may be considered as promising biomarkers for the development of early diagnosis.**
KEYWORDS **Parkinson's disease, tear fluid, patients, experimental models, biomarkers.**
ABBREVIATIONS **DHBA – 3,4-dihydroxybenzylamine; HPLC-ED – high-performance liquid chromatography with electrochemical detection; MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD – Parkinson's disease.**

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized, in particular, by the death of the dopaminergic neurons of the brain nigrostriatal system. Clinically, PD manifests itself many years after the disease onset, after most of the nigral dopaminergic neurons have died, which explains the limitations of the current PD pharmacotherapy [1]. Therefore, the challenge is to develop an early (preclinical) diagnosis of PD, which would enable detection of the disease before the appearance of the first motor symptoms and early start of preventive neuroprotective therapy [1].

In recent years, growing attention has been focused on the investigation of visual system changes in PD and the underlying pathological processes in the eye and accessory visual structures, which may be potential sources of peripheral PD biomarkers [2]. An important role is played by the impaired metabolism of the catecholamines that ensure the transmission of visual information in the retina and regulate the accommodation rate and intraocular pressure [2]. In addition, PD is accompanied by changes in the eyelid tissue containing numerous glands whose secretory products form the tear fluid. The conjunctiva lining the inner surface of the eyelids and conjunctival glands display sympathetic innervation [3]; dysfunction of the conjunctiva occurs in PD as part of a multisystem degeneration affecting both the central and peripheral parts of the autonomic nervous system [4].

Tear fluid sampling is a simple non-invasive procedure, in contrast to the blood or cerebrospinal fluid sampling traditionally used to screen for peripheral biomarkers [5]. However, only a few studies have been

Table 1. Clinical characteristics of patient cohorts

Cohort	N	Gender, m/f	Age, years	PD stage assessment			Disease duration, years
				Hoehn-Yahr scale	UPDRS II (daily activity)	UPDRS III (motor activity)	
PD patients	26	16/10	60.3 ± 2.0	1.8 ± 0.1	8.7 ± 1.0	23.6 ± 2.3	2.6 ± 0.3
Control	19	4/15	57.4 ± 2.9	–	–	–	–

focused on a search for PD biomarkers in the tear fluid, with only the protein composition being analyzed. For example, the tear fluid of PD patients has elevated levels of the tumor necrosis factor [6] and oligomeric α-synuclein [7], as well as a changed proteomic profile in general [8]. These studies indicate that there is a prospect of searching for PD biomarkers in the tear fluid. However, it makes sense to analyze not only proteins, but also the low-molecular substances involved in the PD pathogenesis, in particular the catecholamines that have been actively studied as potential blood and cerebrospinal fluid biomarkers of PD [9].

It should be emphasized that there is no data indicating that the biomarkers found in patients with a diagnosed clinical stage of PD could be related to the preclinical stage of this disease [1]. Therefore, we performed a comparative analysis of catecholamine content changes in the tear fluid of untreated PD patients at the early clinical stage and animal models of the preclinical and clinical stages of PD.

MATERIALS AND METHODS

PD patients and a control group

We analyzed tear fluid samples from 26 PD patients at Hoehn-Yahr stage 1–2 before the start of anti-parkinsonian therapy and from subjects of similar age without motor impairment. All patients gave written consent to participate in the study.

The PD diagnosis was made in accordance with the 2015 Movement Disorder Society (MDS-2015) clinical diagnostic criteria. The control group included individuals of the same age without neurological diseases. Patients with ophthalmic diseases were not included in the study. The key clinical characteristics of the cohorts are presented in *Table 1*.

The tear fluid was collected in the morning using sterile filter paper (5 mm wide) that was placed behind the lower eyelid, as in the Schirmer test. The tear fluid was collected by natural sorption on a test strip, without lacrimation stimulation, for 5 min. The length of the moistened strip was measured to calculate the sample volume, after which the strips were placed in test tubes with 0.1 N $HClO_4$, frozen in liquid nitrogen, and stored at −70°C.

Animals

We used 30 male C57BL/6 mice aged 2–2.5 months and weighing 22–26 g (Pushchino nursery). The animals were kept under standard conditions with free access to food and water. PD at the preclinical stage was modelled by two subcutaneous injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sigma, USA) at a single dose of 8 mg/kg. PD at the clinical stage was modelled by four subcutaneous MPTP injections at a dose of 10 mg/kg. The interval between the injections in both models was 2 h [10]. The control group received 0.9% NaCl according to a similar schedule.

Two weeks after administration of MPTP or 0.9% NaCl, the condition of the mice was assessed by the distance traveled in an open field test using a PhenoMaster animal behavior analysis system (TSE Systems, Germany). Then, the tear fluid was collected from the animals under isoflurane anesthesia, using 2.5-mm wide filter paper strips similar to Schirmer's strips.

After collecting the tear fluid, the anesthetized mice were decapitated and the dorsal striatum was dissected from the brain according to the previously described technique [10]. Striatum samples were weighed, frozen in liquid nitrogen, and stored at −70°C.

High Performance Liquid Chromatography (HPLC)

The concentration of catecholamines (norepinephrine, epinephrine, and dopamine) was measured using high-performance liquid chromatography with electrochemical detection (HPLC-ED). Samples were homogenized using a Labsonic M ultrasonic homogenizer (Sartorius, France) in 200 μL of 0.1 N $HClO_4$ (Sigma, USA) containing an internal standard of 25 pM/mL 3,4-dihydroxybenzylamine (DHBA, Sigma) and centrifuged at 2 000 *g* for 20 min.

HPLC was performed on a ReproSil-Pur ODS-3 reversed-phase column, 4 × 100 mm, 3 μm pore size (Dr. Majsch GmbH, Germany), at a temperature of +30°C and a mobile phase rate of 1.2 mL/min using a LC-20ADsp liquid chromatograph (Shimadzu, Japan), as described previously [11].

Statistics

The HPLC data are presented as percentage means (normalized to control) ± standard error of the mean.

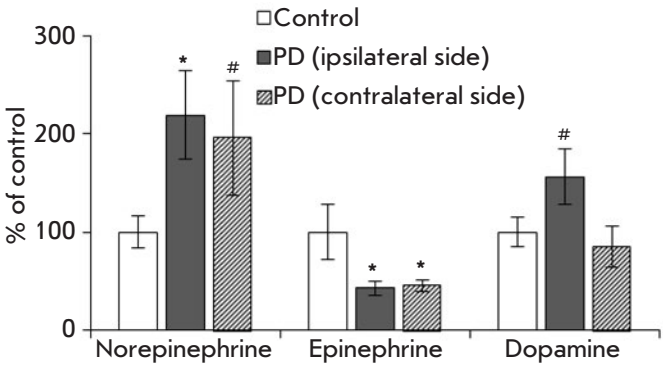

Fig. 1. Catecholamine concentration in the tear fluid of PD patients sampled from the eye on the side of motor symptom appearance (ipsilateral) or on the opposite side (contralateral). * $p \leq 0.05$, # $p \leq 0.05$ relative to the control

Since PD develops asymmetrically, the data collected from the patients were allocated into ipsilateral side data (tear fluid samples collected from the eye on the side where motor symptoms appeared and had greater severity) and contralateral side data (samples collected on the side where motor symptoms were absent or had mild severity). In the control group and experimental animals, the data obtained from the analysis of the tear fluid from the right and left eyes were averaged.

Normality of the data was examined using the Shapiro–Wilk test. Statistical analysis of the data was performed by the parametric Student *t*-test or the nonparametric Mann–Whitney test, using the GraphPad Prism 6.0 software (GraphPad Software, USA). The significance criterion was $p \leq 0.05$.

RESULTS AND DISCUSSION

In this work, the catecholamine concentration in the tear fluid of untreated PD patients was measured for the first time. The norepinephrine and dopamine levels in the tear fluid of patients were shown to be significantly higher than in healthy subjects (controls) of the same age (*Fig. 1*). An important asymmetry was found: the increase in the norepinephrine and dopamine concentrations was more pronounced on the ipsilateral side (on the side of more severe motor symptoms), compared to the contralateral side. Because there were no statistically significant differences in the volume of the collected samples (data not shown), the revealed asymmetry in the dopamine and norepinephrine concentrations in PD patients cannot be explained by asymmetric hypokinesia of the eye muscles with an impaired tear outflow. However, the asymmetry of marker content changes in the tear fluid is in good agreement with the known facts regarding the asymmetric nature of PD development. For example, in the early clinical stage of PD, the threshold degradation of the nigrostriatal dopaminergic system and motor disorders appear only on one side [12]. It should be emphasized that tear composition asymmetry is the first observation of asymmetric PD development in the peripheral organs [12].

Unlike norepinephrine and dopamine, the epinephrine level in the tear fluid decreased on both the ipsilateral and contralateral sides (*Fig. 1*). This response is similar to the previously established decrease in plasma epinephrine in PD patients [9]. It should be noted that the source of catecholamines in the tear fluid is not exactly known. It is known that epinephrine enters the bloodstream from the adrenal glands, while norepinephrine and dopamine mainly originate from sympathetic noradrenergic nerve terminals [13]. Probably, the catecholamines found in the tear fluid are of similar origin.

The catecholamine concentration changes in the tear fluid of PD patients could potentially be used to develop an early diagnosis. However, there is always a risk that the biomarkers detected in patients at the clinical stage are absent at the preclinical stage. In this regard, experimental modeling of PD is of particular value because it may be used to reproduce both stages of the disease [1, 10]. For example, according to our general methodology, matching of the biomarkers found both in patients and in animal models of PD clinical stage indicates a correct reproduction of these aspects of the disease pathogenesis (*Fig. 2*). Some of them may be considered as biomarkers of a PD preclinical stage if they are also detected in a preclinical stage model [9].

An important feature of PD is the precisely defined neurodegeneration threshold, surpassing of which

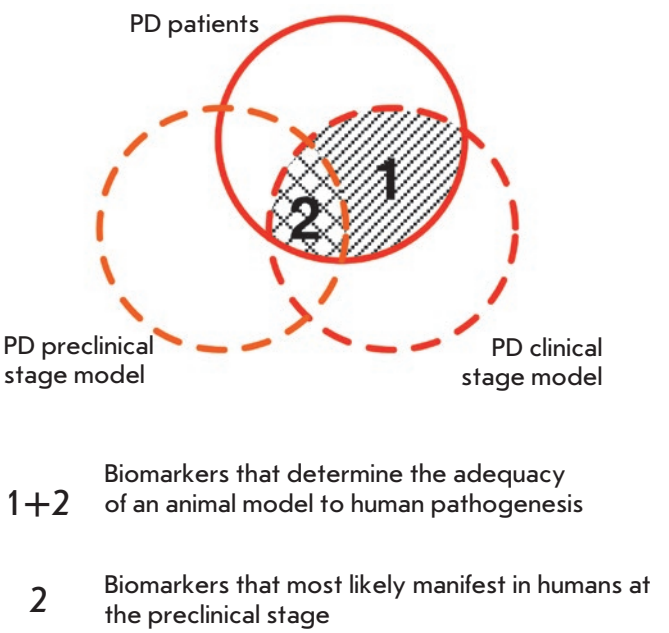

Fig. 2. Schematic of the biomarker test methodology

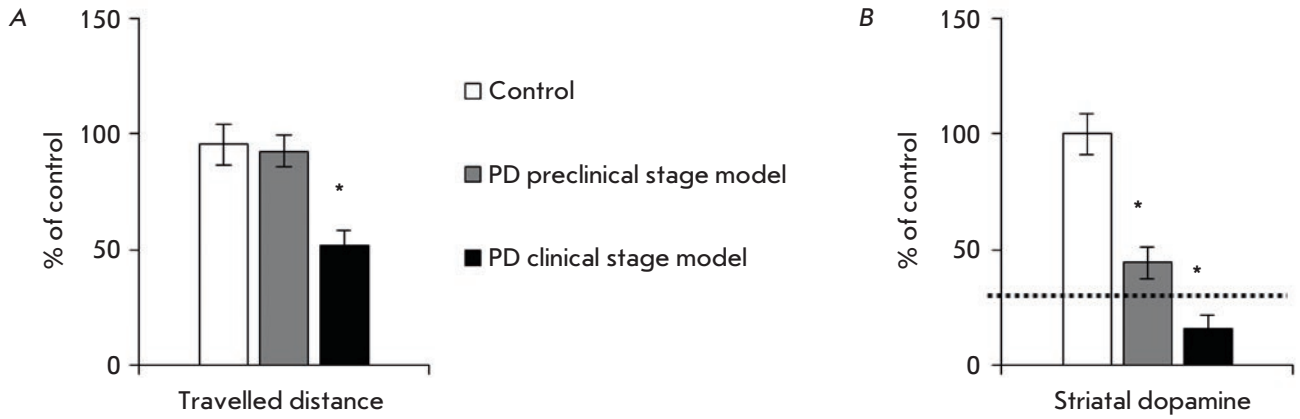

Fig. 3. Total distance in the open-field test (*A*) and the dopamine concentration in the striatum (*B*) of control mice and mice from PD preclinical and clinical stage models. * $p \leq 0.05$ in comparison to control mice; dotted line – threshold of motor symptom appearance

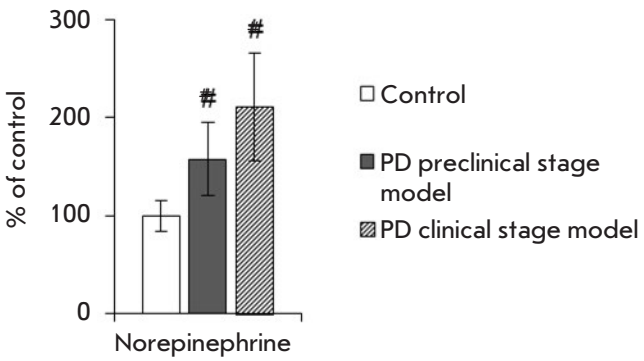

Fig. 4. Norepinephrine concentration in the tear fluid of control mice and mice from PD preclinical and clinical stage models. # $p \leq 0.05$ in comparison to control mice

Table 2. Comparative analysis of biomarkers

Biomarker	PD patients	Mouse PD models	
		Clinical stage	Preclinical stage
Norepinephrine	↑	↑#	↑#
Epinephrine	↓	ND	ND
Dopamine	↑#	ND	ND

Note. ↑, ↓ – an increase and decrease, respectively, in the biomarker concentration in tear fluid compared to the control, ND – not determined, # – tendency ($p < 0.15$).

causes motor symptoms: death of 50–60% of dopaminergic neuronal bodies in substantia nigra, loss of 70–80% of dopaminergic neuron axons in the striatum, and, accordingly, a decrease in the striatal dopamine level by 70–80% compared to the control [1].

According to our findings, 2 weeks after the double administration of MPTP at a dose of 8 mg/kg, mice showed no changes in the open field test, and the striatal dopamine level decreased by 65.6% (*Fig. 3*). In turn, in animals that received four injections of 10 mg/kg MPTP, the traveled distance parameter decreased by almost half and the striatal dopamine level dropped by 83.3% (*Fig. 3*). Therefore, the reproduced models of PD preclinical and early clinical stages fully correspond to the key parameters described above.

The norepinephrine concentration in the tear fluid of mice had a tendency ($p < 0.15$) to increase by 57.6% in the PD preclinical stage model and by 111% in the PD clinical stage model compared with that in the control (*Fig. 4*). The epinephrine and dopamine levels were below the detection limit, which is most likely due to the small volume of the collected samples (1 to 2 μL). There are methods to stimulate lacrimation in animals using cholinomimetics [14], but the composition of stimulated tear fluid is known to vary significantly [15].

Comparison of the changes in the levels of norepinephrine, epinephrine, and dopamine in the tear fluid of PD patients and in mouse PD models revealed an increase in the norepinephrine concentration in all three cases, compared to that in the controls (*Table 2*).

Therefore, an increase in the norepinephrine concentration in the tear fluid was found in both PD patients and mouse models of PD preclinical and clinical stages. This may be considered as a promising biomarker for an early diagnosis of PD. ●

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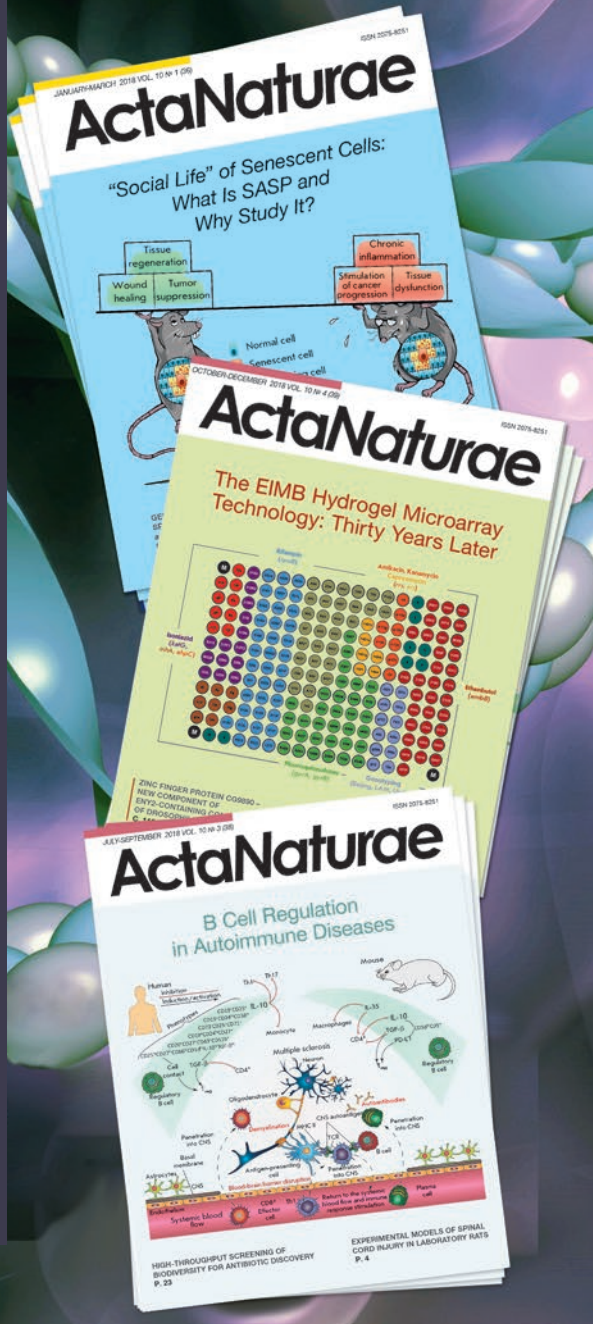

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