Immunological Investigations, 40:130–149, 2011

ISSN: 0882-0139 print / 1532-4311 online
DOI: 10.3109/08820139.2010.513408

informa
healthcare

Antitumor Activity of Glycosylated Molluscan Hemocyanins via Guerin Ascites Tumor

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As observed in most molluscan hemocyanins, high-mannose type glycans were identified in hemocyanins from *Rapana venosa* (RvH), *Helix lucorum* (HlH) and keyhole limpet (*Megatura crenulata*). In addition, a glycan with a branching structure containing xylose, fucose and terminal methyl hexose was identified in β-HlH. We have examined the immuno-adjuvant properties of hemocyanins, their derivatives and conjugates associated with the cell mediated immunity in experimental tumor-bearing animals with ascites tumor of Guerin. After immunization of the animals with the experimental vaccine preparations, the highest values of splenic lymphocytes were observed in groups immunized with the conjugates RvH-TAg, β-HlH-TAg and KLH-TAg (42.3%; 40.8% and 40.58%, respectively) than with the native hemocyanins (36.5%; 35.1% and 32.4%, respectively). The immunization of rats with the hemocyanins β-HlH, RvH and KLH and their conjugates, prolonged the median survival time of tumor-bearing animals compared with non-immunized animals (39, 33, 31 and 7 days, respectively). Both hemocyanins β-HlH and RvH activate the immune system of the experimental animals and therefore could be a good alternative for KLH. For this reason they could be included into the composition of non-specific anti-tumor vaccines to enhance their effectiveness.

Keywords GAT, *Helix lucorum*, hemocyanin, *Rapana venosa*.

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INTRODUCTION

Glycoproteins are abundant in living organisms and are involved in numerous biological processes (Mailliard et al., 2003). There are many glycoproteins that function as hormones, enzymes, inhibitors, etc. Glycosylation, and its relevance to human diseases, is well studied (Hewitt et al., 2009; Michele et al., 2003). A large group of glycoproteins, namely hemocyanins are copper-containing respiratory glycoproteins localized in the hemolymph of mollusks and arthropods (Schnurr et al., 2001; Bonaventura et al., 1980). Keyhole limpet hemocyanin (KLH), isolated from hemolymph of the marine snail *Megatura crenulata*, is one of the most investigated and widely used in experimental immunology and clinical practice (Harris et al., 1999; Donald et al., 2000; Kirkin et al., 1998; Oyelaran et al., 2010).

The high survival rate of experimental mice with bladder carcinoma after treatment with KLH is considered to be due to the cross-reactivity between carbohydrate moieties of the hemocyanin and the tumor cells resulting in strong activation of the immune response against certain tumor antigens (Timmerman et al., 2000; Hartmann et al., 2004; Chiarella et al., 2010). Importantly, KLH was included as a sensitive biomarker for early detection of breast, ovarian, and prostate cancer (Wandall et al., 2000).

The *Helix vulgaris* hemocyanin (HvH) and *Rapana venosa* hemocyanin (RvH), which have recently received particular interest for their immunostimulatory properties, are constituted of different oligosaccharide structures (Salvato et al., 1990; Terwilliger, 1998; Van Holde et al., 2001; Dolashka-Angelova et al., 2009). Studies on HvH revealed that it is very heterogeneously glycosylated carrying preponderantly methylated high mannose-type glycans (Dolashka-Angelova et al., 2004). Methylated sugar residues were also identified in *Helix pomatia* hemocyanin (Gielens et al., 2004; Lommerse et al., 1997) and in KLH. Moreover, a specific branch with a terminal Gal β-1,3-linked to GalNAc was identified (Wuhrer et al., 2004). Several novel types of N-glycans, with an internal fucose connected to one N-acetylhexuronic acid (HexNAc) and one hexuronic acid (HexA), were discovered in the structural subunit RvH1(Sandra et al., 2007).

Studies on the replication of Herpes simplex virus type 1, strain Vic, (HSV-1) and the respiratory syncytial virus (RSV) revealed that there is no inhibitory effect on these viruses for native RvH, structural subunits RvH1 and RvH2, and for non-glycosylated FU-b. However, the glycosylated FU RvH-c does show antiviral effect in the case of RSV replicated in HEp-2 cells. The oligosaccharide structure of the above-mentioned FU, which is exposed on the surface of the molecule could be involved in its antiviral effect (Dolashka-Angelova et al., 2009; Velkova et al., 2009).

Hemocyanins are also effective immunogenic compounds (Dolashka-Angelova et al., 2008). RvH and HvH are effective carriers of non-immunogenic

or poorly immunogenic antigens for developing antibodies against these molecules, as was recently demonstrated for the efficient antibody development against the synthetic C-terminal fragment ProTα[101–109], using RvH and HvH as carriers in comparison with KLH (Dolashka-Angelova et al., 2008).

The difference in the mechanisms of action of the hemocyanins seems to be related to the differences in their carbohydrate side chains as we demonstrate here by the resistance of the experimental animals against the progressive development of Guerin ascites tumor (GAT) after treatment with RvH, β-HlH and KLH in correlation to their specific carbohydrate constituents.

MATERIALS AND METHODS

Materials

The hemocyanins RvH and KLH were isolated from the hemolymph of marine organisms *Rapana venosa* (Dolashka-Angelova et al., 2003) and *Megatura crenulata* (Schütz et al., 2001). The native molecules and their isoforms were purified as described previously (Dolashka-Angelova et al., 2003; Schütz et al., 2001).

Helix lucorum (renamed from *Helix vulgaris*) snails (25 g) were provided from National Snail Breeding Cluster (Bulgaria). The hemolymph was collected from the feet of the snails, centrifuged at 4000 rpm for 20 min at 4°C for removal of rough particles. The crude hemocyanin in the clear supernatant was poured off and diluted with an equal volume of 0.4 M sodium acetate buffer, pH 5.2, precipitated with half-saturated ammonium sulphate and centrifuged at 8000 $\times$ g for 60 min. After decantation of the supernatant liquid, the sediment containing β-heamocyanin (HC) was solubilized at a concentration of about 5% in 0.1M sodium acetate buffer, pH 5.7, containing 0.02% NaN_3 to avoid microbial growth.

The solution was dialyzed at 4°C against the same buffer for 24h, and then against a solution containing 1.5 M NaCl and 50 mM sodium acetate buffer, pH 5.7, for 24h at 4°C in order to eliminate the ammonium sulphate with one renewal at 12 h. After that, another dialysis was performed against 100 mM acetate buffer, pH 5.8, and the protein was purified by anion exchange chromatography on a DEAE Sepharose CL-6B column in 50 mM Tris-HCl buffer, pH 8.0. β-HC were obtained by linear gradient elution of 0.2–1.0M NaCl.

Mass Spectrometric Analysis of Glycoprotein HlH by Nanoflow ESI and μLC/ESI-MS Experiments

Glycopeptides were isolated after trypsinolysis of β-HlH and separated by RP-HPLC on a Nucleosil 7 C18 column as described by Beck et al. (2007).

Several of them were analyzed by nanoflow ESI- and also by nano-electrospray mass spectrometry on a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF; Micromass, Manchester, UK) (Carr et al. 1993).

Animals and Tumors

Wistar rats, 20–30-days-old, were used for tumor transplantation. Guerin ascites tumor induced spontaneously and adapted for growth in rat peritoneal cavity was injected in a suspension of 8×10^6 tumor cells/ml. Tumor cells were collected from the peritoneal cavity 10 days after inoculation in concentrations of 1.2×10^9 cells/ml. The collected ascitic fluid was centrifuged and the cells were pelleted, washed and suspended in saline to a final concentration 2×10^7 cells/ml. Isolation and purification of tumor antigens from the collected Guerin tumor cells were performed after centrifugation of ascitic fluid as described by Pellis et al. (1976). The animals were grown up at the animal facility of IEPP Bulgarian Academy of Sciences at standard conditions accepted from Bulgarian Veterinary Health Control Service. All studies were performed in accordance with the Guide **for** Care and Use of Laboratory Animals, as proposed by the Committee on Care **of** Laboratory Animal Resources, Commission on Life Sciences and National Research Council, a work permit No. 11130006.

Conjugation of Hemocyanins with Tumor Antigen

The method of Zhang et al. (1996) was applied for conjugation of the tumor antigen, superficial soluble protein, with the hemocyanin. Five milligrams of hemocyanin in 1ml borate buffer (pH 10.0) were mixed with 5 mg of TAg. Then, 1 ml of 0.3% glutaraldehyde was added and the mixture stirred at room temperature for 2 h. Free glutaraldehyde was eliminated by adding 0.25 ml 1 M glycine and incubation for 30 min at r.t. The conjugate solution was dialyzed against PBS at 4°C overnight. The electrophoretical mobility of the antigen was determined by SDS-PAGE in 12% gel by the Standard Laemmli Protocol (Laemmli et al., 1970).

Immunization Groups

The protective effect of hemocyanins on the survival of rats with experimental ascitic tumor of Guerin was examined after injection of 0.80 μg/μl of the native RvH, HlH and KLH and their conjugates with the tumor antigen (1:1), supplemented with $Al(OH)_3$ as an adjuvant. Experimental animals were separated in 8 experimental groups of 10 animals each as follows: Group 1) Rats injected with β-HlH; Group 2) Rats injected with RvH; Group 3) Rats injected with KLH; Group 4) Rats injected with HlH-TAg; Group 5) Rats

injected with RvH-TAg; Group 6) Rats injected with KLH-TAg; Group 7) Rats injected with TAg, and for the control tumor bearing rats without treatment were used. They were preliminary immunized threefold for 7 days and then infected with 10^6 tumor cells. Five rats of each group were exsanguinated 10 days post inoculation and the rest were observed to determine the average survival time.

Antibody-dependent, Cell-mediated Cytotoxicity (ADCC) of Splenic Lymphocytes

Antibody-dependent cell-mediated cytotoxicity (ADCC) of the splenic lymphocytes of rats was assessed at the 10th day after tumor transplantation as described by the method of Pearson et al. (1978). Briefly, ascitic Guerin tumor cells, obtained by puncture of peritoneal cavity, were used as target cells. The suspension of tumor cells obtained contained 90–95% viable cells, determined by staining with 0.02% Trypan blue. Guerin tumor cells (10^5 cells/ml) were suspended in RPMI-1640 serum-free medium, seeded in 96-well plate (U-shape), 0.1 ml per well for cell culturing and incubated with 0.05 ml serum in a dilution of 1:50 (immune and control) for 30 min, in a CO_2 incubator (5% CO_2, 37°C and 95% relative humidity).

After incubation, effector cells (spleenic lymphocytes) in a volume 0.1 ml were added to a final ratio of target/effector cells 1:40. After re-incubation for 24 hours under conditions mentioned before, the plate was centrifuged and equal amount of a CP0.04% solution of Trypan blue in PBS was added to each well. The percentage of dead cells was determined using a haemocytometer, and the cytotoxic activity of lymphocytes was determined as follows:

$$\text{ADDC}(\%) = \frac{\begin{array}{c}\%\text{ live tumor cells after} \\ \text{Incubation with} \\ \text{control serum}\end{array} - \begin{array}{c}\%\text{ live tumor cells after} \\ \text{incubation with} \\ \text{immune serum}\end{array}}{\%\text{ live tumor cells after incubation with control serum}} \times 100$$

Determination of Mitogenic and Proliferative Response of Splenic Lymphocytes

The determination of the mitogenic response of the splenic lymphocytes from the treated rats was performed according to the procedure of Masson and Gwanzura (Masson et al., 1990). Lymphocytes (1×10^7 cells/ml) were suspended in growth medium RPMI-1640 (Sigma), supplemented with 20% FCS, antibiotics in usual concentrations, L-glutamine, seeded in 96-well U-shaped

plates (0.1 ml/well) and incubated for 72 hours in a CO_2 incubator (37°C, 5% CO_2, 95% humidity). On the 54th hour of incubation, 1μCi ^{3}H – thymidine (Sigma) was added to each well.

The isolated rat splenic lymphocytes (2×10^6 cells/ml) were seeded in 96-well U-shaped plates (100 μl per well) and incubated for 3 days in the presence of the antigen used for immunization in doses of 5–10 mg/ml under the conditions mentioned above.

Proliferative responses were determined as a measurement of cell-mediated immune responses induced by vaccination. Proliferative responses were calculated as the means of triplicate wells and are expressed as a stimulation index (SI), where SI represents the counts per minute (cpm) in the presence of antigen divided by the cpm in medium alone.

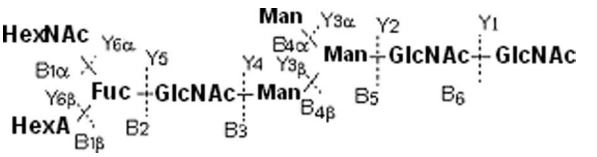
Statistic Data Analysis

Statistical analysis was performed using SigmaPlot for Windows, version 8.0. The results from the experiments were analyzed by the Student's test (*t*-test). Data are presented as mean arithmetical values ± SD, n = 5, and $p < 0.05$ was accepted to be significant.

RESULTS AND DISCUSSION

Characterization of the Glycopeptides Isolated from RvH2 by μLC/ESI-MS and ESI-MS/MS

Molluscan hemocyanins are glycoproteins **with** branching different carbohydrate chains. Several methods and techniques, such as CE-MS/MS and MALDI-MS in combination with exoglycosidase digestions, allowed us to unravel structures found rarely in glycoproteins (Dolashka-Angelova et al., 2004; Sandra et al., 2007; Beck et al., 2007; Dolashka-Angelova et al., 2009). The unusual acidic tetrasaccharide, represents a novel N-glycan motif in RvH1.



Most of the glycans carry at least 1 fucose residue, mainly bound to the carbohydrate chain to the first GlcNAc of the core structure. In RvH1, the presence of hexuronic acid residue (HexA), with a neighboring internal fucose to which also an N-acetylhexosamine (HexNAc) is linked, stands for a novel N-glycan motif (Sandra et al., 2007).

The specific structure Gal(β-1,4)Gal(β-1,4)Fuc(α-1,6) was also identified in KLH, which very likely contributes to the immunostimulatory properties of the hemocyanin (Wuhrer et al., 2004). Mainly high mannose-type glycans and methylated sugars were identified in HpH (Gieliens et al., 2004; Lommerse et al., 1997) and in HvH.

Therefore, we isolated several glycoproteins from β-HlH, and their structures were analyzed by μLC/ESI-MS, nanoES-MS and μLC/ESI-CID-MS. HlH was isolated from the hemolymph of the garden snail *Helix lucorum* after precipitation with half-saturated ammonium sulphate and centrifugation at $8000 \times g$ for 60 min. After decantation of the supernatant liquid, the sediment containing β-HlH was separated by anion exchange chromatography on a DEAE Sepharose CL-6B column in 50 mM Tris-HCl buffer, pH 8.0, and gradient elution of 0.2–1.0M NaCl. Several glycopeptides were isolated after tryptic digestion of β-HC and were analysed as described by Beck et al. (2007).

The glycopeptide-containing fractions, collected from the Nucleosil RP C18 column after elution at 18.5 min time were investigated by μLC/ESI-MS on an ion-trap mass spectrometer. First, the glycopeptides were detected based on the formation of the carbohydrate-specific $[HexNAc]^+$ (m/z 204) and $[Hex\text{-}HexNAc]^+$ (m/z 366) oxonium marker ions obtained aften in source fragmentation in the positive ion mode. The molecular weights of the intact glycopeptides were subsequently determined by a second positive ion mode LC/ESI-MS experiment (identical HPLC conditions) without in-source fragmentation.

Analysis of the fraction by base peak chromatogram (BPC), shown on Panel (A) of Figure 1, reveals several peptides and glycopeptides. Two glycopeptides eluted after 13.7 and 14.1 min (Fig. 1B) caused the formation of intense signals in the carbohydrate-specific m/z 204 and 366 marker ion traces (Figs. 1C, 1D). The carbohydrate chains are composed of a terminal HexNAc residue linked to Asn, followed by a second HexNAc residue and a series of 1–9 Hex residues. The averaged positive-ion mode low cone voltage mass spectrum acquired during the elution of the 13.7 and 14.1 min peaks is shown in Figures 1E and 1F. Surprisingly, a complex mass spectrum with a large number of multiple-charged molecular glycopeptide ions could be observed (data not shown).

Based on these ions, a ladder of homogeneous glycopeptides could be calculated, only differing by 204 and 162 Da, the molecular weights of HexNAc and Hex residues. The glycopeptide eluted at 13.7 min was determined to have a mass of 2907.10 Da and a glycan with **a** structure $Hex_6Man_3HexNAc_2$ is suggested. The second glycopeptide eluted of the 14.1 min has a mass of 2745.12 Da and a glycan with structure $Hex_5Man_3HexNAc_2$.

A series of singly charged ions shows the typical signals for sugars at m/z 204.13 (HexNAc) and 366.22 (HexHexNAc), followed by m/z values of 528.31 ($Hex_2HexNAc$), 690.39 ($Hex_3HexNAc$), 852.52 ($Hex_4HexNAc$) and 1014.62 ($Hex_5HexNAc$) (Fig. 2).

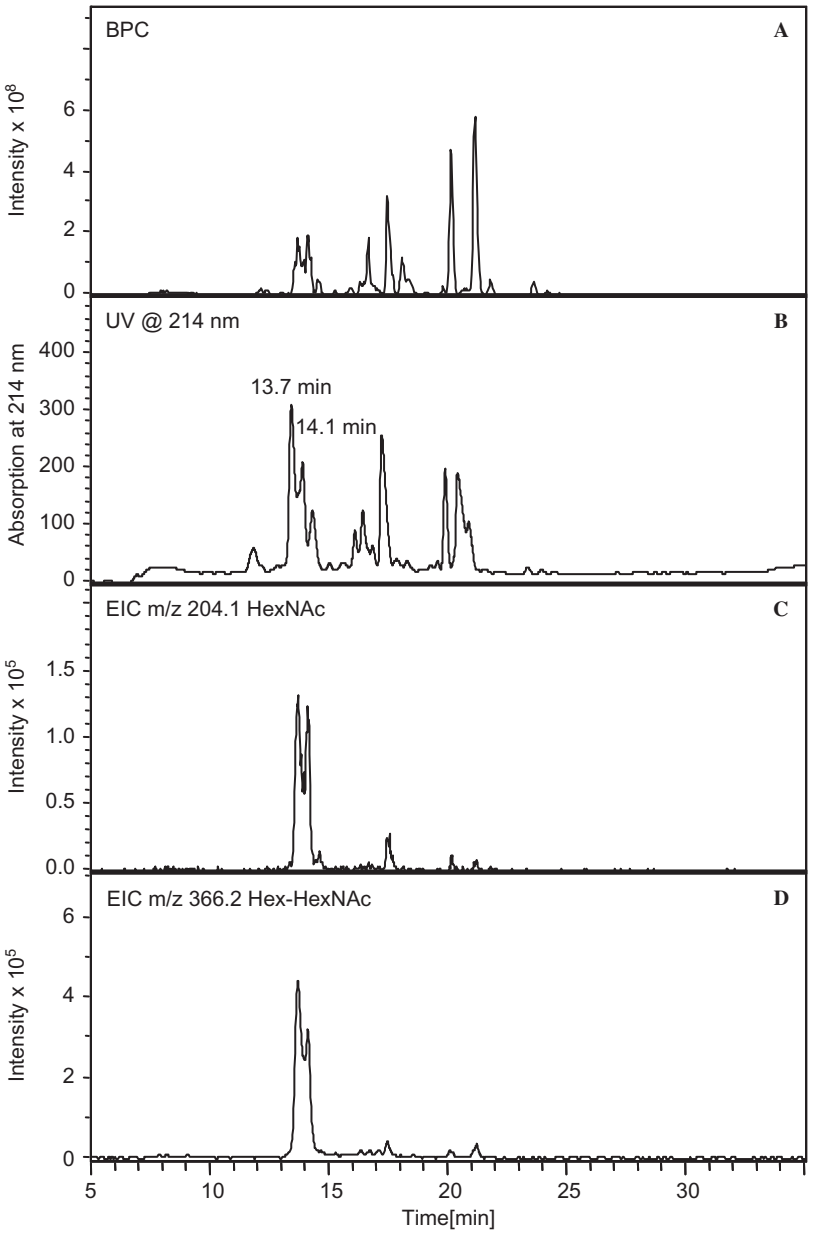


Figure 1: μLC/ESI-MS (no in source fragmentation, low cone voltage and high cone voltage-MS) analysis of the glycopeptide of fraction eluted after 18.5 min in the first dimension RP-HPLC separation of tryptic digested β-HIH. Panel (A) shows the base peak chromatogram (BPC), panel B the UV chromatogram (at 214 nm) and panels C and D the extracted ion chromatograms (EICs) of the diagnostic $(HexNAc+H)^+$ oxonium ion (m/z 204.1) and $((Hex\text{-}HexNAc)+H)^+$ oxonium ion (m/z 366.2), indicating the elution of glycopeptide(s) after 13.7 and 14.1 minutes. Averaged positive ion mode electrospray mass spectra of the glycopeptide (GP) containing fraction eluted after 13.7 min (panel E) and 14.1 min (panel F) acquired during the μLC/ESI-MS analysis (no in source fragmentation, low API-CID).

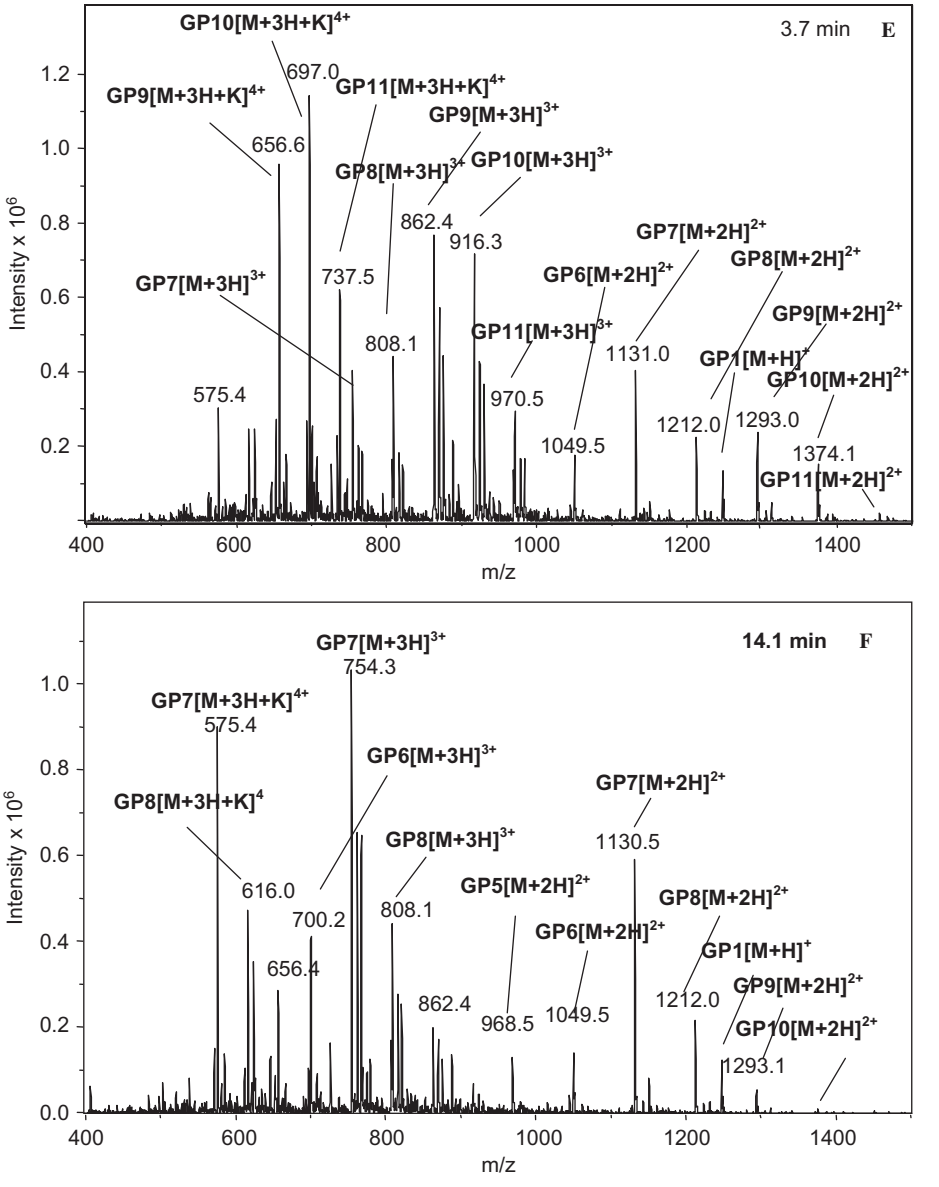


Figure 1: (Continued).

The other glycopeptide was analyzed by nanoflow ESI-MS/MS. Its carbohydrate structure with a molecular mass of 1711.6 was analyzed by the MS/MS spectrum of the glycan. As is shown in Figure 2, the ions at m/z 1711.6 in the MS spectrum coresponds to a structure containing one terminal fucose residue, attached to carbon 6 of GlcNAc. The observed mass difference between the m/z 1079.3 and m/z 933.2 fragment ions amounts to146 mass

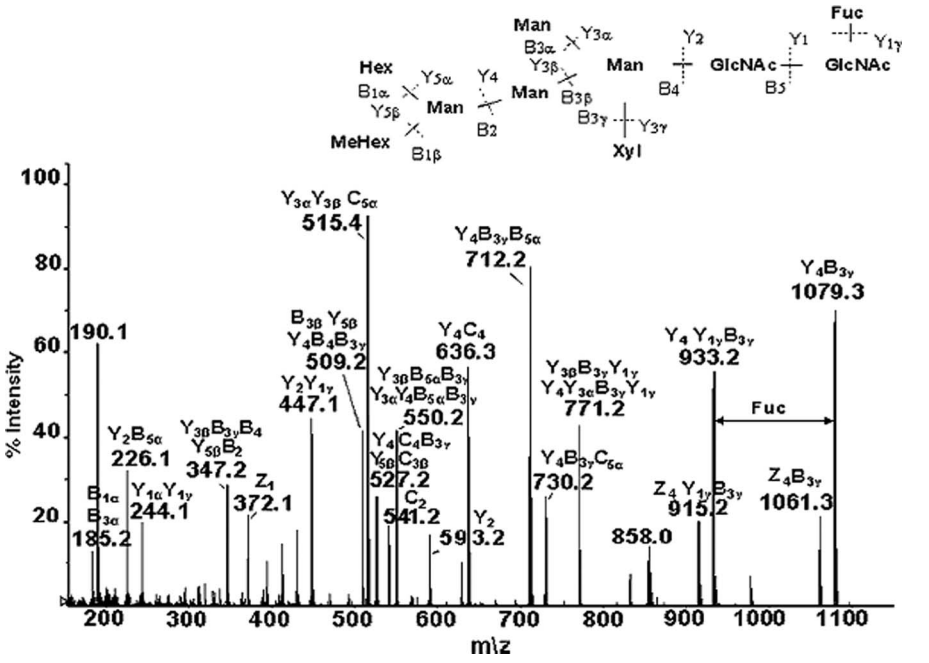


Figure 2: MS/MS spectra and structures with fragmentation nomenclature of the double-charged $(M+Na+H)^{2+}$ of the glycan at m/z 858.5, isolated from β-HlH.

units and is in agreement with a loss of a Fuc unit. Ion Z_4 at m/z 372.1 gives additional information that this Fuc is connected to the N-terminal GlcNAc. Branching of Fuc to the core is confirmed by the Y_2 fragment at 593.2. However, branching of one pentose was identified in glycan at m/z 1711.6. The pentose in the structure is identified by the m/z values 636.3 and 527.2. The occurrence of xylose in *Helix pomatia* HC is considered to be highly immunogenic in mammalian species (Lommerese et al., 1997).

As in most molluscan hemocyanins, some glycans in β-HlH contain terminal methyl hexoses. Branching of methyl Hex to two other Hex was calculated from the ion at m/z 541.2. It is obvious that the glycans at m/z 1711.6 $[M+Na]^{2+}$ have a structure with one xylose, one fucose and methyl hexose.

Effect of the Vaccine Preparations Tested on the Antibody-dependent Cell-mediated Cytotoxicity (ADCC)

It is known that molluscan HCs are powerful immunogens, probably due to their high carbohydrate content and specific monosaccharide composition (Gieliens et al. 2004; Lommerse et al., 1997). Therefore, we studied the effects of vaccine preparation of hemocyanins RvH and β-HlH with different oligosaccharide structure in comparison with KLH (Dolashka-Angelova et al., 2004; Sandra et al., 2007; Wuhrer et al., 2004; Kurokawa et al., 2002; Wirguin et al., 1995).

Table 1: An immunization concept of rats with native and conjugated hemocyanins.

Step 1. Eight groups of 10 rats immunized threefold for 7 days with 0.80 μg/μl of:							
Native molecules			Conjugated molecules			Control	
Group 1 β-HlH	Group 2 RvH	Group 3 KLH	Group 4 HlH-TAg	Group 5 RvH-TAg	Group 6 KLH-TAg	Group 7 TAg	Group 8 without treatment
Step 2. After 7 days:	Rats infected with 10^6 ascitic tumor cells of Guerin.						
Step 3. After 10 days:	Five rats of each group were exsanguinated.						
Step 4.	Determine the average survival time.						

The experiments were performed following the immunization concept as described in Table 1. Seven groups of animals (rats) were treated with native and conjugated hemocyanins. Three of them (Groups 1–3) were treated with purified native hemocyanins: β-HlH, RvH and KLH respectively, dissolved in 50 mM Tris-HCl buffer, pH 8.0. The aggregation state of the hemocyanins used for immunization was as decamers and dimmers (Dolashka-Angelova et al., 2003). The other 3 (Groups 4–6) were immunized with conjugates of the studied hemocyanins with tumor antigens: β-HlH-TAg, RvH-TAg and KLH-Tag, respectively.

The seventh (control) group consisted of untreated rats. The animals from the third and sixth groups were injected with KLH and its conjugate served as a control (compounds with well-known immunostimulating action) (Celluzzi et al., 1996; Herscowitz et al., 1972; Schumacher et al., 2001). In presence of serum from tumor-bearing rats (TBR) and the effect of the hemocyanins and their conjugates on the ADCC and proliferative response of splenic lymphocytes was observed at the 10^{th} day after the tumor transplantation.

As is shown in Figure 3 the highest values of ADCC were observed in the groups immunized with the conjugates RvH-Tag, β-HlH-TAg and KLH-Tag (42.3%; 40.8% and 40.58%, respectively) in comparison to the groups immunized with the native hemocyanins RvH, β-HlH and KLH (36.5%; 35.1% and 32.4%, respectively). The cytotoxic activity of splenic lymphocytes in presence of normal rat serum in all experimental groups showed values close to the control. However, the experimental vaccine containing native hemocyanins or hemocyanins conjugated with Tag, exhibited significantly higher level of ADCC compared with the control and with the vaccine containing Tag alone. It is probably due to the significantly higher molecular weight of the conjugates (TAg-hemocyanin), which favors the stronger activation of the immune system. Therefore we could make the conclusion that the tested hemocyanins RvH, β-HlH and KLH are strong activators of the immune system.

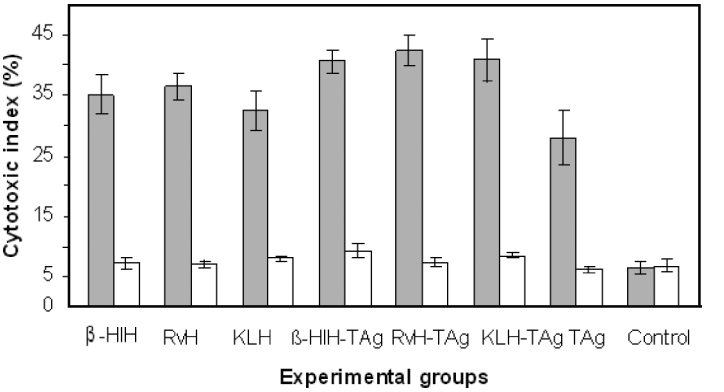


Figure 3: Antibody-dependent cell mediated cytotoxicity (ADCC) of splenic lymphocytes in presence of specific immune serum (dark column) and normal rat serum (white column).

Immunotherapeutic Effect of RvH and β-HlH and Their Conjugates

Immunotherapeutic effect of RvH and β-HlH and their conjugates against experimental GAT-bearing rats were studied as well. The activation of cell-mediated immunity in the experimental groups was examined after immunization with hemocyanins and the consequent effects after application as conjugates with TAgs. The proliferative indexes of splenic T-lymphocytes for each experimental group, given as a chart on Figure 4, exhibited stronger immunogenicity of β-HlH (1.314 ± 0.064; $p < 0.001$) and RvH (1.252 ± 0.065; $p < 0.001$) compared with KLH ($1{,}066 \pm 0{,}023$). These were in agreement with the results from tests for survival rate of experimental animals, presented in Figure 5.

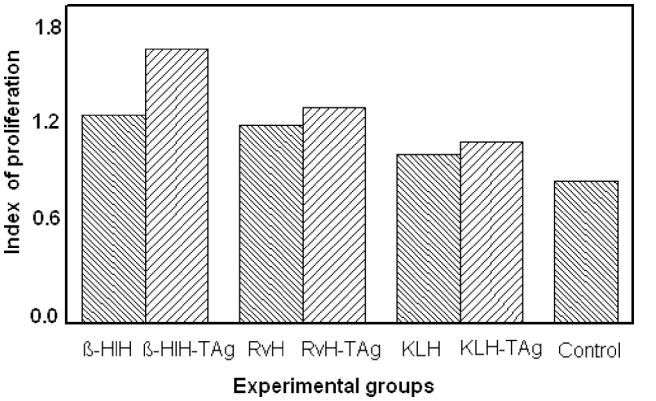


Figure 4: Proliferative response of splenic T-lymphocytes isolated from experimental animals preliminary immunized with the vaccinal medicines researched.

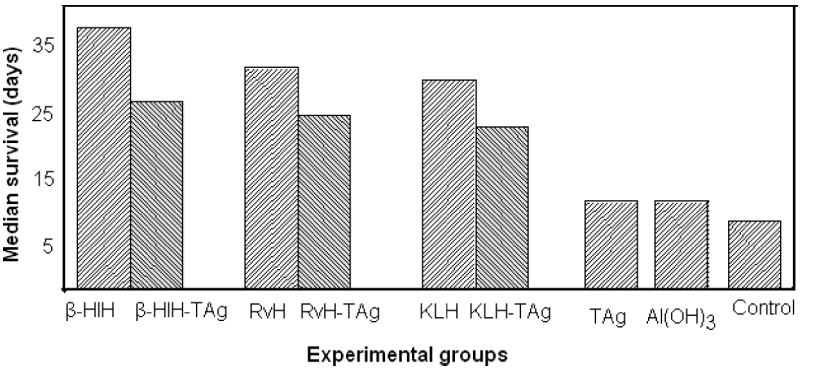


Figure 5: Average survival time (in days after tumor cells application) of experimental animals immunized preliminary with the vaccines studied and subsequently inoculated with Guerin tumor (GAT). $Al(OH)_3$ means neutral adjuvant aluminum hydroxide.

Among the animals preliminary immunized with β-HlH and RvH higher survival rates after infection with GAT (39 and 33 days, respectively) were observed, comparing to those immunized with KLH (31 days) and with the neutral adjuvant aluminum hydroxide $Al(OH)_3$ and the control. Based on the obtained results we are sure to say that hemocyanins, isolated from *H. vulgaris* and *R. venosa* could be a serious alternative of KLH as single inductors of non-specific, cell-mediated immune response and to propose it as a component of non-specific non-conjugated anti-tumor vaccines.

Also, the conjugates of β-HlH, RvH and KLH with tumor antigens against experimental GAT-bearing rats, showed significantly higher levels of determination of splenic lymphocytes: 1.734 ± 0.099 ($p < 0.005$), 1.364 ± 0.043 ($p < 0.05$) and 1.144 ± 0.045 ($p < 0.01$), respectively, contrary to the pure native hemocyanins, but at the same time the ratios between them were kept (Fig. 4). Apparently the higher immune stimulation was observed with β-HlH-TAg followed by RvH-TAg and KLH-TAg. The explanation of the observed higher immunogenicity of conjugates compared with native molecules might be that during the process of conjugation, large multivalent complexes with high molecular weight might be created. The formation of such complexes between tumor antigens and hemocyanin molecules supposes their gradually elimination out of the system, followed by a permanent stimulating of the immune system (O'Brien-Simpson et al., 1997). The exhibited stronger immunogenicity of β-HlH and conjugate of β-HlH could be also explained with the carbohydrate structures dominated with methylated hexoses, as well the complex structure with xylose, one fucose and methyl hexose.

These results correlated with our previously results, about the effect of hemocyanins RvH, HvH and KLH as carriers of a synthetic fragment of ProTα on the development of antibodies for ProTα (Dolashka-Angelova et al., 2008).

Development of antibodies against ProTα-KLH, ProTα-RvH or ProTα-HvH conjugates can be explained by assuming that the KLH, RvH or HvH molecules offer the T-cell epitopes necessary for stimulating humoral immunity in the host animal (Dolashka-Angelova et al., 2008).

However, not in all cases increase of the immune system activation leads to a high level of resistance of the organism against the disease. As shown in Figure 5, the survival rate of the experimental animals, immunized with conjugates, is much lower than that of the immunized with single native hemocyanins only. It is a possible consequence of an autoimmune reaction, targeting the own cells and tissues**.** This "breakdown" of the immune tolerance probably is due to the incomplete purification of the TAg used (Fig. 6). There are a lot of antigens, normal for the native healthy cell surface cells in its composition. That is why the combination of highly immunogenic hemocyanins and non-purified TAg does not influence on the survival rate of the experimental animals as we expected.

The significant antitumor effect was observed for other molluscan hemocyanins, such as KLH and *Concholepas concholepas* hemocyanin (Moltedo et al., 1996). The effect of Concholepas HC was demonstrated in treated mice by decreased tumor growth, prolonged survival and lack of toxic effects. It was found that the hemocyanins increase natural killer cell activity and induce a T helper type 1 cytokine profile. However, there are differences

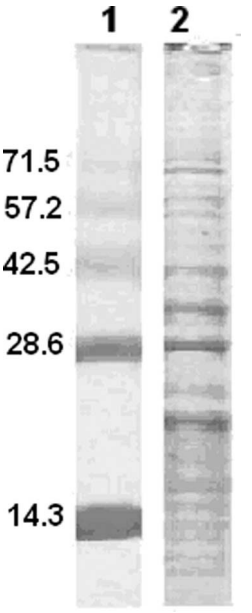


Figure 6: 12% SDS gel electrophoresis of isolated antibody TAg. The electrophoretical mobility of the antigen was determined by the Standard Laemmli Protocol (1970). Line 1 – standard, line 2 – Tag.

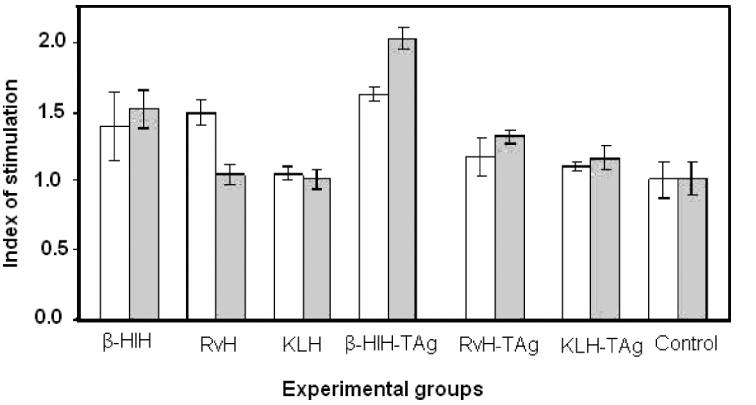


Figure 7: Proliferative response of splenic lymphocytes, isolated from experimental animals preliminary immunized with the vaccinal medicines researched in comparison with T- and B-cell mitogens, PHA (white column) and LPS (dark column), respectively.

in the literature concerning the antitumor effect of KLH on mice (Kafi et al., 2009; Plitnick et. al., 2010; Teitz-Tennenbaum et al., 2008). This may be explained either by differences in the experimental designs or by the presence of lipopolysaccharide in the KLH preparations.

To determine the cell-mediated immunity activated after immunization with the substances studied, we incubated splenic lymphocytes, isolated from the proper group of animals with two different **mitogenic** factors PHA and LPS. These factors activate the two major parts of the lymphocytic population (T- and B-cells) (Lamping et al., 1996; Rabinovitch et al., 1986). The results from this assay are shown in Figure 7. RvH and KLH stimulate stronger the T-lymphocyte population while β-HlH stimulates stronger the B-cell population (humoral immunity). This difference might be a consequence of qualitative and quantitative characteristics of carbohydrate moieties of the two different hemocyanins widely varying.

Studies on the oligosaccharide moieties on these hemocyanins have shown a difference in their structures (Van Kuik et al., 1985; Harris et al., 2000). Contrary to the native hemocyanins, their conjugates have a significantly stronger activating effect on B-lymphocytes. It is hardly likely due to the higher molecular weight of the conjugates, which predominantly activates the B-cells responsible for the humoral immunity (Benjamin et al., 1984).

CONCLUSIONS

We have studied the effects of different glycosylated hemocyanins, isolated from local molluscan species *Rapana venosa*, *Helix lucorum* and *Megatura*

crenulata on mice bearing Guerin ascites tumor, as a function of their chemical structure. As in most molluscan hemocyanins, besides the glycans of high-mannose type specific oligosaccharide structures were identified in RvH, β-HlH and KLH. These differences in the carbohydrate structure of HCs may influence their immunomodulatory properties.

It was found that β-HlH and its conjugate exhibited the highest effect on experimental tumor bearing animals with ascites tumor of Guerin compared to RvH and KLH. Our results are another demonstration of the antitumor effect of hemocyanins besides the investigations with keyhole limpet and *C. concholepas* hemocyanin. It is suggested that this effect is an ancient conserved immunogenic mechanism shared by those hemocyanins enabling to enhance T helper type 1 immunity and lead to antitumor activity.

Finally, the question rises how hemocyanins, such as RvH and KLH, which broadly differ in their origin and subunit organization from HlH, can show similar immunomodulatory properties. We believe that the answer is to be sought in the complex organisation and the conserved molecular architecture of these molecules: gastropod hemocyanins have regions of high sequence homology, as demonstrated by the presence of common conformational or mimic epitopes and revealed by cross-reaction analysis (Moltedo et al., 2006). The results of this work are considered promising and support continuation of relevant studies.

On the other hand, the above results may be considered as preliminary basic information for further investigations on hemocyanins isolated from *Rapana venosa* and *Helix lucorum*, to be used for *in vivo* immunotherapeutics. Recently several therapeutic approaches were offered but one of them, the patient's immune system to recognize the antigens expressed on tumor cells and destroy them, leaving normal cells intact, was found as a reliable scientific rationale. The success of this therapy highly depends on the selection of target antigens that are essential for tumors growth and progression (Arcangeli et al., 2005). Therefore, after conjugation the above-studied hemocyanins KLH, RvH and HlH with different oligosaccharide structures will be used as carrier and immunogens *in vivo* to successfully elicit specific antitumor antibodies.

ACKNOWLEDGMENTS

This work was financially supported by the research grant from the National Research Foundation of Bulgarian Ministry of Education and Science: My-CC 1501/05, UV-L-301/2007, TK01-496/2008 and DFG-01/2008 (Germany). We thank to the National Snail Breeding Cluster (NOC) and "Delta Industry" AD, Sozopol, for providing the snails.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ABBREVIATIONS

ADCC, Antibody-dependent cell mediated cytotoxicity;
cpm, counts per minutes;
CTL, cytotoxic T-lymphocytes;
GAT, Guerin ascites tumor;
HlH, *Helix lucorum* hemocyanin;
HvH, *Helix vulgaris* hemocyanin;
KLH, keyhole limpet hemocyanin;
LPS, lypopolysacharide (B-cell mitogenic factor);
PB, phosphate buffer;
PBS, phosphate-buffered saline;
r.t., room temperature;
RvH, *Rapana venosa* hemocyanin;
SD, standard deviation;
TAg, tumor antigen;
Th-1, T helpers type 1;
PHA, phytohemaglutinine (T-cell mitogenic factor).

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