
REVIEWS

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Ribonucleases as Antiviral Agents

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Abstract—Many ribonucleases (RNases) are able to inhibit the reproduction of viruses in infected cell cultures and laboratory animals, but the molecular mechanisms of their antiviral activity remain unclear. The review discusses the well-known RNases that possess established antiviral effects, including both intracellular RNases (RNase L, MCPIP1 protein, and eosinophil-associated RNases) and exogenous RNases (RNase A, BS-RNase, onconase, binase, and synthetic RNases). Attention is paid to two important, but not always obligatory, aspects of molecules of RNases that have antiviral properties, i.e., catalytic activity and ability to dimerize. The hypothetical scheme of virus elimination by exogenous RNases that reflects possible types of interaction of viruses and RNases with a cell is proposed. The evidence for RNases as classical components of immune defense and thus perspective agents for the development of new antiviral therapeutics is proposed.

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Ribonucleases (RNases) that catalyze RNA cleavage play a key role in the regulation of vital processes in any organism, ranging from virus to human. Without the reactions of RNA degradation, the maturation of mRNA and noncoding RNA, the functioning of global systems of RNA interference, and the epigenetic regulation of gene expression, as well as processes of cell growth and differentiation, apoptosis induction, and protection against viral infection, are not possible. There are approximately 20 exo- and endonucleases of various nucleotide sequence and structure specificity in a cell [1]. Along with intracellular RNases, there are RNases that may be secreted into cultural or tissue fluid. Biological effects of exogenous RNases are of particular interest. The control of blood-vessel growth, toxicity against tumor cells, and antiviral activity are all properties of RNases that may potentially be utilized in medicine and define the current hot points in studies focused on these enzymes. In the review, we consider the best-known RNase that possesses antiviral effects. Molecular structures of these RNases are presented in Fig. 1; obviously, they are not characterized by a significant homology. Therefore, the structural similarity of the catalytically active proteins is not a determining feature for their antiviral effects.

In the 1960s, a group of scientists under the guidance of Prof. P. I. Salganik at the Institute of Cytology

and Genetics (Siberian Branch, Russian Academy of Sciences) demonstrated that increased activity of RNA-cleaving enzymes is observed in the blood and cerebrospinal fluid of tick-borne encephalitis patients [2]. RNases were hypothesized to be directly involved in biological mechanisms of antiviral protection. The activation of intracellular RNases in the presence of antiviral preparations was later detected in plants [3]. In particular, transgenic tobacco plants with increased activity of intracellular *Zinnia elegans* ribonuclease ZRNaseII possessed high stability against the tobacco mosaic virus [4]. Bacterial endoribonucleases specific to certain nucleotide sequences, e.g., the RNase ToxN, provide for the phage stability of the population, inducing death of cells due to cleavage of phage and cellular RNA [5]. Cleavage of tRNA by an anticodon nuclease PrrC of *Escherichia coli* is a mechanism of bacteria protection from infection with T4 phage [6].

The involvement of RNases in the protection of cells and the organism from viruses has been confirmed by ample evidence. A considerable body of data has been accumulated and allows one to consider RNases to be not only components of immune defense, but also the basis for the development of new antiviral preparations.

RNase L

The antiviral effect of RNases is best studied in the case of a signaling system involving RNase L, which mediates the effect of interferon induced by viral infection. The key enzyme of the system is 2',5'-oli-

Abbreviations: MCPIP1, monocyte chemoattractant protein-induced protein 1; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; siRNA, small interfering RNAs.



Fig. 1. Three-dimensional structures of antiviral RNases of various origin obtained using the Jmol program and the protein database (www.jmol.org and www.pdb.org). 1. binase, 2. onconase, 3. RNase from *Rana catesbeiana*, 4. bovine pancreatic RNase, 5. BS-RNase, 6. MCP1P1, 7. ECP, 8. EDN, and 9. RNase L.

goadenylate synthetase polymerizing ATP with formation of RNase L activator, 2',5'-oligoadenylate, of the general formula ppp2',5'-A_n, where $n = 2-10$ adenylate residues. RNase L mechanism of action is well studied, i.e., the active form of RNase L forms a dimer

with endoribonucleolytic activity against both viral and cellular RNA [7, 8]. Products of RNA cleavage less than 200 nucleotide long are recognized by protein factors RIG-I and MDA5. Therefore, the formation of RNA fragments enhanced by RNase L, followed by

their interaction with RIG-I and MDA5, activates transcription factor NF- κ B and triggers transcription of interferon- β gene, which prevents virus replication and stimulates the growth of immune-system cells [9]. However, the system may not completely protect cells from virus. In enteroviruses of group C, a phylogenetically conserved RNA structure resistant to cleavage by RNase L and inhibiting the activity of its endoribonuclease domain has been found inside an open reading frame [10]. A neurotropic Theiler's picornavirus inducing chronic infection of central nervous system and demyelination of nervous tissue produces a species-specific helper protein L* that inhibits RNase L through interaction with its ankyrin domain [11]. These viruses possess mechanisms that provide for their resistance to RNase L, because they may block either its activity or ability for dimer formation. Interestingly, dimerization contributes to the antiviral activity of RNases L, along with the catalytic activity, which is required for viral RNA cleavage a priori.

MCPIP1 PROTEIN

Zinc finger proteins are known to possess an antiviral effect; due to the cleavage of the polyA end of the mRNA, they induce and enhance RNA turnover in cell [12, 13]. The introduction of domains with high catalytic nuclease activity into these proteins promotes enhancement of antiviral properties. For example, a hybrid construct based on a synthetic protein that belongs to this group and staphylococcus nuclease was obtained that prevents the replication of the DNA-containing human papilloma virus, [14].

Monocyte chemoattractant protein-induced protein 1 (MCPIP1) also belongs to zinc finger proteins. The protein contains two conserved domains, i.e., the CCCH sequence (zinc fingers) and NYN nuclease domain. MCPIP1 is involved in the regulation of the inflammatory response in cell, and it is the nuclease domain that binds and destroys the viral RNA [15]. This requires RNase activity and dimerization of the protein. MCPIP1 cleaves viral RNA and cellular mRNA, as well as the microRNA precursor, in a Mg²⁺-dependent reaction [16]. An increased level of the protein induced by inflammatory cytokines (such as tumor necrosis factor α (TNF- α), interleukin-1 β , lipopolysaccharides) inhibits the replication of Dengue fever virus and Japanese encephalitis virus. However, three other proteins of the MCPIP group that contain both the CCCH sequence and nuclease domain, but have no proline-rich domain and thus are unable to form dimers, possess no antiviral activity [15]. These data emphasize the requirement for the dimerization of the MCPIP proteins for manifestation of antiviral activity independently of the presence of the zinc finger domain in the molecule.

EOSINOPHIL-ASSOCIATED RNases

The main RNases associated with eosinophils, the eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), possess antimicrobial, antihelminthic, and antiviral activity caused by their ability to catalytically cleave ssRNA. These RNases are considered as potential agents against viral infections of the lungs [17]. At the level of amino acid sequences, they possess certain homology with bovine pancreatic ribonuclease and belong to a large superfamily of RNase A, the members of which have disulfide bonds in their structures. ECP, or RNase 3, is the most cationic (pI = 11) and the least catalytically active protein of the family, and EDN (RNase 2, pI = 9) possesses approximately 100-fold higher activity [17]. Only EDN that possesses a unique loop of nine amino acid residues (L7) that differs different from a similar loop in ECP at its C-terminus exhibits high activity against the respiratory syncytial virus associated with the contribution of this structure to interaction of RNase with viral capsid and penetration inside the virion [18]. Rosenberg [19], who studies eosinophil-associated RNases, supposes that respiratory viruses containing ssRNA are evolutionarily established mobile targets for EDN, which directly utilizes its ribonucleolytic activity for the antiviral effect. Furthermore, in experiments in vitro, EDN decreased the infecting ability of another ssRNA-containing virus, i.e., human immunodeficiency virus (HIV-1) [20, 21].

AMPHIBIAN RNases

Previously, onconase, an RNases from oocytes of the leopard frog *Rana pipiens*, efficiently suppresses the replication of HIV-1 due to the selective degradation of viral RNA, which exhibits no pronounced cytotoxic effect on infected human cells [22]. Later, onconase was found to be capable of degrading cell tRNA, but not the rRNA and mRNA protected with proteins. This accelerates the tRNA degradation/synthesis cycle and degradation products, which can probably serve as primers for viral replication, are also destroyed by onconase, leading to the inhibition of virus replication [23]. An RNase from the *R. catesbeiana* frog, homologous to onconase, efficiently blocks the replication of an RNA-containing virus of Japanese encephalitis and stimulates activation of caspases 3, 8, and 9, inducing apoptosis of infected BHK-21 cells [24]. Modern studies demonstrated that both onconase and RNase A are inefficient with respect to the respiratory syncytial virus [28]. At the same time, onconase and rAmphinase 2 (a recombinant protein similar to onconase) inhibited the replication of DNA-containing viruses, such as herpes simplex virus types 1 and 2, Epstein-Barr virus, Kaposi sarcoma-associated herpes virus, cytomegalovirus, and roseolovirus, without causing infected cell death; the two latter virus types were the most sensitive to these RNases [25]. Therefore, the antiviral effect, even of

closely related amphibian RNases, does not cover all viruses; certain RNases are active against certain viruses. This is due to the specific features of the structure of various viruses, the versatility of the organization, and the properties of the cell they target, as well as the variability of molecular structures and the level of RNase catalytic activity.

RNase A

The earliest studies on the antiviral activity of RNases were performed using pancreatic RNase as an agent that quickly normalized the state and decreased the symptoms of meningitis and cerebrospinal pleocytosis in patients with tick-borne encephalitis [26]. The first preparation was registered as Amorphous Ribonuclease (registration number 68/333/22, registration date April 30, 1968). Today, cattle pancreatic ribonuclease (RNase A) is produced in Russia by the Samson-Med joint company under the trade name of Ribonuclease (registration number LS-000391, date of registration April 10, 2010) in the form of tablets and lyophilizate for preparing injections and local administration solution. The preparation is advised for inflammatory disorders of the airways (tracheitis, bronchitis, pneumonia, bronchiectatic disease, sinusitis), parodontosis, osteomyelitis, thrombophlebitis, abscesses, viral meningitis, and tick-borne encephalitis [27]. Therefore, the only approved RNase-based antiviral preparation is the preparation obtained from the bovine pancreas.

Today, the improvement of the antiviral agents based on RNase A is ongoing. Conjugates of RNase A with ligand-free human serum albumin that, unlike the initial enzyme, exhibit activity against dsRNA and possess high activity against influenza A and B viruses have been created [28]. RNase A is a component of a complex with gold nanoparticles and oligonucleotides complementary to RNA sequences (nucleotides at positions 322–339) of hepatitis B virus. The complex decreased the content of viral RNA in mice with hepatitis B by 99% [29]. Despite the high probability of inhibiting the catalytic activity of RNase A by cell cytosol inhibitor [30, 31], its functions as one of the major representatives of the wide family of mammalian RNases in the evolutionarily developed system of non-specific immunity are doubtless [32].

BS-RNase

Bovine pancreatic RNase does not possess considerable activity against HIV-1, while bovine seminal RNase (BS-RNase) inhibited the replication of the virus in H9 leukemia cells [33]. It was demonstrated that cleavage of dsRNA by BS-RNase is enhanced in the presence of interferon- γ , which probably contributes to the mechanisms of antiviral immune defense [34]. Let us note that BS-RNase is a natural dimer, monomers of which are linked by two intramolecular

disulfide bonds. This structure imparts the molecule stability against cytosolic RNase inhibitor and decreases its toxic effects compared to the monomer [33]. It should also be noted that many RNases tend to form oligomeric structures, including RNase A lyophilized from a solution in 40% acetic acid forms dimers, trimers, tetramers, and higher order multimers [36]. Dimers are formed via the exchange of the terminal domains between the monomers (either C- or N-terminal dimers). An important factor that promotes oligomerization is the hydrophobic nature of the C-terminal part of the molecule and hydrophilic nature of the N-terminal part [37]. In all studied crystal structures of a microbial RNase binase, there are features that indicate dimer formation, in which active center of one of the subunits is closed due to interactions between the subunits [38]. α -Helices of the molecules of BS-RNase, RNase A, human pancreatic RNase H, and binase contain hydrophobic segments capable of participating in both the interaction with the lipid bilayer and dimerization [39]. Among the RNases discussed thus far, only the BS-RNase is an established natural dimer. However, the data on the need for dimer formation by the monomers of RNase L and MCP1P1 [7, 8, 15, 16] for them to acquire the ability to destroy the viral genetic material evidence that the contribution of supramolecular organization of RNases to their antiviral activity has not yet been sufficiently studied.

MICROBIAL RNases

The clinical application of mammalian RNases is not always efficient, since a specific inhibitor present in practically all tissues and cells and necessary for cell protection from its own RNases [31] blocks their catalytic activity. The inhibitor does not deactivate bacterial RNases, and broad possibilities for the design of simple bioengineered constructs based on microbial RNases make them especially attractive for the development of new therapeutic agents.

A series of experimental works of the end of the 20th century was devoted to the comparison of antiviral activity of pancreatic and microbial RNases, in particular, RNase from *Actinomyces rimosus*. Both native and dextran-modified microbial RNase caused greater and more prolonged effect on a DNA-containing Aujeszky's disease virus than RNase A [40]. Binase, or RNase of *Bacillus intermedius* (modern title *B. pumilus* [41]), well studied by now, exhibited high activity against the RNA-containing street rabies virus in infected Guinea pigs, rabbits, and mice when injected to the site of infection [42, 43]. Therapeutic effect of binase was registered both 2–3 h after infection and 1 day later (57–67% animal protection); the enzyme did not affect the formation of vaccine immunity. A single intraperitoneal injection of binase to rabbits infected with foot-and-mouth disease virus type O and type A22-550 decreased animal mortality by

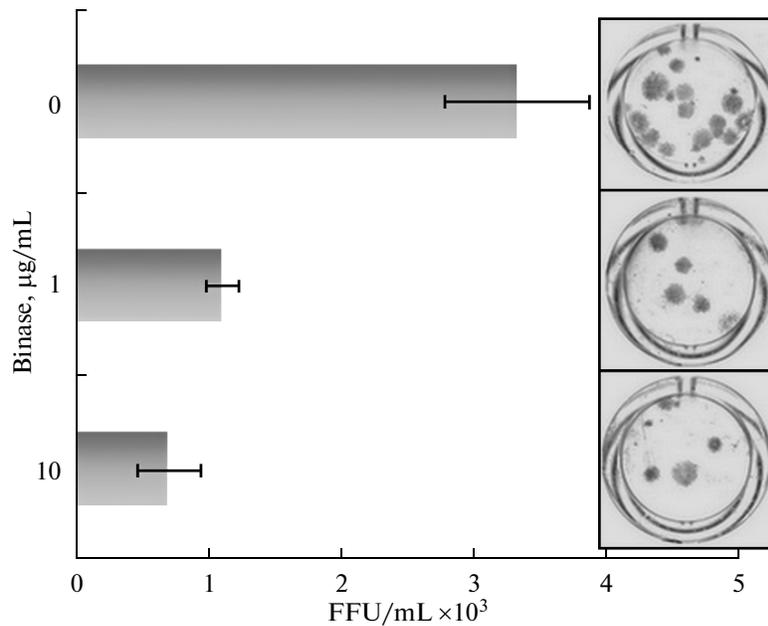


Fig. 2. Decrease of virus infectious particle number (focus forming units, FFU) in A549 cells infected with influenza A (H1N1) virus after treatment with various concentrations of binase (left) and visualization of the process (right). Virus was preincubated with binase for 45 min, cells were infected with the virus upon multiplicity of the infections of 0.01 and grown for 24 h. The experimental procedure has previously been described in detail in [45].

threefold [44]. Binase also possesses activity against influenza A/Bethesda/10/63, A/Odessa/2882/82, and B/Leningrad/369/76 viruses comparable to that of a classic antiviral agent, rimantadine. Binase was found to be active against influenza virus types A and B, while rimantadine is not active against influenza virus type B [45]. Recently, we demonstrated that, at the non-toxic concentrations to epithelial cells, binase exhibited antiviral activity against the pandemic influenza A/Hamburg/04/09 (H1N1) virus, the causal agent of the epidemic in 2009, upon both single- and multicycle virus reproduction. The short-term treatment of virus infectious particles (15–30 min) with binase at increasing concentrations proportionally decreased the viability of the virus, which manifested by weakening its ability to infect lung adenocarcinoma cells A549 by almost an order of magnitude (Fig. 2) [46]. Importantly, binase does not induce expression of specific marker of immune response antigen CD69 and synthesis of interferon- γ in population of CD8⁺ and CD4⁺ T-lymphocytes, which evinces that the enzyme lacks the ability to induce polyclonal T-cell response of the superantigen type [47].

Screening for bacterial RNases that possess new antiviral properties continues. Works on the isolation and characteristics of secreted RNases by the bacteria of the genus *Pseudomonas* are ongoing [48]. The intracellular ribonuclease of *B. cereus* was found to be efficient against the tobacco mosaic virus [49]. Culture fluid of *B. pumilus* isolated from the sea sponge *Petro-mica citrine* possesses antiviral activity with respect to bovine diarrhea virus [50] and the *B. pumilus* strain

var. Pashkov from midland soils exhibits a wide spectrum of antagonist activity, including the antienteroviral activity [51]. Most likely, the antiviral properties of the bacillus cultural fluid are largely caused by the secreted RNases that correspond or are similar to binase.

SYNTHETIC RNases

Starting in the late 1990s of the 20th century, synthetic RNA-hydrolyzing molecules generated from peptides and containing L-lysine, histamine, or histidine methyl ester residues have been developed [52]. Chemical conjugates of lysine moieties with imidazole model the active center of RNase, contain the RNA-binding and RNA-hydrolyzing domains, and may find application for inactivation of RNA in gene targeting therapy [52]. Mimetics of RNases of another class have been created based on the conjugates of diazabicyclo-[2.2.2]-octane with imidazole; the rate of RNA hydrolysis by these mimetics increases proportionally to the number of positive charges in the molecule [53]. Recently, synthetic RNases were found to act not only on RNA-containing viruses through hydrolysis of viral RNA [54], but also on DNA-containing viruses, in particular cowpox virus, through the destruction of the virus envelope [55].

MECHANISMS OF THE ACTION OF ANTIVIRAL RNases

Modern antiviral agents should be used with due regard to the data on mechanisms of action and the

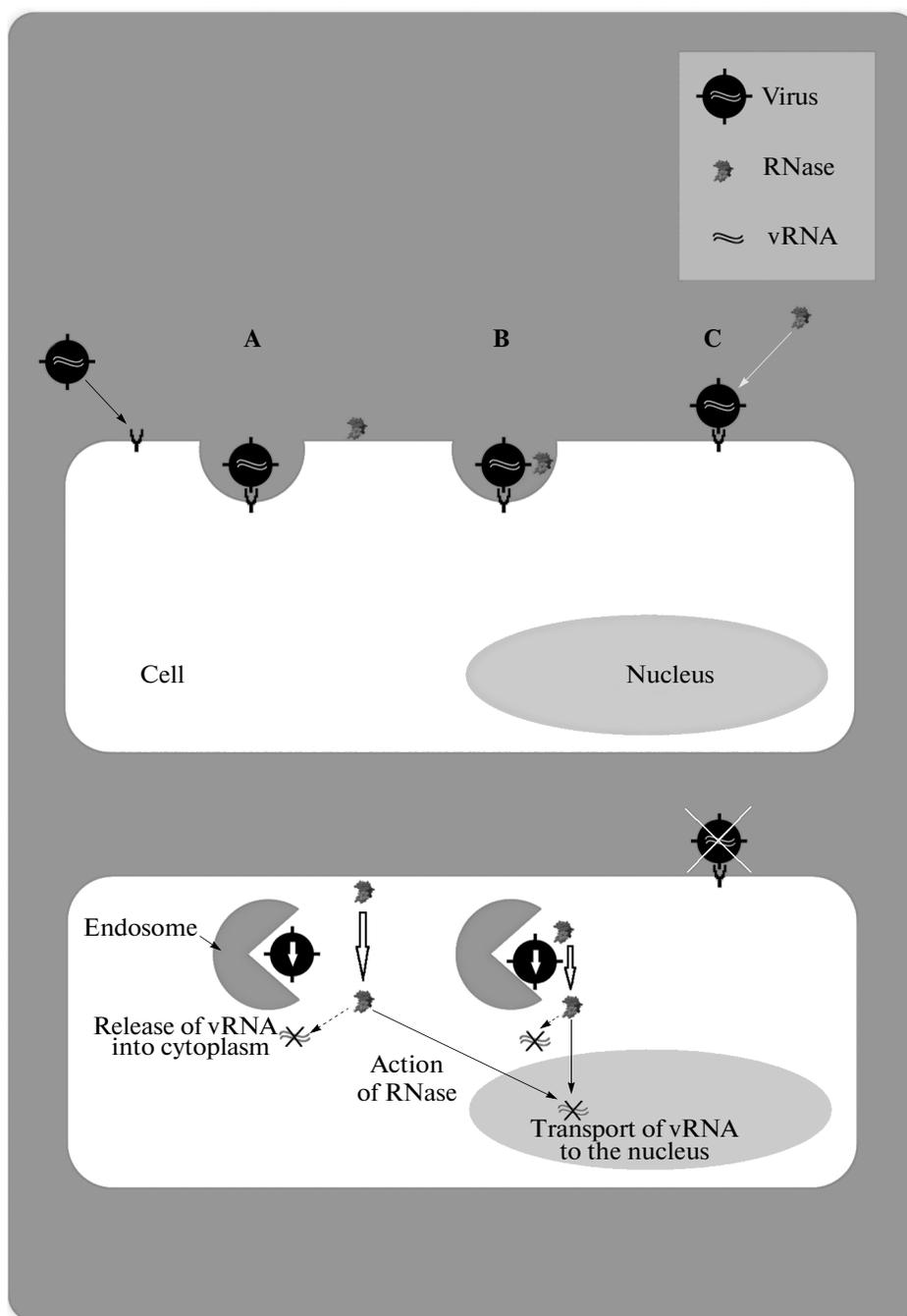


Fig. 3. Hypotheses of the mechanisms of antiviral effect of RNase: (A) independent penetration of virus and RNase into a host cell followed by the release of viral nucleic acid from an endosome into cytoplasm/nucleus and cleavage of viral RNAs by RNase in the nucleus; (B) joint penetration of virus and RNase into an endosome and release of RNase and viral nucleic acid into cytoplasm followed by the cleavage of viral nucleic acid by RNase in the nucleus; and (C) direct effect of the ribonuclease on the virus prior to its penetration into cell.

targets whose damage leads to virus elimination. The most general effect is produced by preparations containing interferon as a stimulator of the cell's natural defense system against the virus or synthetic analogues of nucleosides that block the synthesis of viral nucleic acids. The effect of other agents is selectively targeted against various stages of viral infection development

and the life cycle of the virus, i.e., adsorption, penetration, synthesis of virus components, and the exit of daughter virions from cell. Agents that act upon virus genome are of particular interest; they include anti-sense oligonucleotides [56], ribozymes [57], and RNases discussed here. These agents suppress virus production, but they are probably able to destroy

latent virus infection. Here, we consider three possible mechanisms of the effect of binase on RNA-containing viruses, which may be exerted at any stage of cell infection. At the first stage, when binase meets the virus outside cell, its catalytic activity is not inhibited by the natural RNase and it may destroy viral RNA (Fig. 3, C). Mechanism of binase penetration inside the virion is not clear, however it has been proven that virus infecting ability decreases upon direct treatment of virus particles with binase [46]. At the next stage, binase may interact with the virus inside an endosome since endosomal type of internalization is characteristic for exogenous RNases, e.g. α -sarcine [58], RNase A [59], BS-RNase [60], and binase [61] (Fig. 1). Finally, penetration of binase into cell nucleus is of particular importance: here the enzyme may directly destroy viral RNA (Fig. 3, A and B). Today, localization of exogenous RNases in cell nucleus has been convincingly demonstrated only for BS-RNase [60] and binase [61]. It should be noted that binase penetrated not all cells: for example, the enzyme did not enter the alveolar epithelium MLE-12 cells expressing viral T-antigen on their surface, but rather caused their death [62]. Since a number of facts that indicate the interaction of RNases with cell surface structures has been accumulated [63], the contribution of these structures to the internalization of RNases by infected cells should be taken into account. Therefore, for the development of highly efficient antiviral preparations based on RNases, knowledge of the exact stage at which the enzyme affects the virus is obligatory.

Furthermore, careful attention is focused on the possibility of the intervention of exogenous RNases in the process of RNA interference involved in protection against viruses [64, 65]. The leading role is played by the Dicer RNase producing small interfering RNAs (siRNAs) and using dsRNAs (an intermediate product of virus replication) as a substrate [64]. Furthermore, it has been found that siRNAs specific to a conserved region of influenza virus RNA introduced into a cell decreased the titer of the virus [65]. Antiviral potential of siRNAs is increased upon application in a complex with polycationic carrier [65, 67]. The application of siRNAs in antiviral therapy is not limited to the treatment of the influenza virus. Positive results have been obtained in experiments on laboratory mice infected with a coronavirus causing severe respiratory syndrome and syncytial virus [64]. So far, there are few explicit data on the participation of exogenous RNases in formation and destruction of siRNAs. It was demonstrated that onconase changes the siRNA expression profile in several pleura mesothelioma cell lines through the destruction of precursors of these molecules and thus decreasing the amount of substrate for the Dicer RNase [68]. Therefore, the mechanisms of the antiviral activity of RNases include both the direct effect on the nucleocapsid and nucleic acid and indirect effects, i.e., intervention into the RNA interference, immunomodulation, and induction of apoptosis

in infected cells. Figure 3 demonstrates hypothetical models of the elimination of viral infection by an exogenous RNase in function of the type of interaction with cell: independent penetration (A), joint internalization (B), or outside cell (C).

Considerable economic losses from yearly epidemics cause constant search for new antiviral agents that become useless with time due to high variability of viruses. The study of the molecular mechanism of the action of antiviral RNases is undoubtedly an urgent task, the solution of which may promote the development of novel antiviral preparations capable of protecting the organism independently of changes in the virus genome.

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