

Nuclease Composition with Anti-Rabies Activity

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A composition demonstrating anti-rabies activity was developed on the basis of isoform Sm1 of *Serratia marcescens* endonuclease. Besides the enzyme the composition contained Haemodes-N, magnesium sulfate and sodium chloride. Injection of this composition at a mouse's infection site, two hours after the mouse has been infected with rabies, showed an increase of the infected animals' survival by more than 30% in comparison with the animals of control group.

Key words: Nuclease, *Serratia marcescens*, Sma nuc, Rabies, Postexposure protection.

Rabies is a fatal infectious disease caused by an RNA-containing virus that affects the central nervous system. Incidences of this disease are registered in most countries. Every year about 50,000 people in the world die from the infection, after being bitten by an animal contaminated with rabies^{1,2}.

Currently no treatment for rabies has been developed. Clinically sick animals were put down. In order to prevent people from exposure to the infection, a widely used common tactic consists of immediate local treatment of the wound with subsequent post-exposure anti-rabies preventive and medical treatment vaccinations, based on combined application of anti-rabies vaccine and anti-rabies immunoglobulins³. However, such treatments are successful only if done before the rabies virus penetrates the cells of the central nervous system. Once the virus penetrates into the nervous system, it becomes inaccessible to the action of immune cells, and specific antibodies are unable to exert influence on the infection process.

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Promising anti-rabies effects were found using RNase of gram-positive bacterium *Bacillus intermedius* during experimental infection with rabies. RNase intramuscularly injection in the place of infection after 2 h at the dose of 5 mg/kg showed a 40-70% protection against the rabies disease⁴. However, exceeding this dose the RNase could be toxic and could cause allergic reactions.

Thus, the search of compositions for emergency post-exposure protection against rabies is an important task to solve which the undertaken research aimed to accomplish. For this research, we selected the nuclease of gram negative bacteria *Serratia marcescens*, Sma nuc. This nuclease was earlier found to be similar to *B. intermedius* RNase in its effectiveness in inhibition of RNA-genome bacteriophages⁵. Moreover, due to some antiviral activity, the nuclease was used in beekeeping in order to prevent viral infection of insects^{6,7}. Sma nuc demonstrates a very potent digestive activity towards DNA and RNA^{8,9}. It heads a broad range of homological non-specific nucleases, which widely spread in the world. It is one of the most studied bacterial nuclease. Its structure, mechanisms of action and regulation, as well as physical, chemical and biochemical properties are well known¹⁰⁻¹⁷.

MATERIALS AND METHODS

In the present study, the authors used preparations of yeast DNA ("Sigma", USA). Desintoxicating compound Haemodes-N including (g/l): 0.42- Potassium chloride, 0.5- Calcium chloride, 0.005- Magnesium chloride, 0.23- Sodium hydrocarbonate, 5.5- Sodium chloride, and 60- Povidone 8000, was manufactured by "Drug manufacturer OJSC "BIOSINTEZ" (Russia). Standard CVS virus was kindly provided and characterized by all-Russian state scientific-research control Institute of veterinary compositions (Fgbu VGNI, Moscow, Russia). The viruses were stored at -70° C before use. Contagious activity of the virus was determined by titration using white mice weighing between 6-8 g, by Koprowski's method¹⁸. The virus titer was calculated by the Spearman-Kärber's method or Reed-Manchu's method, expressing it with 50% mouse lethal doses (LD50/0,03 ml).

Sma nuc endonuclease (isoform Sm1) was isolated and characterized as previously noted^{14, 19}. After that, 1 mg of lyophilized Sm1 preparation was dissolved with 1 ml of distilled water and subjected to a column chromatography (1.7 x 17.0 cm) on Sephadex G-25 (coarse) equilibrated with 0.85% water solution of NaCl (physiological saline). Elution was carried out with the equilibrium solution. Then fractions of highest absorption at 280 nm and enzymatic activity were selected to study the biological effects.

Concentration of Sma nuc solution was calculated based on the absorption of protein solution at 280 nm and molar extinction coefficient of 47.292 M⁻¹/cm²⁰.

The nuclease activity was assayed by the previously described method^{8,9}. After addition of Sm1 aliquot to 9-fold volume of assay mixture containing 50 mM Tris-HCl buffer, pH 8.5, 0.3 mM DNA, and 6 mM MgSO₄ the incubation was performed at 37°C for 15 min so that about 15-50% of the substrate was converted to acid-soluble products. The hydrolysis was stopped with an addition of chilled 4% perchloric acid. The precipitate was removed by centrifugation. The absorption of supernatant was monitored at 260 nm. Each experiment was repeated not less than 6 times.

Cytotoxicity of the nuclease solutions

was studied using CellTiter 96®A Queous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, USA) which is widely used to study cytotoxicity of compounds *in vitro*^{21,22}. Initially, human embryonic kidney 293 cell culture, 5x10³ cells/well in 96-well plate (HEK293T; ATCC Number: CRL-11268; Manassas, VA, USA) was incubated in Dulbecco's modified Eagle's medium with high glucose amount (DMEM, Sigma-Aldrich, USA), additionally containing - 10% of Fetal bovine serum (FBS, Sigma, USA), 2 mM L-glutamine (Sigma-Aldrich, USA) and a mixture of penicillin and streptomycin (Sigma-Aldrich, USA) under humid atmosphere containing 5% CO₂ at 37°C. After 24 h the culture medium was gently removed and changed with fresh medium containing endonuclease with final activity 30 U/ml, 120 U/ml or 600 U/ml corresponding respectively to 20, 80 or 400 ng/ml of the protein. After 30 min incubation, a mixture of MTS and PMS (9:1 ratio) was added at 10 µl/well. After 30 min incubation, increased absorption was measured at 490 nm with the multimode reader Infinite M200Pro (Tekan, USA). Each experiment was performed in 4 replicates.

The anti-rabies effect of the examined compositions during experimental rabies was based on an increase in chances of survival of the infected animals in comparison with the control. This was calculated by the difference between the death of animals in the experimental and control groups, expressed by percentage. Experimental compositions were injected intracerebrally by 0.03 ml/mouse; subcutaneously - 0.05 ml/mouse; intramuscular, intraperitoneal and intravenous - 0.1 ml/mouse.

All experiments with animals were performed in accordance with the recommendations of the Physiological Section of the Russian National Committee on Bioethics. The experiments were performed using outbred mice weighing between 6-7 g at various times and modes of injection. The viral suspension at the dose of 4 LD50/0.1ml was injected intramuscularly in the hind leg of animals, 20 per group. This resulted in 60-90% mortality. Then in 2 hours a single dose of 0.1 ml of the examined composition was injected into the place of infection. The period of observation of animals was not less than 2 months.

When determining the safety of Haemodes-N or aqueous solution of magnesium

sulfate, which were taken at 45 – 100% or 0.125 - 25% respectively, the materials were injected once intracerebrally and then their effect on the viability and behavior of the animals was observed.

RESULTS AND DISCUSSION

The result of gel filtration of Sm1 solution is shown in Table 1. Fractions # 4 and 5, most active and concentrated, were selected for further experiments.

Examination of the nuclease cytotoxicity demonstrated its weak influence on the activity of mitochondrial dehydrogenases (MTS test) of human embryonic kidney cells that was verified with slight variation in resulted absorption at 490 nm of the assay mixture (Fig.1). As the cells incubated in the presence of 20, 100 or 400 ng/ml of Sm1 isoform diminished the absorption at 490 nm by 9-14% in comparison with the control (in the absence of Sma nuc), it appeared to decelerate a conversion of MTS reagent to a colored formosan product. This suggested that Sma nuc exerted a little cytotoxic effect.

Despite these observations, we went further and controlled the injurious effect of Sma nuc solutions *in vivo*. Both intramuscular and intracerebral injections of the inspected solutions did not reveal any visible changes in the animals' behavior or tissue morphology at the places of the nuclease injection. A 550-fold increase in the nuclease concentration, and respectively the enzymatic activity (330 000 U/ml), did not influence the result that served as evidence of the absence of injurious effect of the nuclease solutions.

As appeared both intramuscular and intracerebral injections of Sma nuc solution with

the activity of 330 000 U/ml resulted in the absence of influence on rabies infected mice. The mortalities of the infected animals were identical independently on the nuclease injection that concluded the lack of anti-rabies activity of pure Sma nuc nuclease which is in contrast with previous reports on RNase preparation⁴.

Since the enzymatic activity of the nuclease is known to depend on the presence of Mg cations in solutions⁸, and stabilization of the enzymatic activity occurs in the presence of polyvinylpyrrolidone²³, we attempted to develop an effective composition including both Sma nuc nuclease and magnesium salt as well as Haemodes-N compound, which contains 6% polyvinylpyrrolidone and is commonly used as desintoxification compound.

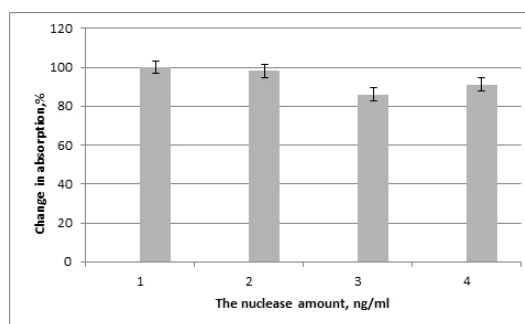


Fig. 1. Cytotoxic effect of Smanuc nuclease on HEK293 cells. Variation of absorption at 490 nm characterizing the activity of mitochondrial dehydrogenases (MTS-test) upon the addition of Smanuc nuclease to final amount 20, 100 or 400 ng/ml (boxes 2,3,4 respectively). The absorption without addition of Smanuc nuclease, taken as 100%.

Table 1. Column chromatography (1.7 x 17.0 cm) of Sm1 water solution on Sephadex G-25 equilibrated with 0.85% water solution of NaCl

Fraction #	Volume, ml	Protein		Activity, U/ml
		A ₂₈₀ /ml	mg/ml	
1	5.2	0.045	0.03	1184
2	5.2	0.03	0.02	544
3	6.0	0.03	0.02	12640
4	5.4	0.29	0.20	331733
5	5.7	0.36	0.25	496000
6	6.3	0.145	0.10	80800
7	5.4	0.125	0.08	73320

The determination of safety dependency on the magnesium sulfate or Haemodes-N solutions concentration upon their intracerebral injections are shown in Fig.2 As shown, 2-25 % solutions of magnesium sulfate as well as undiluted Haemodes-N caused a diminishing viability of experimental mice. Moreover, injection of 15-25% magnesium sulfate resulted in 100% mortality. On the contrary, 0.125 - 1% solutions of magnesium sulfate or 45-50% Haemodes-N did not influence the animals' viability. The data observed at intramuscular injection of magnesium sulfate or Haemodes-N solutions were very similar with the results of intracerebral injections. The found results allowed us to presume the lack of injuries effects of 0.125 - 1% solution of magnesium sulfate or 45-50% solution of Haemodes-N that led us to add them to the nuclease solution in order to enhance and stabilize its enzymatic activity.

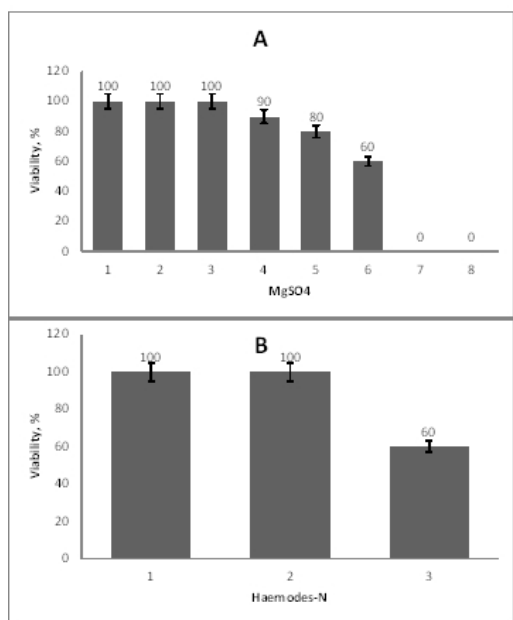


Fig. 2. Variations of the animals' viability upon the injection of water solutions of magnesium sulphate (A) or Haemodes-N (B). A: concentration of MgSO₄ was 0.125 (1), 0.5 (2), 1.0 (3), 2.0 (4), 2.5 (5), 5.0 (6), 15.0 (7), 25.0 (8) %. B: concentration of Haemodes-N was 45 (1), 50 (2), 100 (3) %. The viability without addition of the magnesium sulphate – (A) or Haemodes-N (B) solution was taken as 100%.

The results of anti-rabies activity of the combined compositions are presented on Fig. 3. The compositions were prepared by mixing water solutions with various amounts of magnesium sulfate and Haemodes-N followed by with salt solutions of Sma nuc nuclease in a volume ratio of 1:1:2 respectively. As shown in the pictures, in the absence of at least one of the selected components the anti-rabies activity was not observed. The

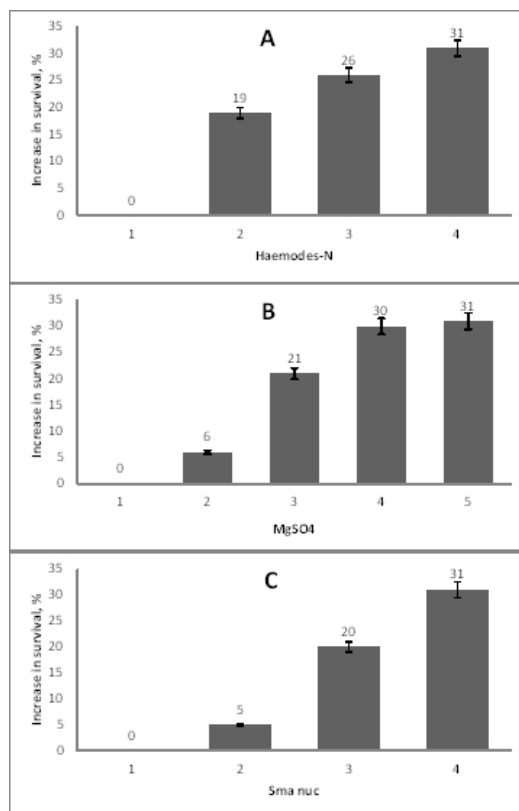


Fig. 3. Change in viability of rabies infected mice after the treatment with combined (in a volume ratio of 1:1:2) compositions, containing magnesium sulfate, Haemodes-N, and Smanuc nuclease and differing by the amounts of each component. (A). Initial Smanuc or MgSO₄ amounts were respectively 0.08 mg/ml or 1%. Haemodes-N concentration was 0 (1), 25 (2), 35 (3), 50 (4)%. (B). The Smanuc or Haemodes-N amounts were 0.08 mg/ml or 50%, respectively. MgSO₄ concentration was 0 (1), 0.001(2), 0.010 (3), 0.125 (4), and 1.000 % (5). (C). The MgSO₄ or Haemodes-N amounts were 1% or 50%, respectively. Smanuc amount was 0 (1), 0.033 (2), 0.066 (3), 0.080 (4) mg/ml.

highest anti-rabies activity followed the rise in survival of the infected mice by more than 30% was observed when we used 1% magnesium sulfate combined with 50% Haemodes-N and Sma nuc solution containing 0.08 mg/ml of the isoform Sm1. If the initial amounts of the combined substances were lower the anti-rabies activity diminished.

Thus a composition demonstrating anti-rabies activity was developed on the basis of isoform Sm1 of *Serratia marcescens* endonuclease. Firstly we have seen that a single salt solution of Sma nuc nuclease is not an effective therapeutic agent for the treatment of rabies infection. On the basis of well studied properties of Sma nuc nuclease the efficient approaches to obtain the nuclease composition with anti-rabies activity was found. Besides the enzyme, the composition has to contain Haemodes-N, magnesium sulfate and sodium chloride, which were mixed at the selected ratio. The injection of this composition at the rabies infection site in mice, 2 hours after infection resulted in an increase of the survival rate by more than 30% of the infected animals, in comparison with that in the control group. The results are protected by the Patent of Russian Federation²⁴. We expect that further increase of the nuclease activity can increase the effect of the developed composition. Therefore, further investigations are in progress.

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