International Immunopharmacology 26 (2015) 162–168

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Helix pomatia hemocyanin — A novel bio-adjuvant for viral and bacterial antigens

Vera Gesheva [a], Stela Chausheva [a], Nadia Stefanova [a], Nikolina Mihaylova [a], Lyuba Doumanova [a], Krassimira Idakieva [b], Andrey Tchorbanov [a,*]

[a] Laboratory of Experimental Immunology, Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. 26, 1113 Sofia, Bulgaria
[b] Institute of Organic Chemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 9, 1113 Sofia, Bulgaria

ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form 26 February 2015
Accepted 11 March 2015
Available online 20 March 2015

Keywords:
Bio-adjuvants
Vaccines
Gastropodan hemocyanins
Helix pomatia
Immunological modulators

ABSTRACT

Background: New generated subunit vaccines are characterized by increased safety and lack of side effects, however they suffer from weak immunogenicity. The adjuvants are substances that have the ability to enhance the magnitude and duration of the immune response and to increase vaccine efficacy, but the different vaccines may require diverse adjuvants. The urgent need of novel adjuvant formulations occurs, thus ensuring protective cellular and humoral responses against infectious pathogens.
The hemocyanins, oxygen binding copper proteins in the hemolymph of molluscs and arthropods, are widely used as peptide carriers and vaccine adjuvants.
Results: In the present study we promote the hemocyanin isolated from the terrestrial gastropod *Helix pomatia* (HPH) as bio-adjuvant, combined with standard antigens. The purified HPH combined with influenza virus hemagglutinin intersubunit peptide (IP) or with tetanus toxoid (TT) were used for immunization.
Administration of tetanus toxoid combined with HPH in mice resulted in an increased number of anti-TT IgG producing plasmocytes and induced a significant increase of B and T cell proliferation. The level of the anti-TT IgG antibodies in mice sera was comparable to the group administered with TT + $Al(OH)_3$. An immunization of experimental animals with IP combined with *H. pomatia* hemocyanin led to generation of strong anti-influenza cytotoxic response.
Conclusion: The vaccination of mice demonstrates that the HPH is acceptable as a potential bio-adjuvant for subunit vaccines and it could be used as a natural adjuvant or protein carrier.

1. Introduction

The vaccine is one of the most successful inventions in the history of medicine, consisting of killed or inactivated pathogens, subunit viral proteins, or processed bacterial toxins. The aim of the vaccination is to induce specific Th1/Th2 immune response generating memory B and cytotoxic T-cells specific to a certain antigen, therefore preventing disease development. Whole cell vaccines produced from live or killed whole organisms are able to induce strong and potent immune response without additional compounds but it is connected with increased potential risk and high reactogenicity [1]. Next, with new vaccine development, they have become safer, but less effective. Pure recombinant or synthetic subunit vaccines have almost no side effects but reduced immunogenicity makes them unusable without adjuvants [2]. Their aim is to enhance the immune response to weak antigens and to reduce the number of immunizations needed for protective immunity, therefore optimizing immunization protocols. For this reason many adjuvants have been developed and studied in the last century [1].

Aluminum derivatives are the most widely used adjuvants for human use [3]. The regulatory limit for aluminum in biological products (including vaccines) is 0.85 mg/dose in the US and 0.125 mg/dose in Europe [4]. Although aluminum salts are well tolerated adjuvants and generate strong humoral immunity they induce feeble cellular immune response [5–7]. In addition alum can cause a number of side effects which can occur after vaccination, including granuloma formation, increased IgE production, and neurotoxicity. The most serious observation is the possibility of aluminum to accumulate in the organism and to cause neurological syndrome and even to be associated with Alzheimer's disease [1]. These facts point to the increasing need for development of effective, safe, biodegradable, and inexpensively produced adjuvants.

Currently many clinical trails of adjuvants are under evaluation. MF59, an oil-in-water microemulsion adjuvant, is part of a subunit influenza vaccine licensed in Europe [8,9]. Adjuvant System 04 (AS04), an adjuvant that contains aluminum hydroxide and monophosphoryl

Abbreviations: HPH, *Helix pomatia* hemocyanin; TT, tetanus toxoid; IP, influenza virus hemagglutinin intersubunit peptide.

* Corresponding author at: Stefan Angelov Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street, Block 26, 1113 Sofia, Bulgaria. Tel.: + 359 2 979 6357; fax: +359 2 870 0109.
E-mail address: tchorban@microbio.bas.bg (A. Tchorbanov).

http://dx.doi.org/10.1016/j.intimp.2015.03.011
1567-5769/

lipid A (MPL), is part of a vaccine which aims to prevent infection with hepatitis B for specific high-risk patients [10].

The interest to different natural products has increased tremendously in the last decades and various new applications for their use have been developed. Bioactive substances have been used as protein carriers, stimulating and enhancing an adequate immune response to particular antigens as compounds of different vaccines — tetanus toxoid, diphtheria toxoid, CRM197 (a nontoxic mutant of diphtheria toxin), *Haemophilus influenzae* protein D and *Neisseria* outer membrane protein [11]. Another group of protein carriers such as, *Pseudomonas aeruginosa* exotoxin A, flagellin and KLH (keyhole limpet hemocyanin, isolated from *Megathura crenulata* species), is extensively studied and deserves attention.

The hemocyanins (Hcs) are giant (4–8 MDa) respiratory protein complexes, freely dissolved in the hemolymph of molluscs and arthropods, and perform very promising properties as protein carriers and effective bio-adjuvants. KLH is the most studied hemocyanin for its immunomodulatory properties [12,13]. It has been extensively explored as a protein carrier, conjugated to haptens and small model antigens, as well as for tumor associated carbohydrate antigens (TACAs) with glycolipid and glycoprotein structure. Livingston et. al. have demonstrated that KLH is the most effective carrier for TACAs [14]. Another hemocyanin isolated from Chilean gastropod *Concholepas concholepas* was found to be an alternative substitution of KLH, based on its immunomodulatory and anti-cancer activity [15]. Studying the adjuvant properties of *Rapana thomasiana* hemocyanin (RtH) we also found an effective immune stimulator, generating protective long lasting humoral and cellular immunity against viral and bacterial model antigens [16].

It is well known that Hcs stimulate the immune system non-specifically by interaction with various immune cells including macrophages, polymorphonuclear cells, CD4+, CD8+ and B lymphocytes. The exact mechanism of activation of the immune system by the Hcs is unknown yet, but it results in potentiation of the humoral and cellular immune responses. Some authors speculate that carbohydrate content (3–12% of the Hcs molecules) is a key factor for the strong antigenicity of Hcs [12]. Beside the large size and a foreign biochemical structure, the specific carbohydrates additionally expand the immunogenicity of these molecules and make them potentially successful candidates for adjuvants. As biomolecules, Hcs are also biodegradable and do not accumulate in the organism, thus escaping the risks derived from heaping. Therefore, it might be possible to use Hcs as bio-adjuvants and protein carriers due to their immunogenicity, safety, and lack of side effects.

In the present study we have analyzed the ability of hemocyanin isolated from terrestrial snail *Helix pomatia* (HPH) to generate protective immune response in Balb/c mice when administered as bio-adjuvant combined with model viral and bacterial antigens. The HPH consists of three components: a β-component (β-HPH) and two α-components (αD- and αN-HPH) [17]. The β-HPH differs from the α-HPH by its ability to precipitate or crystallize during dialysis at pH 5.3 (the isoelectric pH of β-HPH) at low ionic strength. This Hc consists of only one type of polypeptide chain (β subunits) compared with two types (α and α′ subunits) in each of the two α-Hcs [18]. Because of this subunit homogeneity, structural and immunological investigations have mainly been performed on β-HPH.

The observed results revealed the potency of HPH to obtain vigorous humoral response stimulating generation of specific antibody producing B lymphocytes as well as long lasting cellular immunity with antigen specific CTLs. The preclinical studies for testing of new adjuvants include induction of inflammation, local tolerance, pyrogenicity, hypersensitivity and anaphylaxis, systemic toxicity, genotoxicity, reproduction toxicity, and carcinogenicity. Obtained immunological data suggests that HPH could be assumed as an alternative adjuvant to conventionally used alum with similar effectiveness and potentially less side effects.

2. Materials and methods

2.1. Isolation and purification of H. pomatia hemocyanin

HPH was isolated from the hemolymph of terrestrial snails *H. pomatia* as described [19]. The Hc was solubilized in 100 mM sodium acetate buffer (pH 5.7) and the β-isoform of the HPH was precipitated by dialysis against 10 mM sodium acetate buffer (pH 5.3) as described elsewhere [20]. Next, β-HPH was solubilized in 100 mM sodium phosphate buffer (pH 6.5) and further purified by gel filtration chromatography on a Sepharose 4B column (90 × 2.4 cm), and equilibrated and eluted with 50 mM PBS buffer (pH 7.2). The purity of isolated β-HPH was controlled by SDS and native PAGE [21,22] as well as by transmission electron microscopy [22]. The protein concentrations were determined spectrophotometrically using the specific absorption coefficient $A_{278}^{0.1\,\%} = 1.416\ mg^{-1}\ ml\ cm^{-1}$ (20 °C) for β-HPH [23].

Hc solution was passed through a Detoxigel column (Thermo Fisher Scientific Inc., Rockford, IL, USA) in order to remove endotoxin contaminations. The level of the remaining endotoxin was determined by Limulus Amebocyte Lysate (Coatest Gel) (LAL, Chromogenix AB, Molndal, Sweden).

2.2. Animals

Female 8-week old Balb/c mice were obtained from Harlan Farm (Blackthorn, UK). The animals were kept under specific pathogen free (SPF) conditions and the manipulations were approved by the Animal Care Commission at the Institute of Microbiology in accordance with the international regulations.

2.3. Construction of conjugated molecule bovine serum albumin-influenza peptide (BSA-IP)

The hemagglutinin intersubunit peptide (IP) Ac-MVTGLRNIPSI-QSRGLFGAIAGFIE-Ahx-K-NH_2 (containing T and B cell epitopes) from the influenza virus strain A/PR/8/34 was used to make the construct. The peptides Ac-(coding region HA317–341)-Ahx-K-NH_2 were purchased from Caslo Laboratory (Lyngby, Denmark) and were purified (≥98% purity) by high-performance liquid chromatography.

The covalent coupling of the bovine serum albumin (BSA, Sigma-Aldrich, Taufkirchen, Germany) to the peptides was performed by the classical EDC (1-ethyl-3(3′-dimethylaminopropyl) carbodiimide·HCl, Fluka AG, Buchs, Switzerland) cross-linking technique using a spacer (Ahx-K-NH_2) in the C-end of the peptides as described [16,24]. Briefly, BSA (in a concentration of 0.1 mg/ml in 0.1 M sodium phosphate buffer, pH 6.0) was mixed with a 20-fold molar excess of the peptide (dissolved in 10% (v/v) N,N-dimethylformamide (Sigma-Aldrich)) in the same buffer to 0.02 mg/ml final concentration.

The reaction was started by addition of carbodiimide at 60-fold molar excess over the BSA and the mixture was stirred overnight at 4 °C, dialyzed against phosphate buffered saline (PBS) and concentrated by ultrafiltration (XM10). The construct BSA-IP was used for coating of plates in ELISA for measuring the level of anti-flu antibody formation.

2.4. Treatment schedule

Groups of female 10-week old Balb/c mice (5–8 mice per each group) were administered intraperitoneally (i.p.) with PBS only or with three different amounts of HPH (16 μg, 40 μg, and 100 μg/mouse). Mice were boosted 21 and 35 days later with the same doses of HPH and with PBS as described above.

Three other groups were injected i.p. either with 50 μg/mouse of unconjugated IP, IP emulsified in an equal volume of Freund's complete adjuvant (CFA, Sigma) [25] or with IP mixed with 100 μg/mouse $Al(OH)_3$. Three more groups of mice were immunized with

unconjugated IP combined to different amounts of β-HPH (16 μg, 40 μg and 100 μg per mouse).

Mice were boosted 21 and 35 days later with the same doses of PBS, IP (without adjuvant), IP + $Al(OH)_3$, and with IP + HPH as described above. The re-immunization of the animals treated with CFA + IP was done with IP emulsified in an incomplete Freund's adjuvant (IFA).

Other groups of mice were immunized i.p. with 20 μg/mouse of tetanus toxoid (TT, from Bulbio, Sofia, Bulgaria) alone, or with TT emulsified in an equal volume of CFA, or with TT mixed with $Al(OH)_3$. Three more groups were administered with TT combined with different amounts of β-HPH (16 μg, 40 μg and 100 μg per mouse). Second and third immunizations were performed following the same schedule as described above.

The mice were bled before each immunization and after the last treatment. Collected sera were kept frozen at −70 °C before testing for antibodies and cytokines.

2.5. Enzyme linked immunosorbent assay (ELISA) for anti-IP, anti-TT or anti-HPH IgG antibodies

BSA-IP, TT or β-HPH diluted to 20 μg/ml in coating buffer ($NaHCO_3$, pH 9.6) was coated on 96-well plates (Nunc, Roskilde, Denmark) by incubation overnight at 4 °C. After washing with PBS/0.05% Tween 20 and blocking with 1% BSA, serum samples diluted 1:100 in PBS/0.05% Tween 20 for measuring of IgG antibodies were added and incubated for 1 h at room temperature. The plates were then washed and incubated for 1 h at room temperature with alkaline phosphatase-labeled goat anti-mouse IgG (Sigma-Aldrich). After washing, Sigma 104 phosphatase substrate was added and the absorbance was measured at 405 nm. The obtained ELISA results were presented as relative units (RU), corresponding to the titer of anti-IP, anti-TT, or anti-β-HPH standard antibodies used for ELISA.

2.6. ELISpot assay for counting anti-TT antibody-secreting cells

Splenocytes (2×10^6 cells/ml) were isolated from Balb/c mice six months after immunization either with TT + $Al(OH)_3$, TT + 40 μg HPH, or TT + 100 μg HPH, as well as with PBS only. Cells were cultured in complete RPMI (Roswell Park Memorial Institute medium) 1640 culture medium (Gibco, CA, USA) containing 10% FCS (fetal calf serum), 4 mM L-glutamine, 50 μM 2-mercaptoethanol and antibiotics at 37 °C in 5% CO_2 for 4 days in the presence of 10 μg/ml lipopolysaccharide (LPS, from *Escherichia coli*; L-2630, Sigma-Aldrich). Cells from each treated animal, cultured in medium only, were used as controls.

Furthermore, ELISpot 96-well plates (Millipore, Bedford, MA, USA) were activated with 35% ethanol, coated for 16 h with TT (10 μg/ml) at 4 °C, washed with PBS, and blocked with 1% gelatin in PBS. The pre-cultured cells were transferred to ELISpot plates with the TT-coated membranes and were further cultured for 4 h in a humidified 5% CO_2 atmosphere at 37 °C.

Further, the plates were washed and incubated with an alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich) for 1 h and developed by NBT-BCIP substrate (Sigma-Aldrich). The number of spots corresponding to cells producing IgG anti-TT antibodies was counted by C.T.L Immunospot S5 Versa Analyzer (Bonn, Germany).

2.7. Cytokine detection

IFN-γ and IL4 levels were measured in the mouse sera using ELISA sets (BD OptEIA™, BD Biosciences Pharmingen) according the manufacturer's instructions.

2.8. MTT proliferation assay

Spleen cells from mice immunized either with TT + CFA, TT + $Al(OH)_3$, TT + HPH (40 μg or 100 μg), or with PBS only, were isolated 6 months after the first immunization and used in MTT colorimetric assay for cell viability. Cells (2×10^6 cells/ml) were washed and cultured in 96-well cell culture plate (Nunc) in complete RPMI 1640 without Phenol Red (GE Healthcare, Hatfield, UK) supplemented with 10% FCS at 37 °C/5% CO_2 for 5 days. Splenocytes from each experimental group were incubated in the presence of LPS (10 μg/ml), or ConA (Sigma-Aldrich) (10 μg/ml), or culture medium only. After 5 days of cultivation MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue; Sigma] was added to each well (20 μl, 5 mg/ml) and the cells were incubated for additional 4 h. Next, the supernatant was removed and an MTT solvent (4 mM HCl, 0.1% Nondet P-40) was added (150 μl/well) to the wells to dissolve the insoluble purple formazan into a colored product. Absorbance of converted dye was measured at 595 nm.

2.9. Cytotoxic assay

The influenza A/Aichi/2/68 (H3N2) strain (the collection of the Stephan Angeloff Institute of Microbiology, Sofia, Bulgaria) was grown on MDCK cells and after pelleting, the virions were concentrated by ultracentrifugation and suspended in PBS.

Confluent 3T3 cell line (mouse embryo fibroblasts) monolayer was cultured in DMEM (5% FCS) with 10^2/ml virus particles at 37 °C, 5% CO_2 for 18 h. Later on the cells were washed, trypsinized and transferred to 96-well tissue culture plate (2×10^5 cells/ml). Freshly isolated spleen cells from mice immunized with IP peptide alone or in combination with alum, or with HPH (16 μg, 40 μg, and 100 μg/mouse), were used as effector cells in a non-radioactive cytotoxic assay at a ratio between target and effector cells 1:40 (4 weeks after the last immunization). Splenocytes obtained from mice immunized with IP + CFA or with PBS only were used as controls. After 4 h of incubation at 37 °C/5% CO_2 the cells were centrifuged (110 ×*g*, 4 min, 4 °C) and the lactate dehydrogenase (LDH) concentration released in the supernatant was determined by CytoTox assay (Promega, USA) according to the manufacturer's instructions. The percentage of specific lysis was calculated as follows: specific lysis (%) = (experimental release − spontaneous release / (maximum release − spontaneous release) × 100. Maximum or spontaneous release was obtained by the incubation of the target cells with 1% Triton or with medium only.

2.10. Statistical analysis

Values in the figures correspond to mean ± SD. The two-way ANOVA test was used to determine differences between each two groups. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Anti-IP, anti-HPH and anti-TT IgG antibody production

Blood samples were collected by retro-orbital puncture and mouse sera were tested for IgG anti-IP, anti-HPH and anti-TT IgG antibodies by ELISA. Groups of mice treated with IP peptide-$Al(OH)_3$, TT-$Al(OH)_3$, and PBS only were used as controls and significant differences were registered between the test and control groups.

The ability of *H. pomatia* hemocyanin to stimulate immune response was evaluated after immunization of Balb/c mice with three different amounts of HPH (16 μg, 40 μg and 100 μg/mouse). The results observed have shown generation of high titers of anti-HPH IgG antibodies even after the first immunization and presence of elevated levels of antibodies for at least 4 months after the first immunization (Fig. 1). The different HPH quantities exhibit similar patterns of generated antibodies which are in accordance with obtained immunogenicity data of the hemocyanin isolated from marine gastropod *R. thomasiana* [26] and indicated the potential bio-adjuvant properties of HPH.

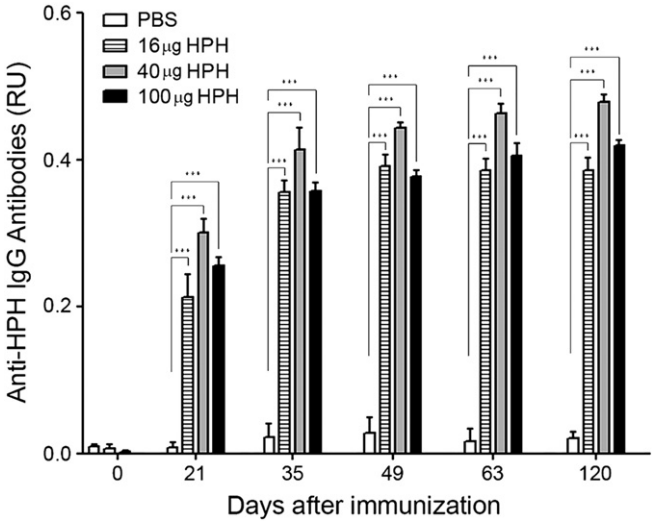

Fig. 1. Serum anti-HPH IgG antibody levels after i.p. immunization of the experimental animals with PBS or with different doses (100, 40 or 16 μg per mouse) of HPH. The animals were bled before each and 15 days after the last immunization and the sera analyses were performed by ELISA. The results are presented as Relative Units (RU), calculated using standard polyclonal IgG antibodies against HPH. The titers obtained from the standard's dilutions were used to create a curve from 0.01 to 0.6 RU. The data are represented as mean ± SD from individual sera collected (n = 5–8). The experiments were repeated twice. p values are calculated using the two-way ANOVA test (***$p < 0.005$) in comparison to PBS treated controls.

In order to study the ability of HPH to induce immune response to model antigens we administered Balb/c mice with HPH combined with viral (IP peptide) or bacterial (TT) antigens. The results from ELISA revealed that the complex of non-immunogenic small viral peptide (without chemical conjugation) with HPH is unable to generate high titers of anti-IP IgG antibodies compared to IP peptide administered with conventional adjuvant $Al(OH)_3$, although they are higher than control unimmunized mice (Fig. 2A).

When bacterial antigen TT was immunized with HPH, the complex induced high anti-TT humoral response and after the second reimmunization, the level of the anti-TT IgG Ab was comparable to the group administered with TT + $Al(OH)_3$. TT combined with different amounts of HPH has shown dose-dependent differences in respect to anti-TT IgG antibody production only after the first immunization and further reached the flatten levels (Fig. 2B).

3.2. Anti-TT IgG antibody-secreting plasma cells are affected by HPH hemocyanin

Balb/c mice were immunized with TT combined with $Al(OH)_3$ or with different amounts of HPH. The number of anti-TT producing B lymphocytes was determined by ELISpot assay. We found that cultivation of splenocytes from TT + 100 μg HPH and TT + $Al(OH)_3$-immunized mice in the presence of LPS resulted in an increased number of anti-TT IgG producing plasmocytes (Fig. 3). Low levels of anti-TT IgG antibody producing plasma cells were detected in the same groups of mice without LPS stimulation.

3.3. Cytokine profile

The cytokine levels were measured in mice sera after different immunizations using ELISA kits. To investigate whether HPH administration corresponds to the Th1/Th2 type of immune response we examined IL4 and IFN-γ levels after immunization with bacterial or viral proteins combined with HPH or with $Al(OH)_3$. The groups of Balb/c mice treated with IP + 100 μg HPH produced high serum levels of IFN-γ as compared to the PBS-injected mice after the second

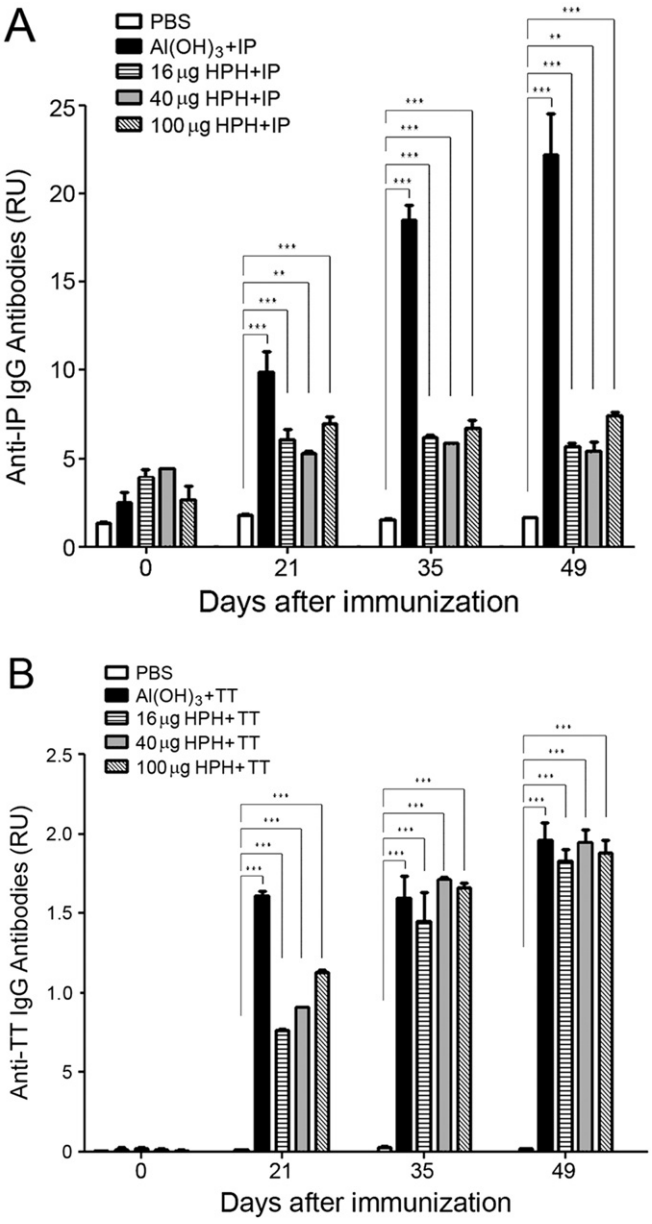

Fig. 2. Serum anti-IP and anti-TT antibody levels. Serum anti-IP and anti-TT antibody titers in mice injected i.p. with PBS or 2A. with 50 μg of IP mixed with 100 μg/mouse $Al(OH)_3$. Three other groups of mice were injected with IP combined to different amounts of β-HpH (16 μg, 40 μg and 100 μg per mouse); 2B. with 20 μg of TT mixed with 100 μg/mouse $Al(OH)_3$. Three other groups of mice were injected with TT combined to different amounts of β-HpH (16 μg, 40 μg and 100 μg per mouse). Mice were boosted 21 and 35 days later with the same doses. The animals were bled before each and 15 days after the last immunization and the sera analyses were performed by ELISA. The results are presented as Relative Units (RU), calculated using standard polyclonal IgG antibodies against IP or TT. The titers obtained from the standard's dilutions were used to create a curve from 1.0 to 25.0 RU (for IP) and from 0.1 to 2.5 RU (for TT). The data are represented as mean ± SD from individual sera collected (n = 5–8). The experiments were repeated twice. p values are calculated using the two-way ANOVA test (**$p < 0.01$; ***$p < 0.005$) in comparison to PBS treated controls.

immunization, while IP + 40 μg HPH treated mice produced high IFN-γ levels starting from the last immunization (Fig. 4A). As expected, IP + $Al(OH)_3$ immunization had no effect for IFN-γ production.

We observed a generation of high IL4 levels obtained after immunization with IP + 100 μg HPH, while the levels obtained after treatment with IP + $Al(OH)_3$ were moderate (Fig. 4B). In the other treated groups administered with IP + HPH the cytokine production measured was comparable or lower than the levels obtained after treatment of the control group.

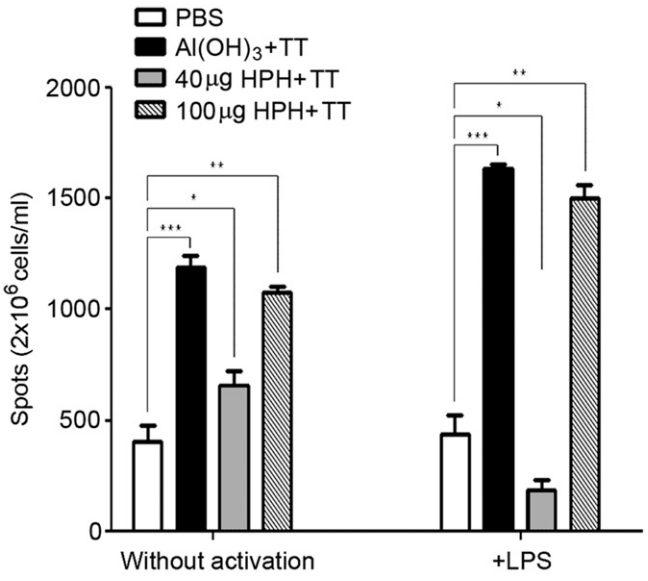

Fig. 3. ELISPOT assay for detection anti-TT IgG antibodies from plasma cells. HPH hemocyanin immunization up-regulates anti-TT IgG antibody-secreting plasma cells. Splenocytes from Balb/c mice immunized with TT (20 μg/mouse) combined either with $Al(OH)_3$ or with different amounts of HPH (40 μg or 100 μg per mouse) were cultured in the presence of LPS or in medium only. The number of plasma cells secreting anti-TT specific IgG antibodies was determined using ELISpot assays. Results are expressed as the mean value ± SD of triplicates. Splenocytes from each mouse were tested individually. The numbers of spots in the test wells were compared with control wells containing cultured splenocytes from untreated mice (ANOVA test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$). Data are representative of at least 4 experiments.

We did not observe significant differences in the serum levels between the groups of TT + HPH and TT + $Al(OH)_3$ immunized animals compared to PBS-treated mice.

3.4. HPH hemocyanine treatment increases B and T cell proliferation

Splenocytes from mice injected with TT + $Al(OH)_3$, or with TT + 40 μg HPH, or with TT + 100 μg HPH, or with PBS only, were isolated and stimulated in vitro with LPS (B-cell) and ConA (T-cell) lymphocyte activators. Cells isolated from the same animals without stimulation were used as controls. A significant increase of cell proliferation was observed in the TT + 100 μg HPH immunized group with or without in vitro stimulation and in the TT + $Al(OH)_3$ immunized group stimulated with LPS compared to PBS treated group (Fig. 5). No significant differences among the groups immunized with TT + 40 μg HPH and PBS were found.

3.5. Generation of cytotoxic cells

We tested the bio-adjuvant properties of *H. pomatia* hemocyanin by its ability to generate specific CTLs against the model viral antigen after immunization of IP + HPH in Balb/c mice. Freshly isolated spleen cells from all immunized animals were examined for CTL activity against influenza virus-infected 3T3 cells 4 weeks after the last immunization. The cytotoxic effect in the IP + 100 μg HPH immunized group was significantly stronger than the effect in other treated groups, while the effects of IP + 40 μg HPH, IP + 16 μg HPH and IP + $Al(OH)_3$ were moderate (Fig. 6). Immunization with IP + CFA, IP alone, or PBS only induced a low cytotoxic activity.

4. Discussion

Killed or subunit viral and bacterial vaccines are able to induce specific immune response, but unfortunately, they are weakly immunogenic and require adjuvants. The effective protection after vaccination

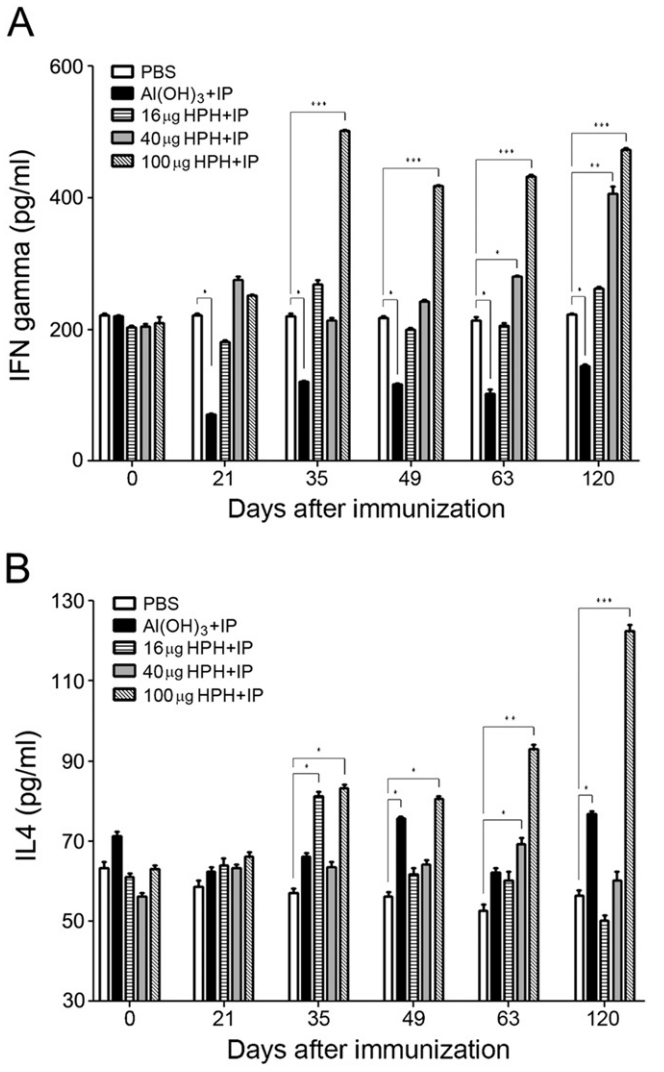

Fig. 4. Cytokine production after administration of HPH combined with IP to the experimental animals. The animals were immunized as described in the legend of Fig. 2A. Serum levels of IL4 and IFN-gamma were measured by sandwich ELISA using commercial cytokine assays. The data represent mean ± SD from individual sera collected. The experiments were repeated twice. p values are calculated using the two-way ANOVA test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$) in comparison to PBS treated controls.

should have a result induction not only of humoral immunity characterized by antibody production, but also generation of cellular immune response mediated by cytotoxic T cells. The mostly used aluminum salts, aluminum hydroxide and aluminum phosphate are able to provoke protective antibody titer activating B lymphocytes' function, however, they fail in stimulating specific CTLs [27]. A significant number of adjuvants are clearly more potent than alum, and generally have higher levels of toxicity, which have been the main reasons that they have not been used as adjuvants for human vaccine formulations. Therefore, development and identification of effective and safe vaccine adjuvants are urgent and remain to be an area of extensive investigations.

The interest in developing new vaccine adjuvants has grown for several reasons. Many of the traditional vaccines developed to induce antibodies against bacterial or viral surface molecules were adjuvanted with aluminum-containing compounds, and these vaccines stimulated primarily T helper type 2 (Th2) responses (IgG1, IgE and the production of certain cytokines) rather than Th1 responses [1,28,29]. However, many new vaccine candidates for prevention of more challenging infectious diseases (e.g., malaria and tuberculosis), cancer, fertility, and allergic and autoimmune diseases contain sophisticated antigen/adjuvant combinations (e.g. AS04 and AS01) intended to protect through the

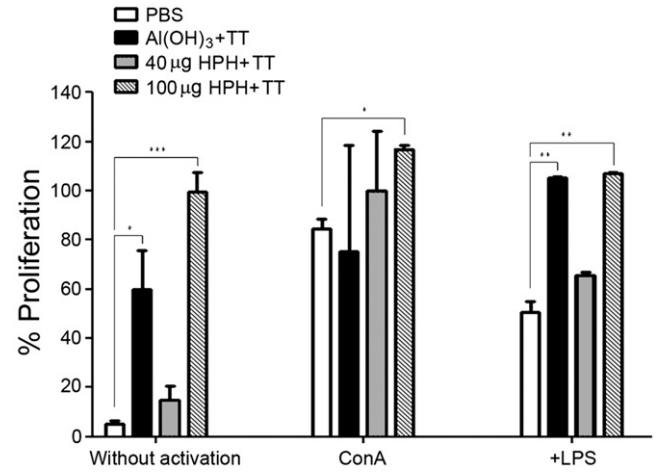

Fig. 5. Proliferation assay for detection of B and T cells. HPH immunized mice show increased B and T-cell proliferation. Spleen cells from mice immunized with TT + $Al(OH)_3$, or with TT + HPH (40 μg or 100 μg), or with PBS only, were incubated in presence of LPS, ConA, or culture media only. After 5 days of cultivation MTT solution was added to each well and cell proliferation was evaluated by measuring the converted dye spectrophotometrically. Results are expressed as the mean value ± SD of triplicates. Splenocytes from each mouse were tested individually. The results in the test wells were compared with control wells containing cultured splenocytes from untreated mice (two-way ANOVA test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$). Data are representative of at least 4 experiments.

generation of cellular immunity, particularly Th1 responses for optimal effectiveness [30].

The hemocyanins are very immunogenic in mammals and stimulate immune response because of their xenogenic origin, giant size, and complex structure. They are widely used as immune modulators. In the present study we promote the hemocyanin isolated from the terrestrial gastropod *H. pomatia* (HPH) as bio-adjuvant. It was expected that immunization of Balb/c mice with different amounts of HPH will generate high titers of anti-HPH IgG antibodies, similar to the results observed after the immunization of Balb/c mice with the hemocyanin isolated from marine gastropod *R. thomasiana* (RtH) [26].

We have shown that RtH as well as *Keyhole Limpet* hemocyanin (KLH) used as adjuvants to commercial anti-flu vaccine are potent to stimulate the production and long-lasting persistence of cytotoxic T lymphocytes specific for the influenza virus. Instead of whole anti-flu vaccine in the present study we introduced small hemagglutinin intersubunit peptide (IP) (containing T and B cell epitopes) from the influenza virus strain A/PR/8/34 with HPH as adjuvant. The level of generated antibodies to IP peptide in the sera of immunized mice was lower compared to IP mixed with alum (Fig. 2A) or with CFA (data not shown). No anti-IP IgG antibodies were detected in the sera of Balb/c mice injected with IP alone without adjuvant. The presence of IP-specific plasmocytes was not detected in mouse spleens by ELISpot assay, which explained the absence of anti-IP antibodies in the sera (data not shown).

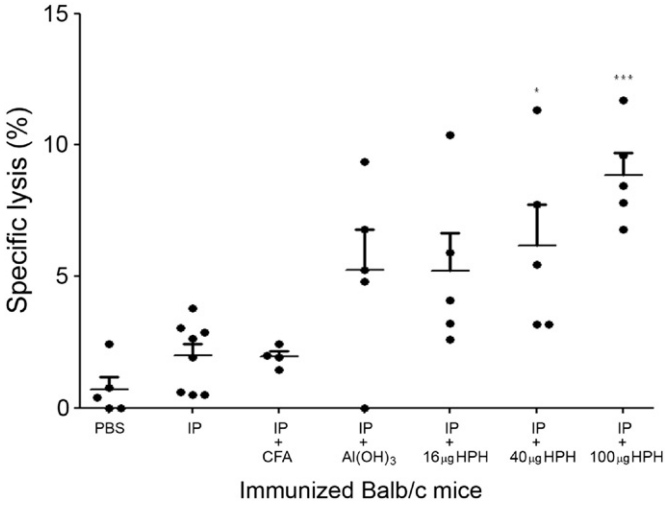

Fig. 6. CytoTox assay. CTL activity of splenocytes isolated at day 30 after the last immunization of all test-groups. 3T3 cells pulsed with the influenza virus were cultured with effector spleen cells from individual mice at a ratio 1:40 for 4 h at 37 °C. LDH concentration in the supernatants was determined in triplicates by a commercial CytoTox assay. Each icon indicates result obtained from individual mice as a part of the treated group. Results are expressed as the mean value ± SD comparing immunized and PBS only-treated mice using the two-way ANOVA test (*$p < 0.05$; ***$p < 0.005$). Data are representative of at least 3 independent experiments.

The results obtained from the cytotoxic assay reveal that HPH is a strong inductor of specific cytotoxicity to influenza virus. The IP + HPH immunization generated specific T lymphocytes (CTLs) and respectively, specific lysis of virus infected cells, higher than those induced by IP + alum, IP + CFA, and IP peptide alone (Fig. 6). These data are in accordance with the results obtained after the immunization of Balb/c mice with RtH combined with viral model antigens [26].

The tetanus toxoid is a whole protein and it is much bigger than IP peptide. The TT + HPH treated Balb/c groups produced high levels of anti-TT IgG antibodies after the second immunization and further reached the level registered in Balb/c group injected with TT + $Al(OH)_3$ (Fig. 2B). The TT + PBS immunized Balb/c group produced moderate levels of IgG antibodies after the second immunization, while in the group vaccinated with TT + CFA IgG antibody levels were comparable with HPH and $Al(OH)_3$ groups (data not shown).

In different countries the standard vaccines consist of 125–850 μg $Al(OH)_3$ per dose, but to obtain adjuvant properties many authors used higher quantities, resulting in severe reactions of inflammation and IgE synthesis [31,32]. HPH hemocyanin exhibited high immunogenicity by itself but when it is introduced with antigens it generates potent humoral and cellular immune responses. We have shown in previous experiments that RtH and KLH hemocyanins used as adjuvants to standard anti-flu vaccine in higher doses for immunization (250 μg/mouse) did not provoke any side effects, IgE synthesis or life span changes detected during six months of observation [26]. It confirms the data reported from other authors about the lack of side effects after use of KLH as bio-adjuvant and immunological modulator for different types of vaccines, including bacterial, viral, and anti-cancer ones in animal models and humans. No detectable differences have been found between KLH treated and control animals and patients such as number of CD4 + cells and lymphocyte count; development of germinal centers in axillary lymph nodes, macrophage accumulation and granuloma formation/capsule in the site of the injection, or anti-KLH antibodies of the IgE-isotype [33,34]. In the present study we have used only up to 100 μg HPH hemocyanin per mouse, which supports the expectation for low risk of side effects.

The Th1 cytokine IFN-γ is important for the efficient cellular immune response after immunization with anti-viral vaccines. High levels in mouse serum correlate with the generation of virus-specific CD8 + cells. IFN-γ levels indicated the prevalence of Th1 immune response in the groups immunized with IP + 100 μg HPH. The cytokine production observed in IP + HPH groups supports the results from the cytotoxic assay.

IL4 plays opposing role to IFN-γ and IFN-γ/IL4 ratio characterizes Th1/Th2 responses. The IL4 levels detected in IP + alum group could explain the higher anti-IP antibody titer obtained in mouse sera. Interestingly, the IL4 amount in the group immunized with TT + 100 μg HPH did not correlate with the very low anti-IP antibody levels in the same animals.

Administration of TT alone or combined with adjuvants did not result in an increased production of IL4 and IFN-γ in any group.

Splenocytes from the same groups immunized with TT combined with different adjuvants were isolated for in vitro studies and subjected to ELISpot assay. We found that cultivation of spleen cells from

TT + HPH-immunized mice resulted in an increased number of anti-TT IgG producing plasmocytes compared to PBS treated mice with or without LPS activation (Fig. 3).

The most important increase of the number of plasmocytes secreting TT-specific IgG antibodies was observed in the group of mice immunized with TT + 100 μg HPH which was comparable with the result obtained from TT + $Al(OH)_3$ group.

The performed proliferation assay confirmed this observation. Incubation of splenocytes from TT + 100 μg HPH immunized mice in the presence of either B cell stimulation (LPS), T cell stimulation (ConA) or without stimulation leads to increased cell proliferation compared to PBS treated group. The detected proliferation was even higher than the one from TT + $Al(OH)_3$ treated animals.

These findings provide reasons to believe that *H. pomatia* hemocyanin could be used as potential bio-adjuvant for different types of viral or bacterial subunit vaccines.

Acknowledgments

This study was supported by the Bulgarian National Science Fund — grant DTK 02/78.

References

[1] N. Petrovsky, J.C. Aguilar, Vaccine adjuvants: current state and future trends, Immunol. Cell Biol. 82 (5) (2004) 488–496.
[2] A.S. McKee, M.K.L. MacLeod, J.W. Kappler, P. Marrack, Immune mechanisms of protection: can adjuvants rise to the challenge? BMC Biol. 8 (2010) 37.
[3] E.B. Lindblad, Aluminium adjuvants, in: D.E.S. Stewart-Tull (Ed.), The Theory and Practical Application of Adjuvants, John Wiley & Sons Ltd, Chichester, 1995, pp. 21–35.
[4] W. Wang, M. Singh, Selection of adjuvants for enhanced vaccine potency, World J Vaccines 1 (2011) 33–78.
[5] R. Schirmbeck, K. Melber, T. Mertens, J. Reimann, Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implications for the pathogenesis of HBV-induced hepatitis, J. Virol. 68 (1994) 1418–1425.
[6] P. Traquina, M. Morandi, M. Contorni, G. Van Nest, MF59 adjuvant enhances the antibody response to recombinant hepatitis B surface antigen vaccine in primates, J. Infect. Dis. 174 (1996) 1168–1175.
[7] J.M. Brewer, M. Conacher, A. Satoskar, H. Bluethmann, J. Alexander, In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production, Eur. J. Immunol. 26 (1996) 2062–2066.
[8] A. Podda, G. Del Giudice, MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile, Expert Rev. Vaccines 2 (2) (2003) 197–203.
[9] D. Sesardic, R. Dobbelaer, European Union regulatory developments for new vaccine adjuvants and delivery systems, Vaccine 22 (19) (2004) 2452–2456.
[10] N. Garcon, S. Morel, A. Didierlaurent, D. Descamps, M. Wettendorff, M. Van Mechelen, Development of an AS04-adjuvanted HPV vaccine with the adjuvant system approach, BioDrugs 25 (4) (2011) 217–226.
[11] M. Knuf, F. Kowalzik, D. Kieninger, Comparative effects of carrier proteins on vaccine-induced immune response, Vaccine 29 (31) (2011) 4881–4890.
[12] J.R. Harris, J. Markl, Keyhole limpet hemocyanin (KLH): a biomedical review, Micron 30 (1999) 597–623.
[13] J. Markl, B. Lieb, W. Gebauer, B. Altenhein, U. Meissner, J.R. Harris, Marine tumor vaccine carriers: structure of the molluscan hemocyanins KLH and HtH, J. Cancer Res. Clin. Oncol. 127 (2001) R3–R9.
[14] E. Kagan, G. Ragupathi, S.S. Yi, C.A. Reis, J. Gildersleeve, D. Kahne, H. Clausen, S.J. Danishefsky, P.O. Livingston, Comparison of antigen constructs and carrier molecules for augmenting the immunogenicity of the monosaccharide epithelial cancer antigen Tn, Cancer Immunol. Immunother. 54 (2005) 424–430.
[15] B. Moltedo, F. Faunes, D. Haussmann, P. De Ioannes, A.E. De Ioannes, J. Puente, I.M. Becker, Immunotherapeutic effect of *Concholepas* hemocyanin in the murine bladder cancer model: evidence for conserved antitumor properties among hemocyanins, J. Urol. 176 (2006) 2690–2695.
[16] V. Gesheva, K. Idakieva, N. Kerekov, K. Nikolova, N. Mihaylova, L. Doumanova, A. Tchorbanov, Marine gastropod hemocyanins as adjuvants of non-conjugated bacterial and viral proteins, Fish Shellfish Immunol. 30 (2011) 135–142.
[17] G. Préaux, Gielens C. Hemocyanins, Copper Proteins and Copper Enzymes, in: R. Lontie (Ed.), vol. II, CRC Press, Boca Raton, 1984, pp. 159–205.
[18] R. Lontie, Components, functional units, and active sites of *Helix pomatia* hemocyanin, Life Chem. Rep. (Suppl. 1) (1983) 109–120.
[19] K. Heirwegh, H. Borginon, R. Lontie, Separation and absorption spectra of α- and β-haemocyanin of *Helix pomatia*, Biochim. Biophys. Acta 48 (1961) 517–526.
[20] C. Gielens, L.J. Verschueren, G. Pre aux, R. Lontie, Gel chromatographic separation of the haemocyanins of *Helix pomatia*. Further electrophoretic and immunological characterization of the components, Comp. Biochem. Physiol. 69B (1981) 455–462.
[21] K. Idakieva, S. Severov, I. Svendsen, N. Genov, S. Stoeva, M. Beltramini, G. Tognon, P. Di Muro, B. Salvato, Structural properties of *Rapana thomasiana* hemocyanin: isolation, characterization and N-terminal amino acid sequence of two different dissociation products, Comp. Biochem. Physiol. 106B (1993) 53–59.
[22] K. Idakieva, H. Schwartz, N. Genov, W. Voelter, S. Stoeva, *Rapana thomasiana* hemocyanin (RtH): dissociation and reassociation behaviour of two isoforms. RtH1 and RtH2, Micron 33 (2002) 7–14.
[23] E.J. Wood, M.F. Chaplin, C. Gielens, J. De Sadeleer, G. Preaux, R. Lontie, Relative molecular mass of the polypeptide chain of βc-haemocyanin of *Helix pomatia* and carbohydrate composition of the functional units, Comp. Biochem. Physiol. B 82 (1985) 179–186.
[24] S. Bauminger, M. Wilchek, The use of carbodiimides in the preparation of immunizing conjugates, Methods Enzymol. 70 (1980) 151–159.
[25] D.E. Stewart-Tull, Freund's complete and incomplete adjuvants, preparation, and quality control standards for experimental laboratory animals use, Vaccine 24 (15) (2006) 3018–3025.
[26] A. Tchorbanov, K. Idakieva, N. Mihaylova, L. Doumanova, Modulation of the immune response using *Rapana thomasiana* hemocyanin, Int. Immunopharmacol. 8 (7) (2008) 1033–1038.
[27] E. Agger, J. Cassidy, J. Brady, K. Korsholm, C. Vingsbo-Lundberg, P. Andersen, Adjuvant modulation of the cytokine balance in mycobacterium tuberculosis subunit vaccines: immunity, pathology and protection, Immunology 124 (2008) 175–185.
[28] N. Burdin, B. Guy, P. Moingeon, Immunological foundations to the quest for new vaccine adjuvants, BioDrugs 18 (2) (2004) 79–93.
[29] E.B. Lindblad, Aluminium adjuvants — in retrospect and prospect, Vaccine 22 (2004) 3658–3668.
[30] N. Garcon, P. Chomez, M. Van Mechelen, GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives, Expert Rev. Vaccines 6 (5) (2007) 723–739.
[31] A. Abadie, A. Prouvost-Danon, Specific and total IgE responses to antigenic stimuli in Brown-Norway, Lewis and Sprague–Dawley rats, Immunology 39 (1980) 561–569.
[32] M. Akahira-Azuma, M. Szczepanik, R.F. Tsuji, R.A. Campos, A. Itakura, N. Mobini, et al., Early delayed-type hypersensitivity eosinophil infiltrates depend on T helper 2 cytokines and interferon-γ via CXCR3 chemokines, Immunology 111 (2004) 306–317.
[33] D.R. Roth, D. Roman, P. Ulrich, A. Mahl, U. Junker, E. Perentes, et al., Design and evaluation of immunotoxicity studies, Exp. Toxicol. Pathol. 57 (2006) 367–371.
[34] P. Boelens, J. Fonk, A. Houdijk, R. Scheper, H. Haarman, S. Meijer, et al., Primary immune response to keyhole limpet haemocyanin following trauma in relation to low plasma glutamine, Clin. Exp. Immunol. 136 (2004) 356–364.