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Marine gastropod hemocyanins as adjuvants of non-conjugated bacterial and viral proteins

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ABSTRACT

Killed viral vaccines and bacterial toxoids are weakly immunogenic. Numerous compounds are under evaluation as immunological adjuvants and peptide-carriers to improve the immune response. The hemocyanins, giant extracellular copper proteins in the blood of many mollusks, are widely used as immune stimulants. In the present study we investigated the adjuvant properties of hemocyanins isolated from marine gastropods *Rapana thomasiana* and *Megathura crenulata.* An immunization with Influenza vaccine or tetanus toxoid combined with *Rapana thomasiana* hemocyanin (RtH) and Keyhole limpet hemocyanin (KLH) in mice induced an anti-influenza cytotoxic response lasting at least 5 months and an antibody response to viral proteins. The IgG antibody response to the tetanus toxoid (TT) combined with RtH or KLH was comparable to the response of the toxoid in complete Freund's adjuvant. The results obtained demonstrate that the both hemocyanins are acceptable as potential bio-adjuvants for subunit vaccines.

1. Introduction

Many of the vaccines that are in use today consist of mainly killed viruses and microbial agents, or, alternatively – viral subunits or bacterial toxoids. The desired immune response to vaccines is the production of antibodies and/or generation of cytotoxic T-cells, and this is enhanced by adding certain substances to the vaccines. Previously used vaccines made from live or killed whole organisms were effective, but suffered from high reactogenicity. As vaccine manufacturers developed safer, less reactogenic subunit vaccines, they found that with lower reactogenicity came reduced vaccine effectiveness. Adjuvants are vaccine additives that enhance the elicited levels of antibodies and specific T lymphocytes. According to their chemical nature, adjuvants are a highly heterogenous group of compounds with only one thing in common: their ability to enhance the immune response. The mode of action of adjuvants is formation of an antigen depot with slow release at the site of inoculation, presentation of antigen to immunocompetent cells and production of various cytokines [1]. In the conventional vaccines, adjuvants are used to elicit an early, high and long-lasting immune response. The newly developed purified subunit or synthetic vaccines using biosynthetic, recombinant and other modern technologies are poor immunogens and require adjuvants to evoke the immune response [2–4]. There are several types of adjuvants; today the most common adjuvants for human use are aluminium hydroxide, aluminium phosphate and calcium phosphate. However, there are a number of other adjuvants based on oil emulsions, products from bacteria, endotoxins, paraffinic and vegetable oils. Alum has been widely used in human vaccines for 70 years, but the molecular mechanism of its action and the target cells are still unknown. It has been proposed that absorption to it increase antigen availability at injection site allowing an efficient uptake by antigen-presenting cells (APCs) [5]. Alum could also increase antigen uptake by dendritic cells in vitro, further supporting an antigen delivery function [6].

The desire for new and improved adjuvants stems not only from the need to make existing inactivated vaccines more potent, but also to gain features such as antigen-spreading ability, more rapid seroprotection, stimulation of T-cell immunity, and longer-lasting protective immunity. Safety and tolerability are critical regulatory issues confronting the new adjuvants, and pose the greatest barrier to new adjuvant approvals. Therefore, the benefits of incorporating any adjuvant into vaccines must be balanced against any increased reactogenicity or risk of adverse reactions. Unfortunately, in most cases, increased adjuvant potency is associated with increased reactogenicity and toxicity. The best example for this is complete

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Freund's adjuvant (CFA). While it remains the gold standard in terms of adjuvant potency, its extreme reactogenicity and toxicity precludes its use in human vaccines.

The hemocyanins (Hcs) are a multigene family of giant extracellular copper proteins which serve as oxygen carriers in the blood of many mollusks [7,8]. Their large molecular size (4 to 8 MDa), xenogenic character and carbohydrate content have been implicated in inducing strong immune response in mammals. Hcs have been applied as hapten carriers, as adjuvants in immunocompetent tests and as experimental antigens in studies of the immune system. Keyhole limpet hemocyanin isolated from marine gastropod *Megathura crenulata* is a powerful immunogen and therefore has been widely used as a carrier for peptides inducing antibodies with specificity for the native proteins from which they were derived [1,9–14]. Recently, Hc obtained from the Chilean gastropod *Concholepas concholepas* was reported to possess adjuvant immunostimulatory effect [15–18].

In our previous studies on the hemocyanin isolated from *Rapana thomasiana* – a marine gastropod living along the west coast of the Black Sea – we demonstrated its high immunogenicity as a single model antigen and also its properties as a strong protein carrier for viral peptides from Influenza hemagglutinin [19]. These results suggested a potential role of RtH as an acceptable compound needed for adjuvanicity of standard vaccines without covalent binding to the antigen.

The aim of the present work was to investigate the adjuvant properties of RtH and KLH in an experimental murine model and to use them as non-conjugative carriers of viral or bacterial proteins.

2. Materials and methods

2.1. Hemocyanins preparation

Isolation of Rapana thomasiana hemocyanin: Living marine snails *Rapana thomasiana* were caught near Bulgarian coast of Black Sea (Varna) and stored in sea water. The hemolymph was collected by bleeding through several diagonal slits made on the foot of the mollusc and filtered through gauze. Phenylmethanesulphonyl fluoride (PMSF) was added (1mM) to the crude material to avoid possible proteolysis of the hemolymph. Hemocytes and other cells were removed by centrifugation at $5000 \times g$ for 30 min at 4 °C. Native RtH was isolated from freshly obtained hemolymph by ultracentrifugation at $180\,000 \times g$ (an ultracentrifuge Beckman LM-80, rotor Ti 45) for 4 h at 4 °C and stored in the presence of 20% sucrose (w/v) at −20 °C until used.

RtH was further purified by gel filtration chromatography on a Sepharose 4B column (90 × 2.0 cm), equilibrated and eluted with 50 mM PBS, pH 7.2. The purity of the isolated Hc was controlled by SDS- and native PAGE as described previously [20,21]. Protein concentration was determined spectrophotometrically using the absorption coefficient $A_{278}^{0.1\%} = 1.36\ \mathrm{mg^{-1}\ ml\ cm^{-1}}$ [20].

Hc solution was passed once through a purification column to remove endotoxin contaminations (Detoxigel column, Pierce). The level of the remaining endotoxin was determinated by Limilus Amebocyte Lysate coatest gel (LAL) (Chromogenix AB, Molndal, Sweden).

KLH was purchased from Calbiochem (Darmstadt, Germany).

2.2. Transmission electron microscopy (TEM)

The sample of native RtH was adsorbed to a glow-discharged Poloform/carbon coated support film, washed with distilled water to remove the buffer salts and negatively stained with 5% (w/v) ammonium molybdate containing 1% trehalose at pH 7.0. Sample was viewed in a Philips CM 10 transmission electron microscope at 60 kV acceleration voltage and an instrumental magnification of 52 000.

2.3. Construction of conjugated molecules Bovine serum albumin – influenza peptide (BSA-IP)

The hemagglutinin intersubunit peptide IP (containing T and B cell epitopes) from the influenza virus strain A/PR/8/34 was used to make the construct. The synthesis of Ac-(coding region HA317-341)-NH-$(CH_2)_6$-NH_2 was carried out using a Fmoc-based manual solid phase peptide synthesis protocols on 2-Cl-Trt resin. The peptides Ac-(coding region HA317-341)-Ahx-K-NH_2 were purchased from Caslo Laboratory (Lyngby, Denmark) and were purified (≥98% purity) by HPLC [22].

The covalent coupling of the 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 [23]. The BSA (in concentration 0.1 mg/ml in 0.1M 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 PBS and concentrated by ultrafiltration (XM10). The construct BSA-IP was used for coating of plates in ELISA measuring the level of anti-flu antibodies formation.

2.4. Animals

Balb/c mice were obtained from Iffa-Credo, L'Arbresle, France (Charles River Company) and bred under specific-pathogen-free conditions in our animal facility. Female mice aged 8 to 10 weeks were used for immunization.

2.5. Immunization protocols

The protocols used were approved by the Animal Care Commission at the Institute of Microbiology in accordance with the International laws. Control groups of Balb/c mice (6 to 8 mice each) were injected i.p. with PBS only or with commercial anti-flu vaccine Influvac (2007/2008, Solvay Pharmaceuticals, The Netherlands) alone or with the same vaccine emulsified in an equal volume of Freund's complete adjuvant (CFA, Sigma). Further in the text the commercial vaccine is abbreviated as Vac or when administered in CFA as Vac + CFA. The vaccine dose contained 15 μg influenza hemagglutinin (HA) per mouse. Two other groups of mice were immunized with Vac combined to RtH or KLH (100 μg per mouse). Two more groups were treated with RtH or KLH alone. Mice were boosted 21 days later with the same doses of PBS, Vac (without adjuvant), Vac + KLH, Vac + RtH, RtH or KLH as described above. The reimmunization of animals treated with Vac emulsified in CFA was done with Vac emulsified in an incomplete Freund's adjuvant (IFA). The last treatment was administered 14 days later under the same schedule.

Other groups of mice were administered with 20 μg per mouse of tetanus toxoid (from Bulbio, Sofia, Bulgaria) alone or with tetanus toxoid emulsified in an equal volume of CFA, or with the same toxoid combined to RtH or KLH (100 μg per mouse). Second and third immunizations were performed under 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.

2.6. Enzyme linked immunosorbent assay (ELISA) for anti-flu and anti-TT IgG and IgM antibodies

BSA-IP or TT diluted to 20 μg/ml in coating buffer ($NaHCO_3$, pH 9.6) was used for coating of microplates (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 for measuring of IgG or IgM 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-labelled goat anti-mouse IgG or IgM (Pharmingen BD, San Diego, USA). 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 or anti-TT standard antibodies used for ELISA.

2.7. Cytokine detection

IL4 and IFN-γ levels were measured in mouse sera using commercial ELISA kits (BD Biosciences, USA).

2.8. Cytotoxity 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 (mouse embryo fibroblasts) cell monolayer cultured in DMEM with 5% FCS was incubated with 10^2/ml virus for 18 h at 37 °C. Then cells were washed, trypsinized and transferred to the wells of a 96-well tissue culture plate (2×10^5 cells/ml). Freshly isolated spleen cells from immunized mice were used as effector cells in a non-radioactive cytotoxic assay at a ratio between target and effector cells 1:40. After incubation for 4 h at 37 °C the cells were centrifuged and the lactate dehydrogenase (LDH) concentration in the supernatants was determined by the CytoTox assay (Promega, USA) according to the manifacturer's instructions. The percentage of specific lysis was calculated as follows: specific lysis (%) = (experimental release-spontaneous release/(maximum release-spontaneous release) × 100. Maximal or spontaneous release was obtained by incubating of the target cells with 1% Triton or with medium only.

2.9. Statistical analysis

Values in the figures correspond to mean ± SD. All ELISA, cytokine and cytotoxity samples were triplicated. The unpaired Student *t*-test was used to determine differences between each two groups. The two-tailed Mann–Whitney U test was used when appropriate. A value of $P < 0.05$ was considered as statistically significant.

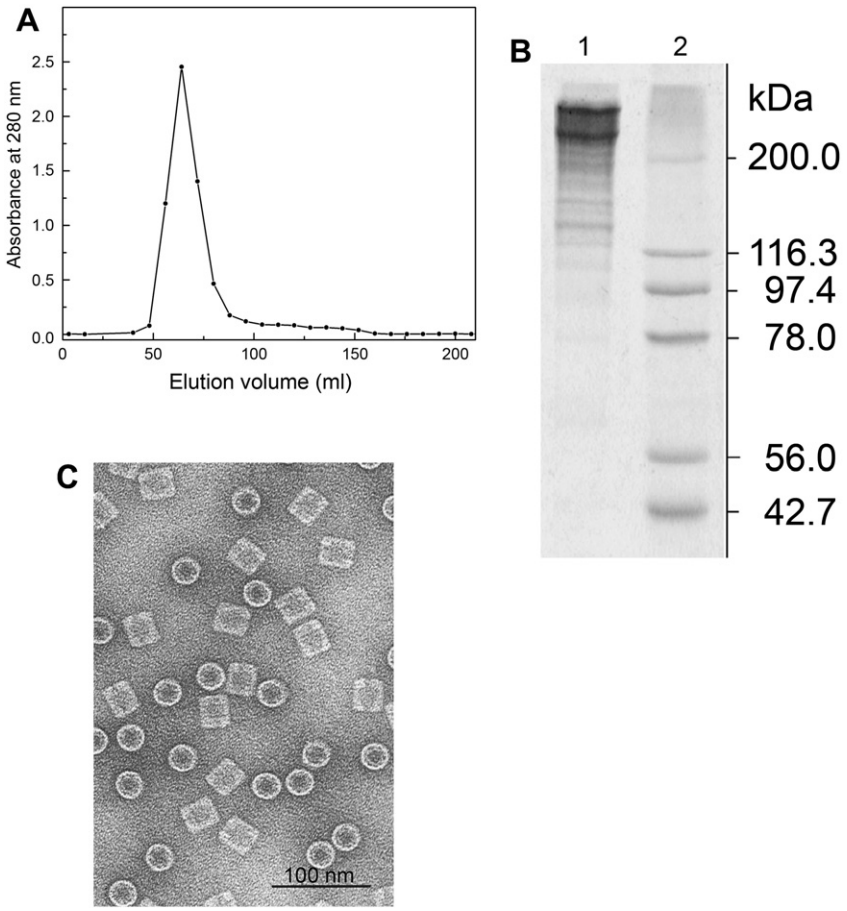

Fig. 1. A. Gel filtration of RtH on a Sepharose 4B column (90 × 2.0 cm) equilibrated and eluted with 50 mM PBS, pH 7.2. B. SDS-PAGE on 7.5 % running gel of RtH under reducing conditions. Line 1, isolated RtH; Line 2, protein markers with the following molecular masses (from the top): myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase b, 97.4 kDa; ovotransferrin, 78 kDa; glutamate dehydrogenase, 56 kDa; ovalbumin 42.7 kDa. C. Electron micrograph of specimen prepared from native RtH. The specimen was negatively stained with 5% ammonium molybdate, containing 1% trehalose. The scale bar indicates 100 nm.

3. Results

3.1. Preparation of Rapana thomasiana hemocyanin

The purification of Hc from the hemolymph of marine gastropod *Rapana thomasiana* yielded a highly purified protein preparation as assessed by gel filtration chromatography on a Sepharose 4B column and PAGE (Fig. 1A–B). The electron micrograph of isolated RtH showed a homogeneous preparation of solitary didecamers, typical for gastropodan Hcs (Fig. 1C). The evaluation of purity and chemical characterization of the RtH molecule by SDS-, native-PAGE, and N-terminal sequence analysis was described in details in our previous works [20,21]. The final purified and sterile Hc solution contained 0.48 EU/mg protein and was used for further study of its adjuvant properties.

3.2. Adjuvant activity of RtH and KLH for anti-flu IgG and IgM antibodies formation

Blood samples were collected by retro-orbital puncture and mouse sera were prepared and tested for IgM and IgG anti-IP antibodies by ELISA. The Vac + KLH groups have produced high levels of IgM antibodies after the second immunization. In the group vaccinated with Vac + CFA and Vac + RtH IgM antibody formation started after the last immunization and serum IgM levels further reached the level registered in the group injected with Vac + KLH (Fig. 2, lower panel). Induction of IgG anti-flu antibodies was not observed in the groups immunized with Vac, Vac + CFA and Vac + RtH, but an increase in anti-IP titers were observed in the Vac + KLH treated group (Fig. 2, upper panel).

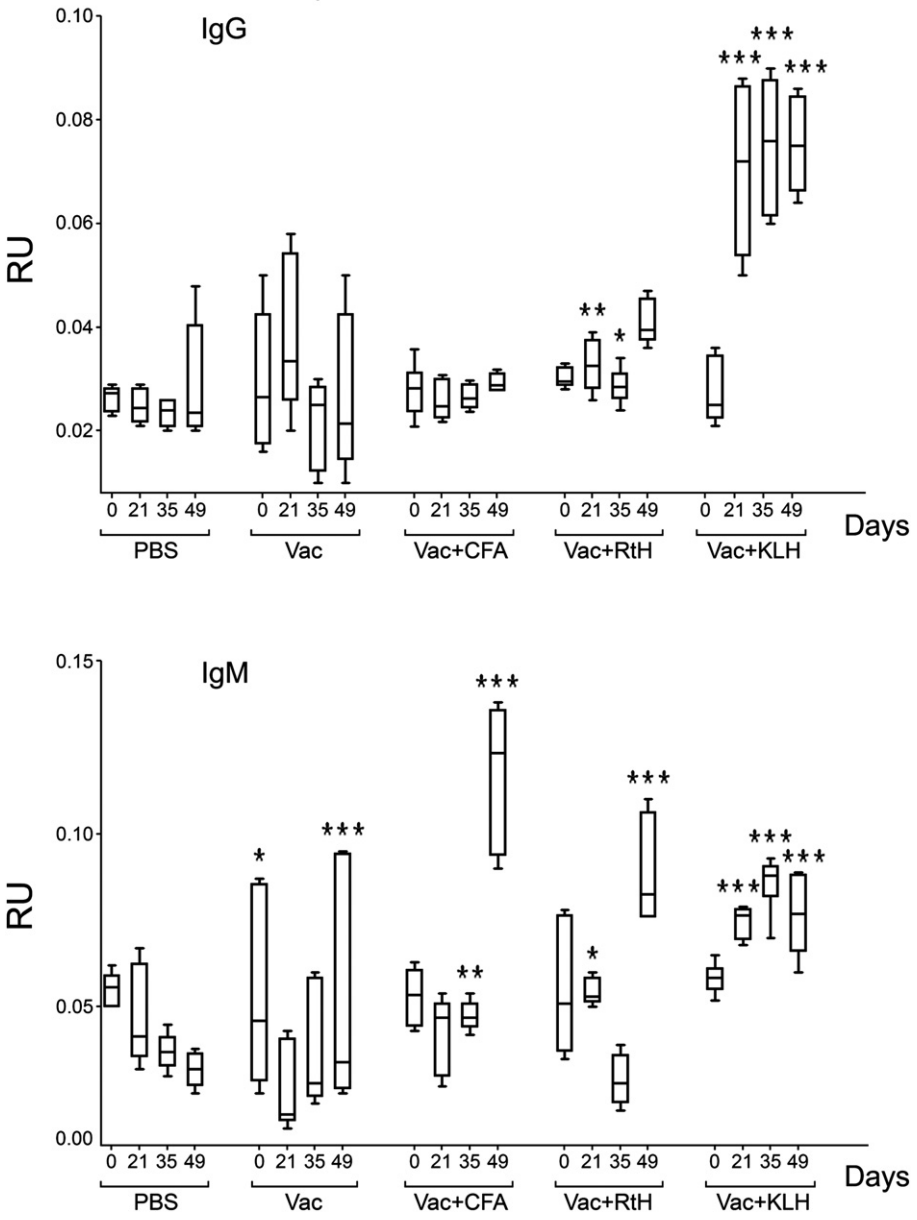

Fig. 2. Serum anti-IP antibody titers in mice injected i.p. with PBS or with 15 μg of commercial Influenza vaccine alone (Vac) or vaccine in CFA (Vac + CFA). Two other groups of animals were immunized with Vac (15 μg per mouse) combined to 100 μg RtH or KLH. Mice were boosted 21 days later and once again 14 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 and IgM antibodies against IP. The titers obtained from the standard's dilutions were used to create a curve from 0.01 to 0.2 RU. The data are represented as mean ± SEM from individual sera collected ($n = 6–8$). The experiments were repeated twice. *P* values are calculated using the Mann–Whitney U test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$), in comparison to PBS treated controls.

3.3. Adjuvant activity of RtH and KLH for anti-TT IgG and IgM antibodies formation

Testing of the sera for specific anti-TT IgG and IgM antibodies by ELISA showed that the groups immunized with TT, TT + KLH and TT + RtH produced high levels of IgG antibodies even after the second immunization (week 5) (Fig. 3, upper panel). RtH showed better adjuvant property than KLH at this point, similar to the antibody response to TT + CFA. After the third immunization TT + RtH and TT + KLH reached the maximal values obtained by TT + CFA. The group immunized with TT alone also showed high levels of anti-TT IgG antibodies after first and second booster.

For all immunized groups IgG class switching was observed. In the group treated with TT + KLH and TT + CFA high levels of IgM antibodies were developed even after booster vaccinations (Fig. 3, lower panel).

3.4. Cytokine detection

IL4 and IFN-γ levels were measured in mice sera using ELISA kits. To investigate whether the anti-hemocyanin response corresponds to the Th1/Th2 type of immune response we examined the cytokine levels after immunization with bacterial or viral proteins with RtH or KLH. We observed a high correlation between the IL4 and IFN-γ levels obtained after immunization with Vac + KLH and Vac + RtH (Fig. 4). In both cases the cytokine production measured was lower than the levels obtained after treatment with the control groups.

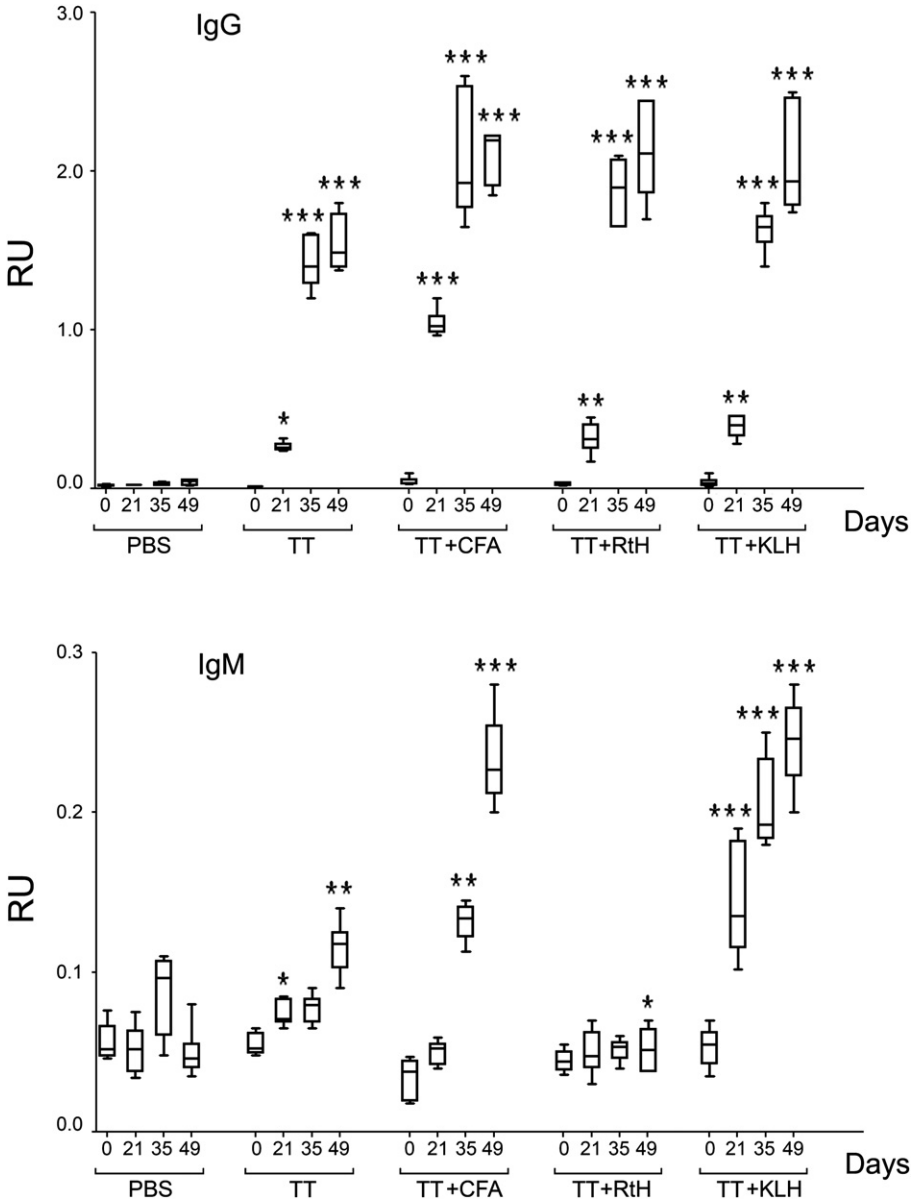

Fig. 3. Anti-TT IgG and IgM levels induced by TT immunization with or without adjuvant. The mice were injected i.p. with PBS or with 20 μg of TT alone or TT in CFA (TT + CFA). Two other groups of mice were immunized with TT (20 μg per mouse) combined to 100 μg RtH or KLH. The treated groups were boosted 21 days later and once again 14 days later with the same doses. The animals were bled before each and 15 days after the last immunization. The results are presented as Relative Units (RU), calculated using standard polyclonal IgG and IgM antibodies against TT. The titers obtained from the standard's dilutions were used to create a curve from 0.01 to 0.5 RU for IgM measurement and from 0.1 to 5.0 RU for IgG determination. The data are represented as mean ± SEM from individual sera collected ($n = 6–8$). The experiments were repeated twice. P values are calculated using the Mann–Whitney U test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$), in comparison to PBS treated controls.

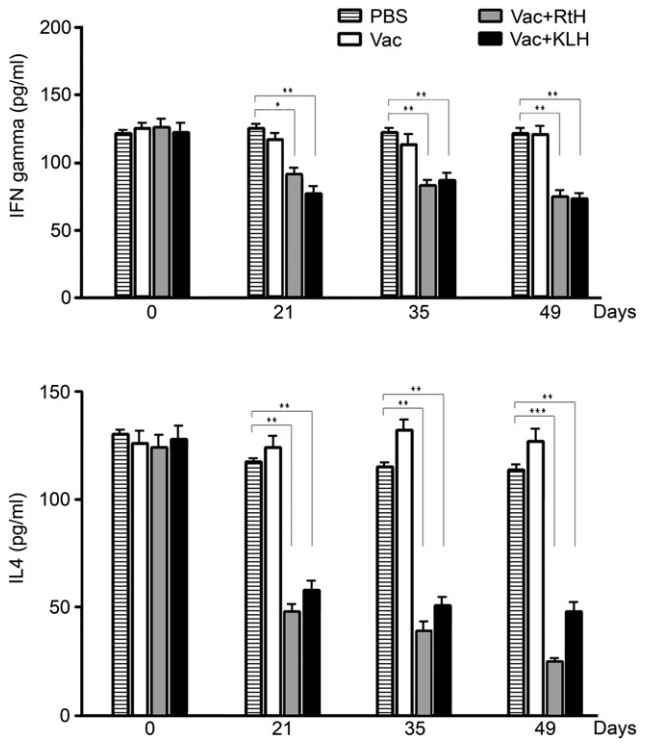

Fig. 4. Cytokine profile of mice vaccinated with Influenza Vac. The animals were immunized as described in legend to Fig. 2. Serum levels of IL4 and IFN-gamma were measured by sandwich ELISA using commercial cytokine assays. The data represent mean ± SD from individual sera collected. *P* values are calculated using the Mann–Whitney U test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$), in comparison to PBS treated controls.

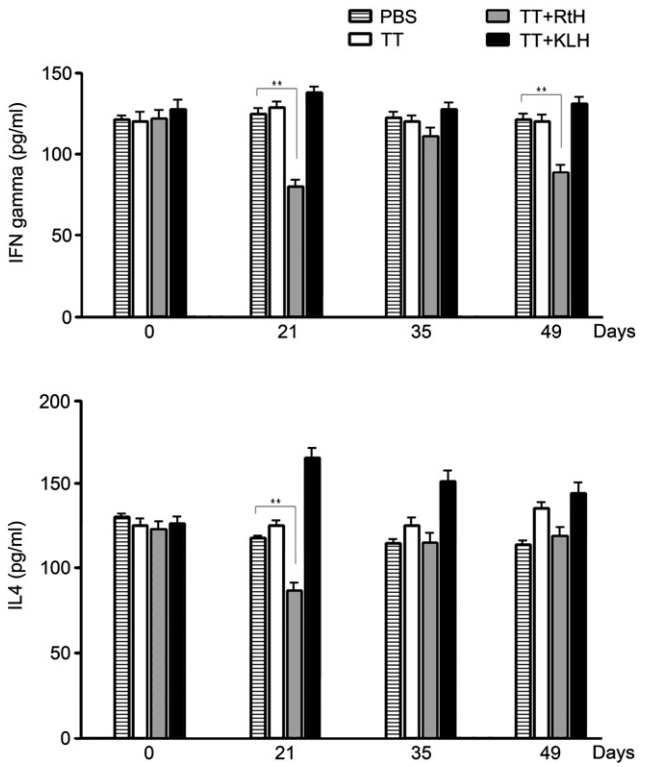

Fig. 5. Cytokine production after administration of TT to the experimental animals. The mice were immunized as described in legend to Fig. 3. Serum levels of IL4 and IFN-gamma were measured by sandwich ELISA using commercial cytokine assays. The data represent mean ± SD from individual sera collected. *P* values are calculated using the Mann-Whitney U test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$), in comparison to PBS treated controls.

Vaccination with TT did not result in an increased production of IL4 and IFN-γ in any group except in the TT + KLH treated group where an increase of IL4 synthesis was observed (Fig. 5). However, IL4 and IFN-γ levels were modestly decreased after treatment with TT + RtH.

3.5. Generation of cytotoxic cells

We examined the CTL activity of freshly isolated spleen cells from all animals against influenza virus-infected 3T3 cells three months after the last immunization. Immunization with RtH or KLH alone induced a low cytotoxic activity, while the effects of Vac and Vac + CFA were moderate. The effector cells obtained from the Vac + KLH group lysed 7.5 % of the infected cells (Fig. 6). The cytotoxic effect in the Vac + RtH immunized group was significantly stronger than the effect in other treated groups.

4. Discussion

Adjuvants are substances which are added to vaccines to boost immune response, but the major unsolved challenge in their development is how to generate a potent adjuvant effect while avoiding reactogenicity or toxicity. The development of recombinant DNA technology presents a new way to design vaccines that no longer have side effects. The goal of vaccine development is to produce multivalent vaccines with built-in adjuvanicity, and the engineering approach may satisfy that need. Such a hybrid DNA molecule was constructed by us, encoding a T- and B-cell epitope-containing influenza hemagglutinin peptide and a single-chain (scFv) antibody fragment binding to mouse complement receptors 1 and 2. A single immunization with a plasmid containing the described construct induced a strong anti-influenza cytotoxic response lasting for more than 6 months [24,25].

The use of Hcs from different sources as carriers of haptens and peptides for production of monoclonal antibodies and as carriers of vaccines against infectious diseases is generally accepted. Despite the increasing use of sophisticated small antigenic fragments, containing defined B and T cell epitopes, distinct adjuvants and vaccine preparations are desirable. We have tested RtH and KLH as non-conjugated adjuvant for standard vaccine preparations – commercial tetanus toxoid and flu vaccine.

Side effects of the KLH have been studied a lot due to its large use as bio-adjuvant and immunological modulator for different types of vaccines, including bacterial, viral and anti-cancer ones in animal models and humans. In their study concerning immunotoxicity Roth et al. have shown that only the levels of KLH specific antibodies have been elevated in the group, immunized with KLH (50 μg/rat in aluminium hydroxide) compared to the control non-treated animals and there was no detectable difference between them in the following parameters: number CD4+ cells and lymphocyte count; development of germinal centers in Axillary lymph nodes and macrophage accumulation and granuloma formation/capsule in the site of the injection [26]. Boelens et al., examine the antibody response to KLH in healthy and trauma patients and did not find neither in the patients nor in the healthy controls anti KLH antibodies of the IgE-isotype [27].

In previous experiments we have used higher doses of RtH for immunization (250 μg/mouse) and we observed the animals for six months [19]. There weren't any side effects, IgE synthesis or life span changes detected (data not shown).

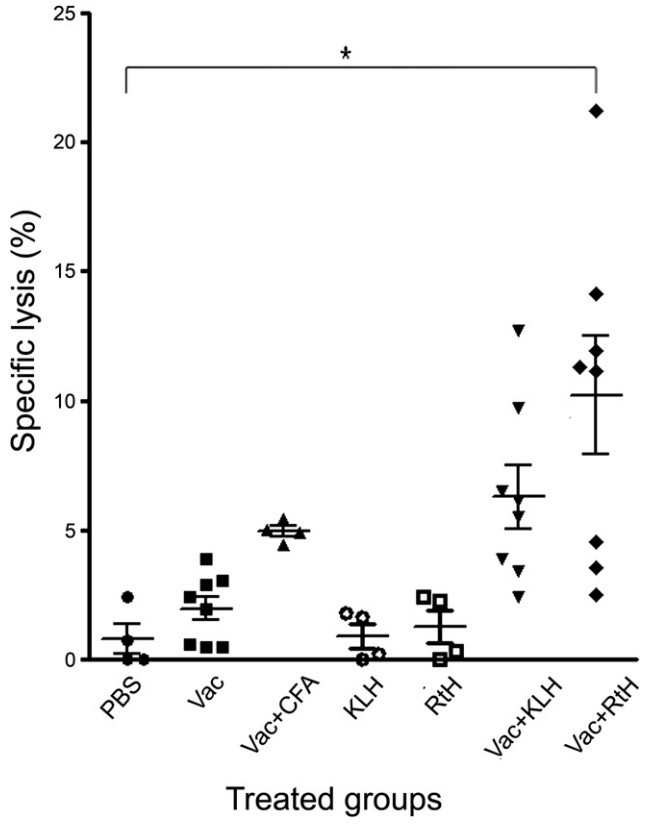

Fig. 6. CTL activity (% Specific lysis) of splenocytes isolated at day 150 after the first immunization of all test-groups. 3T3 cells pulsed with the influenza virus were cultured with effector spleen cells from individual mice at a ratio 1:40 for 4 h at 37 °C. LDH concentration in the supernatants was determined in triplicates by a commercial CytoTox assay as described in Section 2.8. 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. $^{*}P < 0.05$; Student *t*-test. Data are representative of at least 3 independent experiments.

The usual adjuvant quantity used for immunization is much higher then we have used in our experiments. In standard vaccines $Al(OH)_3$ is 170–200 μg per dose, but many authors used even higher quantities to obtain adjuvant properties. Using huge amounts of adjuvants could lead to severe reactions, IgE synthesis and significant alteration in the cytokine profile [28,29]. In the present research we have used very low doses of both hemocyanins – 100 μg per mouse. The cytokine profile does not show any indication for anaphylactic reactions and support the conclusion for low risk of side effects.

In our previous experiments we have used various flu vaccines in different models [24]. In general, the conventional vaccines provoked high antibody titers when administered in CFA. In this experiment the small quantity of Vac combined with CFA resulted in much lower antibody levels than in previous studies [24]. Using the RtH as a protein carrier of a T- and B-cell epitope-containing influenza hemagglutinin peptide leads to generation of high anti-influenza IgG antibody levels [19]. In contrast, repeated immunization with an influenza vaccine without covalent conjugation to RtH did not lead to a significant antibody production. The present results show high levels of IgG and IgM antibodies in the animals immunized with Vac combined with KLH (Fig. 2).

A similar immunostimulatory effect was observed by both Hcs that differ in their origin and structure. We compared the serum levels of IL4 and IFN-gamma characterizing Th1/Th2 responses. Either hemocyanins combined with Vac resulted in the reduction of IL4 and IFN-gamma values after vaccinations (Fig 4).

Vaccination with Hcs combined with Vac appears to be very effective at inducing long-lived cytotoxic memory. Our data show that high CTL anti-influenza activity was present 5 months after the first immunization with Vac + RtH or KLH (Fig. 6). These results are in accordance with data, showing that immunization with DNA vaccine coding influenza-peptide generates CD8+ cytotoxic T lymphocytes persisting for at least 6 months [24]. The CTL response after the administration of Vac + RtH was much stronger than that observed after immunization with the Vac alone. Exogenous antigens can prime some CD8+ cytotoxic T cells when administered in CFA [30–32]. In our hands the conventional vaccine in CFA induced weak anti-influenza cytotoxic immunity. Hcs used as hapten-carriers stimulate major histocompatibility complex class I CD8 and also class II CD4 T-cell responses [16,33]. These data support the results obtained of a CTL activity after immunization with Vac-RtH and Vac + KLH.

More powerful antibody production is achieved after repeated administration of a non-conjugate TT with RtH or KLH (Fig. 3). Our results showed that immunization with a tetanus toxoid without adjuvant, leads to a significant antibody production and it might be concluded that the effect was due to the self-adjuvant properties as a result of the big size of the toxoid molecule. In some cases tetanus toxoid is even used as a protein carrier for peptides derived from influenza hemagglutinine [1]. Vaccination with TT+RtH and TT+KLH appears to be effective at inducing a humoral response. High IgG anti-TT titers were found after the first booster and following the second one they reached the levels, obtained after administration of TT + CFA. Production of IL4 and IFN-gamma was reduced after the first immunization in the group treated with TT + RtH and that became normalized before third immunization (Fig. 5). It could be explained with the limited protein quantity immunized in the animals and the different mechanism of adjuvanicity enhanced by hemocyanins.

5. Conclusion

In general, immunization with Hcs as adjuvants leads to immune response without any dramatic effects as a local or general inflammation, storm cytokine production and other connected side reactions. These extremely foreign for mammals substances stimulate the immune system for generation of CTL and antibody formation and their protein origin make them acceptable as a potential bio-adjuvants for subunit vaccines. The results obtained demonstrate that RtH and KLH are able to induce a humoral and CTL immune response when they are used as adjuvants of non-conjugated bacterial and viral proteins.

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