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Antiviral Activity of Hemocyanin *Rapana venosa* and Its Isoforms Against Epstein-Barr Virus

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Abstract: The native molecule of *Rapana venosa* hemocyanin (RvH) consists of two structural subunits RvH1 and RvH2 and each of them contains 8 functional units with different carbohydrate content. In this study, the antiviral effect of the native molecule of RvH and its isoforms against Epstein-Barr virus (EBV) is presented. This virus is the etiologic agent of acute form of the disease infectious mononucleosis. The persistence of the virus in the human organism leads to the development of lymphoproliferative disease, the formation of various carcinomas and to the affection of the peripheral and central nervous system. Therefore, the antiviral activity against Epstein-Barr virus was studied *in vitro* by a PCR method. We have shown that all preparations of hemocyanin RvH have low toxicity; CC_{50} was about 700µg/ml. The antiviral activity was determined in preparations of a concentrations range: 1, 10 and 100 µg/ml. The analysis of the obtained data allowed to determine the concentration, oppressing the replication of the virus of about 50 % in reducing the number of genomic equivalents of EBV DNA on a cell. ID_{50} for the hemocyanin RvH was determined up to 1 µg/ml. At the same time the FU RvH1-a inhibited the EBV at concentrations of 10 and 100 ìg/ml, what is in contrast to native RvH. A similar effect was observed for RvH2 and its functional unit RvH2-e. For the investigated hemocyanins it was found that they have low toxicity and their effective doses were determined. Proceeding from the index of selectivity, which is 700 for hemocyanins isolated from *R. venosa*, we can conclude about their availability for future research as drugs for the treatment of Epstein-Barr virus.

Key words: Epstein-Barr Virus · Functional Units · Hemocyanin · *Rapana venosa* · Viruses

INTRODUCTION

Molluscan hemocyanins (Hcs) have recently received particular interest due to their significant immunostimulant properties [1-4]. This is mainly related to their high carbohydrate content and specific monosaccharide composition. Hcs act as oxygen transporting glycoproteins in many arthropodan and molluscan species [5-7]. There are large differences in the molecular mass, structure, carbohydrate content and monosaccharide composition of Hcs [7-10]. It was also found that hemocyanins are active against viruses [11-14]. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura Syndrome Virus (TSV) infection showed the increased expression of hemocyanin [11]. The activity, against spot syndrome virus (WSSV) and Singapore grouper iridovirus (SGIV) is assigned to the protein of the arthropod *Penacus monodon* [13]. Also the Hc of the gastropod *Rapana venosa* has antiviral properties, in particular against Respiratory Syncytial Virus (RSV) and Herpes simplex virus type 1, strain Vic (HSV-1) [13, 14]. The property seems to be associated with the glycosylation of functional units but the mechanism is not known. Therefore, the oligosaccharide structures of *Rapana venosa* hemocyanin (RvH) were very well studied using different approaches [15-17].

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In this article, the cytotoxicity of the native RvH and its isoforms in different cultures of lymphoblastoid cells of B-phenotype, namely, Raji, B95-8 and Namalwa is investigated in context with preliminary information about its carbohydrate structure and antiviral activity.

EBV virus was chosen as a representative of the family *Herpesviridae*. Infectious mononucleosis is a clinical form of primary human EBV infection [18, 19]. EBV is one of the etiologic agents that cause Burkitt's lymphoma and nasopharyngeal carcinoma. Lately, Epstein-Barr virus is potentially linked to various clinical syndromes (X-linked lymphoproliferative syndrome (Duncan syndrome), B-and T-cell lymphomas, including lymphomas in immunodeficient hosts [20, 21], a wide variety of other diseases, including chronic fatigue and autoimmune disorders, for example, Sjogren's syndrome and rheumatoid arthritis. EBV, along with other herpes viruses, affects the central and peripheral nervous system. EBV infection often develops as an opportunistic infection at immunodeficiency states of different genesis, including, after organ transplantation, the use of immunosuppressive drugs, or AIDS [22-24].

At present, there are two known approaches for the treatment of this viral infection, namely, the use of effective antiviral chemotherapy, serotherapy and seroprevention which supposes the use of specific human immunoglobulins and vaccines [25, 26].

Antiviral drugs may be divided into acyclic nucleoside analogues (Aciclovir, Ganciclovir, Penciclovir), acyclic nucleotide analogues (Cidofovir and Adefovir) and substances of natural origin [27, 28]. All submitted drugs are potent inhibitors of viral reproduction, but not all of them are promising for clinical application, since they are very different in terms of toxicity. This aspect is quite relevant, especially for children and young people. In drug-immunomodulators (Alizarin, Likopid, Polyoxidonium) have immunostimulant properties in relation to cellular and humoral immunity and are involved in redox processes and cytokines synthesis. Interferons and their inductors are drugs that successfully combine etiotropic immunomodulating effects and induce the formation of endogenous interferon (IFN) - α/β, γ- by T-and B-lymphocytes, enterocytes, hepatocytes [29].

Thus, the modern arsenal of antiherpetic means is broad enough. However, despite of it, herpes as viral respiratory infections remains until now a hardly controllable infection. It is due to genotype features of the agent, long-term persistence of the virus in the organism and formation of resistant strains to anti-viral preparations. Therefore, search of new anti-EBV preparations remains topical.

MATERIALS AND METHODS

Isolation of RvH and its Isoforms: *Rapana venosa* hemocyanin (RvH) and its two structural subunits RvH1 and RvH2 were isolated from the hemolymph of marine snails living in the Black Sea as described previously [30]. After treatment of the structural subunit RvH1 and RvH2 with trypsin in a ratio of 400:1 and incubation at 37 °C for 4 hours, FUs of RvH1 and RvH2 were isolated on an ion-exchange “Resource Q 6 ml” (Pharmacia) column using an FPLC system by elution with a 50 mM Tris/HCl buffer, pH 8.2, using a 0.0-0.5 M NaCl gradient as described by Dolashka-Angelova *et. al.* (5).

Pyridylethylation and Enzymatic Digestions of Rvh1-e: Three milligrams of RvH1-a were dissolved in 1.0 ml of 0.25 M Tris/HCl, pH 8.5, 6 M guanidine-HCl and 1 mM EDTA. An ethanolic solution of 30 Wµl DTT was added and the mixture was incubated under nitrogen for 2 hrs at room temperature in the dark. Neat 4-vinylpyridine (100-fold molar excess of the expected cysteinyl residues) was added and the mixture was incubated under nitrogen for 2 hrs at room temperature in the dark. The pyridylethylated protein was desalted by reverse phase HPLC on an Aquapore RP-300 column (2.1x30 mm; Applied Biosystems, Weiterstadt, Germany).

A volume of 50 µl of a trypsin solution (1 mg/ml) was added to 0.50 ml of 25 mM Tris/HCl, pH 9.0, containing 1 mg pyridylethylated SOD (E:S 1:50); the reaction mixture was incubated overnight at room temperature. The digest was applied to an HPLC Hypersil column (250x4.6 mm; 5 µl HyPURITY C_{18}, Thermo Quest), eluted with eluent A (0.1% TFA in water) and eluent B (80% acetonitrile in A), using a gradient program of 0% B for 5 min and then 0-100% B in 60 min; the flow rate was 0.7 ml/min. The UV absorbance of the elution was monitored at 214 nm.

Mass Spectrometric Analyses of the Peptides: Peak fractions were dried and, after dissolving in 0.1% (v/v) TFA, were analysed by MALDI-TOF-TOF mass spectrometry on a 4700 Proteomics Analyser (Applied Biosystems, Framingham, MA). The mass spectrometer uses a 200 Hz frequency-tripled Nd-YAG laser operating at a wavelength of 355 nm. For analysis, about 50 pmol of the HPLC fractions were dissolved in 0.1% (v/v) TFA and applied to the target. Analysis was carried out using α-cyano-4-hydroxycinnamic acid as a matrix. The instrument was calibrated using a peptide mixture solution provided by the manufacturer.

Cultures of Cells and Virus: The following cultures of cells were used in the work:

B95-8-leukocytes of monkeys-marmaset, transformed by Epstein-Barr virus and Raji-human B-lymphocytes transformed by Epstein-Barr virus, which incorporate 63 copies of viral genome and produce only separate early antigens, but not virus particles. Cells have been given by cells cultures Bank of Institute of Virology of RAMS (Russia). Namalwa-human B-lymphocytes producing γ-interferon, which have been given by Bank of cultures of cells of Institute of Experimental Pathology, Oncology and Radiobiology of NASU (Ukraine).

Cultures of cells were cultivated in 24-wells plates in the growth medium that contained 90 % of RPMI-1640 (Sigma, USA), 10 % fetal bovine serum (Sigma, USA) and antibiotics at 37° C in atmosphere of 5.0% CO_2.

Epstein-Barr virus was isolated from B95-8 lymphoblastoid cell culture which produced of this virus. The virus purification was carried out by Wolls, Kroford method [31].

Research Substances: Working dilutions of structural subunits RvH1 and RvH2 and functional units of RvH1 (FU 1, FU2, FU3, FU5 and FU6) and RvH2 (FU2, FU3 and FU5) and Gancyclovir ("Cimevene»), were dissolved in RPMI-1640 and filtered through sterilizing filters "Millipore" with the size of pores 0,22 μm.

Definition of Viability of Cells by Trypan Blue: Viability of culture of cells was determined by them coloring with 0.4 % trypan blue solution ("Sigma", USA). Analysis was performed with using a light microscope with magnification x10. The analysis was carried out according to the instruction of the manufacturer.

Evaluation of Antiviral Activity: DNA from cell suspension was isolated by the DNA-sorb-B DNA kit ("AmpliSens", Russia). Concentration of DNA was measured using Biophotometer ("Eppendorf", Germany). EBV was detected by PCR using "AmpliSens® EBV-EPh" kit ("AmpliSens", Russia) according to the manufacturer's recommendations. Each PCR mix contained 50 ng DNA.

The amplified products and GeneRuler™ DNA Ladder Mix ("Fermentas", Lithuania) were loaded in 1.7% (w/v) agarose gel containing 0.01% (v/v) ethidium bromide. Results were visualized in a UV chamber and processed using program Gel Imager ("DNA-technology", Russia).

Statistical processing of data was performed according to standard approaches to the computation of statistical errors (standard deviation) and by study of correlation, using the computer program Origin 6.0.

RESULTS AND DISCUSSION

Molluscan and arthropodan hemocyanins (Hcs) have recently received particular interest due to their significant immunostimulatory and antibacterial properties [32-35]. This is mainly related to their high carbohydrate content and specific monosaccharide composition. We have analysed the properties, oligosaccharides and the carbohydrate linkage sites of the *Rapana venosa* hemocyanin (RvH) using different approaches [36]. Moreover, the antiviral effects of the native molecules of RvH, of its structural subunits and of the glycosylated and the non-glycosylated functional units on Herpes simplex virus type 1, strain Vic (HSV-1) and against the respiratory syncytial virus (RSV) were investigated [13,14]. We show that the glycosylated FU RvH-c has antiviral properties against the respiratory syncytial virus (RSV), whereas native RvH and the non-glycosylated FU RvH-b have not. This was the first report of the fact that also molluscan hemocyanin functional units possess antiviral activity.

Isolation of Rvh and its Isoforms: *Rapana venosa* hemocyanin (RvH) and its two structural subunits RvH1 and RvH2 with molecular masses of 400 and 420 kDa were purified, as described by Dolashka *et al.* (5). Several functional units with molecular masses of 50 kDa were isolated on an ion-exchange "Resource Q 6 ml" (Pharmacia) column after treatment of the both structural subunit RvH1 and RvH2 with trypsin in a ratio of 400:1 and incubation at 37 °C for 4 hours. The native proteins have two absorption bands at 278 nm and at 348 nm and the "ratio of purity" (R_z) was $R_z = A_{347}/A_{280} =$ 0.21, which confirmed that the proteins are in the native forms.

The Study of Cytotoxicity of the RvH Preparation *in Vitro*: It was performed a comparative analysis of the cytotoxicity of two isoforms RvH in different cultures of lymphoblastoid cells of B-phenotype, namely, Raji, B95-8 and Namalwa.

Influence of the hemocyanin was studied in within of concentrations 400-100 µg/ml. The tested substance did not influence on viability of Raji cells in the range of studied concentrations, in fact the percent of dead cells made up 30 % only in concentration 400 µg/ml for RvH1 and 34%-for RvH2, at that the growth of a cellular population was enough intensive.

In B95-8 cells line, transformed by EBV, under influence of different concentrations of the RvH1 hemocyanin the percent of dead cells was 15-24 %. The addition of RvH2 resulted in the death of 27-44 % of cells.

The tested preparation was nontoxic for Namalwa cells. In a range of studied concentrations, the percent of dead cells did not exceed 18 % and evident influence on proliferative activity of cells was not observed. Thus, the cytotoxicity of both structural subunits RvH1, RvH2 and some FUs (RvH1-a and RvH2-a), isolated from *Rapana venosa* hemocyanin was investigated in several lines of lymphoblastoid cells of the B-phenotype and concentration, which on 50 % reduces viability of cells, was determined. For Namalwa cells it is 1000 µg/ml in relation to RvH1 and 1358 µg/ml for RvH2; for Raji cells it is 700 µg/ml in relation to both preparations; for B95-8 cells-710 and 1352 µg/ml for RvH1 and RvH2, accordingly.

Antiviral activity against EBV was studied using PCR analysis. Investigations were carried out in concentrations of 1, 10 and 100 µg/ml. It was detected that the isoform RvH1 reduced the reproduction of the virus to 57% while the preparation, which contains a functional unit 6 (FU6), inhibited the reproduction of the EBV upon 48% in the lowest tested concentration of 1 µg/ml and upon 100% in the two other concentrations. Concerning isoform RvH2, maximal inhibitory concentration is 10 µg/ml results in 46% inhibition of viral reproduction. For a RvH2-e, 100% inhibition was found at concentrations of 1 and 10 µg/ml with reducing of examined index to 34 when 100 µg/ml of substance was added.

Consequently low toxicity of hemocyanin isolated from *R. venosa* was shown and its antiviral activity against Epstein-Barr virus was detected. A functional unit RvH1-a in the structure of hemocyanin RvH1 has antiEBV activity and RvH2-e - in preparation RvH2 too. It is known that the both FUs are glycosilated. Perhaps exactly the carbohydrate group of these functional units interacts with glycoproteins of Epstein-Barr virus. Probably, the carbohydrate chains of the FU interact with specific

Table 1: Level of viability of cell cultures Raji, B95-8 and Namalwa (%)

	The percent of viable cells in various cell cultures at addition of RvH *					
	RvH1			RvH2		
Concentration (µg/ml)	Raji	B95-8	Namalwa	Raji	B95-8	Namalwa
100	87	85	93	87	78	93
200	85	82	89	76	66	91
400	70	76	77	66	83	82

* standard error $p \leq 0.05$

Table 2: Isolated peptides of RvH1-a and obtained AA sequences by MS/MS spectra

N	[M +H]+	AA Sequence	Position
1	1147.7494	FNKRNPLCR	40-48
2	1535.9010	RTVGVPYWDWTR	86-97
3	2070.2136	RYYDPLFYLHHSSTDR	201-240
4	1191.3196	LWAPWAYER	220-240
5	2196.2890	EPLQPFSFGSPYNLNDNTR	244-262
6	1349.5842	HSTPKSLFNYR	268-278
7	1678.9637	LQDYLNQQKEEDR	296-326
8	1852.1220	FFVLGGPLEAPWAYER	344-359

regions of glycoproteins of EBV, through van der Waals interactions in general or with certain amino acid residues on the surface of the both FUs. This proposal is based on our previous study of the antiviral activity of RvH and a non-glycosylated and a glycosylated FUs inhibit the replication of Herpes simplex virus type 1 (HSV-1) and the respiratory sencivial virus (RSV), where we suggested that oligosaccharide structures of the above mentioned hemocyanins could be involved in their antiviral effect [13,14].

Until now the complete gene sequence of *Rapana hemocyanin* is not known and different N-terminal sequences were published about isolated FUs of RvH1 and RvH2 after trypsinolysis [30]. In order to identify the FUs with antiviral activity they were treated with trypsin and the obtained fractions were analysed by mass spectrometry.

Structure of RvH1-a: The primary structure of RvH1-a was elucidated by N-terminal sequencing of the intact protein combined with the determination of the amino acid sequences of a set of overlapping peptides generated by proteolytic cleavage and sequenced by MALDI-MS/MS (Table 1). The spectrum of the peptide with mass 2070.21 is shown in Figure 2. It allows to deduce the sequence RYYDPLFYIHHSSDTR from the series of y-and b-ions. As for all the other peptidesof the protein sequence in which the isobaricresidues Leu or Ile might occur, the assignment was made on the basis of sequence homology

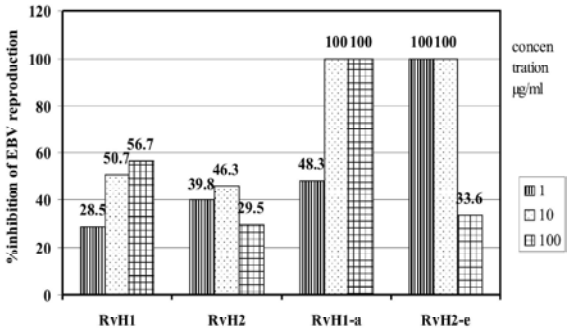

Fig. 1: The level of EBV inhibition under action of different concentrations of examined preparations. PCR method

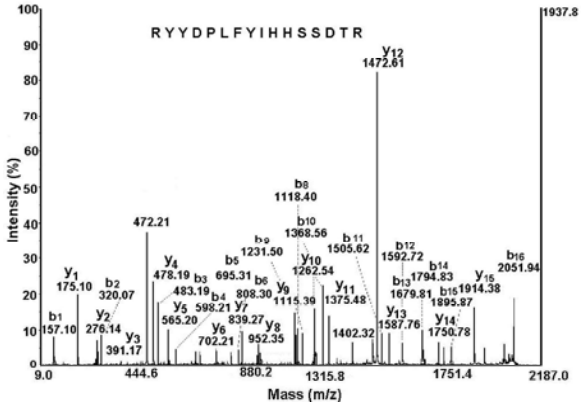

Fig. 2: MS/MS spectrum of the peptide with molecular mass 2070.2136

with other known sequences of FU's from RvH (date not shown).Very high homology was observed in several regions in comparison with the known sequences of few FU's from RvH. Arg48, Asn41, Val44, Leu45 and Ser215 are replacing the conserved residues Pro48, His41, Gly44, Gly45 and Asn215, respectively. Based on MALDI-MS/MS analyses of the peptides two FUs were recongnised as RvH1-a and RvH2-e.

It is known that both FUs are glycosilated and the carbohydrate content of the N-terminal functional unit RvH1-a is 7%, represents as two oligosaccharides N-glycosidically attached to Asn262 and Asn401. The primary structures of two biantennary N-glycans of the N-terminal functional unit RvH1-a were also determined. Two oligosaccharide chains with molecular mass 1609 and 1653, containing $(SO_4)MeGalGlcNAc_4Man_3$ and $MeGal_2GlcNAc_4Man_3$ were found to be connected to the peptides Glp1 and Glp2, respectively [15]. However, using tandem mass spectrometry a complex glycan represents a novel N-glycan motif with an internal fucose was proposed for the glycan with mass 1609, which itself is decorated with a hexuronic acid (HexA) and an N-acetylhexosamine (HexNAc) [15].

The importance of these findings resides in the fact that, *in vitro*, hemocyanin displays antiviral effects on several types of viruses, e.g. on poliovirus type 1, coxsackie virus B4 and respiratory syncytial virus and that these effects are associated with either one or two glycosylated functional units of RvH [10]. Native RvH, however, does not show any antiviral activity against the viruses mentioned and so does non-glycosylated FUs. The original therapeutic concept for suppressing enveloped viruses is presented that is based on a specific interaction of carbohydrate-binding agents (CBAs) with the glycans present on viral-envelope glycoproteins [37]. In this study we confirmed the previous suggestion [38] and we now found that glycosylated FU RvH1-a has the same capacity. Finally, we propose that the reason why the complete molecule of RvH does not have antiviral activity is due to the fact that the carbohydrate chains are buried in between the structural subunits of the global proteins and therefore, are unable to interact with viral glycoproteins.

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