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Hemocyanins as Immunostimulators

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Abstract: The effect of the immunization of BDF1 mice with molluscan hemocyanins from *Rapana venosa*, *Helix vulgaris*, Keyhole Limpet (KLH) and arthropodan *Carcinus aestuarii* hemocyanin and selected fragments on the proliferation of spleen lymphocytes to T and B cell mitogens as well as to the respective hemocyanin were studied. Parallel Il-2 production in the culture of lymphocytes and the serum levels of IL-2 of animals were assayed. It was established that the *Carcinus* hemocyanin produced stimulating effect on the mitogen reactivity of the spleen lymphocytes of the animals. An increase of stimulation indeces of lymphocytes from immunized mice in the presence of immunization hemocyanin of 3.45 and 3.24 (CaH native form and fragment respectively) were determined. Serum Il-2 production was better expressed in animals immunized by *Helix vulgaris* hemocyanin and CaH and by native molecule KLH. Increased IL-2 production in supernatants of *in vitro* cultivated lymphocytes was observed in animals immunized with native *Carcinus* hemocyanin and KLH as well as by their selected fragments. Compared to tested hemocyanins, *Carcinus* hemocyanin causes increased specific and non-specific proliferation of spleen lymphocytes and Th1 associated cytokin production in BDF1 mice.

Key words: hemocyanins · dendritic cells · immunostimulation · lymphocytes · molluscs

INTRODUCTION

In aerobic organisms the reversible binding of dioxygen and its transport from the environment to the tissues are performed by three types of respiratory proteins: hemoglobins/erythrocruorins, hemerythrins and hemocyanins (Hcs). The members of the latter group of dioxygen carriers are giant multisubunit proteins, freely dissolved in the hemolymph of invertebrates of two phyla: molluscs and arthropods. Although similar in function, the two types of Hcs are oligomeric proteins whose molecular mass and quaternary structure are completely different in the two phyla [1, 2]. Molluscan hemocyanins are more complex proteins with molecular masses ranging from $3.6x10^6$ Da (cephalopodan Hcs) to $43.4x10^6$ Da (gastropodan Hcs). The basic building block is a cylinder made up of ten structural subunits (350-450 kDa). Each subunit has seven (cephalopods) or eight (gastropods and some cephalopods) functional units (FUs) of 50-55 kDa [3].

Molluscan hemocyanins from *Rapana venosa*, prosobranch gastropod, appear as hollow cylinders, described as decamers or didecamers of subunits with molecular masses of 400 or 420 kDa [4]. Two physicochemically distinct isoforms of *Rapana* Hc were isolated and characterized [5]. In recent publications we have also determined the arrangement of the FUs [6, 7].

In contrast, arthropodan Hcs are oligomers of basic hexameric units resulting from the aggregation of structural subunits, with molecular mass of 65-90 kDa

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each and forming a trigonal antiprism. The arthropodan hemocyanin isolated from the crab *Carcinus aestuarii* is composed of 75 kDa subunits [8].

The hemocyanin from the giant keyhole limpet *Megathura crenulata* (commonly abbreviated as KLH) has been a subject of biomedical interest for more than 30 years because of its remarkable immunostimulatory properties in experimental animals and man [9-11]. Both, the humoral and cellular immune responses are activated. It was suggested that KLH is a non-specific immunostimulant/adjuvant with a low level non-specific blastogenic and lympho-proliferative action [12]. In addition, KLH was used as a hapten carrier for small molecules, such as peptides, hormones, polysaccharides, lipids, oligonucleotides etc. [12].

A promising approach in the immunotherapy of cancer is the use of DCs-based vaccines where DCs are highly effective antigen-presenting cells with unique capability of inducing primary immune responses against tumor-associated antigens [13]. Most important is the use of KLH as a compound co administrated with DC vaccine [14, 15]. T cells from the cocultures with KLH-pulsed DCs proliferated to a higher extent and lysed tumor cells more efficiently. KLH can serve as a helper antigen augmenting tumor-specific immune response [16].

KLH is applied in the immunotherapy of patients with superficial bladder carcinoma (superficial transitional cell carcinoma-TCC) extending trough the 1980s [17-18]. In more recent clinical trials KLH subunit product Immucothel has been applied [18-20]. Possible benefits for bladder carcinoma in experimental animals and man was shown as early as 1974 [21]. The possible direct immunotherapeutic action of KLH for the treatment of superficial bladder carcinomas has been demonstrated by the study of Wirguin *et al.* [22] It was found that oligosaccharide side chains of KLH have a terminal galactose (β1-3) N-acetyl-galactosamine epitope (cross-reactive with the TF/Thomson-Friedrich antigen) which induces *in vivo* protective antibodies against this carbohydrate sequence along with a cytotoxic T-cell response [22]. The clinical success of intravesical administration of KLH to patients was ascribed to the presence of the disaccharide epitope Gal (β1-3) GalNAc. This KLH epitope is cross-reactive with an equivalent epitope on bladder tumor cell surface. The cumulative humoral and cellular immune response to KLH is expressed as a cytolytic reduction of tumor growth [22]. The oligosaccharide analyses of KLH1 and KLH2 confirm the multi-epitope potential of these molecules.

Several reports on treatments of bladder carcinoma using Immunocyanin/Immucothel [23-25] showed that a beneficial antibody response to KLH is paralleled by an increased Natural Killer (NK) cell activity. Investigations of Satoskar *et al.*[26] showed that immunization of NK-T$^+$mice with KLH induces an efficient Th1 response and produces significant levels of IFN-γ but results in markedly reduced or absent antigen specific IgG2a production. It was shown that NK cells are involved in the induction of specific IgG2a production *in vivo*.

Due to the observed immunotherapeutic effect of KLH, we started comparative studies with hemocyanins from different sources in order to establish their effect on mitogen reactivity and IL-2 production of spleen lymphocytes in mice and to develop improved therapeutic concepts.

MATERIALS AND METHODS

Experimental animals: Male BDF1 mice (parents: mother C57BL/6 and father DBA_2 mice) weighing 18-20 g were used as source of spleen lymphocytes and sera. The animals were grown up at standard conditions accepted by the Bulgarian Veterinary Health Service in the animal house of the Institute of Experimental Pathology and Parasitology, Sofia. The animals were distributed in 10 groups of 10 animals each as follows:

- Group 1-mice immunized with *Carcinus* hemocyanin (CaH)
- Group 2-mice immunized with *Rapana venosa* hemocyanin (RvH)
- Group 3-mice immunized with RvH fragment
- Group 4-mice immunized with *Helix vulgaris* hemocyanin (HvH)
- Group 5-mice immunized with HvH fragment
- Group 6-mice immunized with CaH fragment
- Group 7-mice immunized with KLH
- Group 8-mice immunized with KLH fragment
- Group 9-mice injected with Complete Freund's adjuvant (CFA)
- Group 10-healthy control mice

Preparation of R. venosa, Helix vulgaris, keyhole limpet and Carcinus aestuarii hemocyanin samples: Marine snails, *Rapana venosa* grosse, were caught near to the Bulgarian coast of the Black Sea. Hemolymph was collected from specimens of 20-35 g. Hemocyanin was

isolated by preparative ultracentrifugation in a Beckman L-80 Ultracentrifuge using a Ti 45 rotor at 24 000 rpm for 4 h at 4°C.

The two subunits, RvH1 and RvH2, were isolated by the procedure specified in Dolashka *et al.* [6]. Multiunit fragments as well as individual FUs were obtained through limited proteolysis by trypsin, separated and purified by FPLC using a Resource Q 6 ml column. All fragments and FUs were characterized by SDS polyacrylamide gel electrophoresis.

Carcinus aestuarii (previously called *Carcinus mediterraneus*) Hc was purified from the hemolymph of crabs following the procedure previously described [8]. To isolate structural subunits from *Carcinus aestuarii* hemocyanin, the protein, previously stored at -20°C in the presence of 18% sucrose, was dialysed at 4°C against the desired buffer. The native protein was dissociated into subunits by dialysis for 24 h against 100 mM sodium bicarbonate buffer pH 10.0 containing 20 mM EDTA and 2 M urea. The subunits were isolated by FPLC ion exchange chromatography as described previously [8].

KLH was obtained from Biosyn (Fellbach, Germany) with a protein concentration of 6.9 mg ml^{-1} in Tris/HCl buffer, pH 7.4. The sample was dialysed for 24 h against 0.1 M Tris/HCl buffer, pH 6.5, containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. The structural subunit KLH2 and functional unit KLH2-c were purified as described previously[27]. Purity control and identification of the isolated functional unit KLH2-c were performed using SDS-PAGE and N-terminal sequence analysis.

Helix vulgaris hemolymph was collected from garden snails and centrifuged at 5000 x g for 15 min to remove hemocytes. Hemocyanin was sedimented in a Beckman L-80 ultracentrifuge as described above. The blue pellet of native hemocyanin was resuspended in 50 mM Tris-HCl buffer, pH 7.5 containing 20 mM $CaCl_2$ and 10 mM $MgCl_2$. Dissociation of the native Hc molecule was achieved by dialysis for 24 h against 50 mM Tris-HCl buffer, pH 8.5, containing 10 mM EDTA (25°C). For isolation of the functional subunits the crude Hc was purified on DEAE-Sepharose 6 CL and DEAE Cellulose 52 columns (12×2.5 cm), equilibrated with 50 mM Tris-HCl buffer, containing 10 mM EDTA, pH 8.5. Multiunit fragments as well as individual FUs were obtained through limited proteolysis by trypsin, separated and purified by FPLC, using a Resource Q 6 ml column.

The protein concentration was determined using the absorption coefficient at 278 nm. The percentage of oxy-Hc was determined by considering the absorbance ratio of A_{337}/A_{280} 5 0.21 for a preparation containing 100% oxy-Hc.

Immunization protocol: Hemocyanins *Rapana venosa*, *Helix vulgaris*, *Megathura crenulata* and *Carcinus aestuarii* as well as selected fragments were mixed with equal volumes of Complete Freund's Adjuvant (CFA). Groups of mice were injected subcutaneously (s.c.) in the inter-scapular space with 10 μg of the respective hemocyanin or fragment of hemocyanin, dissolved in 0.2 ml of PBS. Booster immunizations were performed two weeks later using the respective antigen+Incomplete Freund's Aduvant (IFA). Two weeks after the booster immunization the mice were killed under ether narcosis.

***In vitro* proliferation of spleen lymphocytes in presence of mitogens:** The spleens of the mice were exstirpated in an aseptic way and the proliferation responses of lymphocytes in the presence of mitogens were determined by the method described by Stamm *et al.* [28]. Briefly, the spleens were carefully homogenized to receive a single cell suspension in RPMI-1640 medium (Fluka) supplemented by 2 mM L-glutamine, 100 E ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% fetal calf serum (Fluka). The obtained cells were centrifuged at 200 x g for 5 min. The erythrocytes were lysed by the solution of Boyle (0.17 M Tris+0.16 M ammonium chloride). The obtained lymphocyte suspension was washed twice by centrifugation. The viability of the cells was examined by the Trypan blue exclusive test. Suspensions of $5x10^5$ live cells in 0.1 ml of complete RPMI-1640 medium were distributed in triplicate in 96 wells of flat bottom tissue culture plates. The cells were stimulated by 20 μg ml^{-1} phytohemagglutinin (Sigma), 20 μg ml^{-1} *Escherichia coli* lipopolysaccharide (LPS) (Sigma) or the respective hemocyanin (fragment) in given concentration. The cells were cultivated in a CO_2 incubator at 37°C for 72 h. 1 μ Ci of ^3H thymidine was added to each well 18 h before the end of the incubation. The cells were collected on nitrocellulose filters using a manual cell harvester and were distributed in scintillation flasks. After a supplementation with 5 ml scintillation mixture, the isotope uptake of each sample was determined in a Beckman scintillation counter. Stimulation Index (SI) of lymphocytes was calculated according to the formula: SI = Number of cpm of lymphocytes incubated with mitogen: Number of cpm of lymphocytes incubated without mitogen. In parallel experiments, cell-free supernatants were harvested after 72 h of culture for quantitation of IL-2.

Determination of Interleukin 2 in supernatants and serum: Culture supernatants from cultivated *in vitro*

lymphocytes in the presence of hemocyanins and sera from animals immunized with native hemocyanins and selected fragments from *Rapana venosa, Helix vulgaris, Carcinus aestuarii* and *Megathura crenulata* were examined for their IL-2 levels. A mouse IL-2 ELISA set (BD Biosciences Pharmingen, San Diego, USA) was used for the ELISA test. The determinations of the IL-2 contents of supernatants and sera were performed according to the standard procedure, using an ELISA reader (Organon Tekknika). The concentrations of IL-2 are presented in pg ml^{-1} serum.

Human cells: PBMCs from healthy blood bank donors positive for HLA-A*0201 and HCMV were isolated by standard gradient centrifugation (Lymphocyte Separation Medium, PAA Laboratories GmbH, Pasching, Austria). Monocyte derived human DCs were generated as previously described [29] and activated with 10 ng ml^{-1} human TNF-α (R and D Systems, Wiesbaden, Germany) + 20 μg ml^{-1} poly (IC) (Sigma Aldrich, Steinheim, Germany) for 3 days. For restimulations after priming with DCs, cryopreserved autologous PBMCs were used.

Peptides, recombinant MHC molecules and fluorescent tetramers: Peptides were synthesized by standard Fmoc-chemistry using the Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany). Peptides in this study were NLVPMVATV from HCMV pp65 495-503 and ELAGIGILTV from Melan-A 26-35.

Biotinylated recombinant MHC class I molecules and fluorescent MHC tetramers were produced as described earlier [30]. Briefly, fluorescent tetramers were generated by coincubating biotinylated HLA monomers with streptavidin-PE or streptavidin-APC (Molecular Probes, Leiden, The Netherlands) at a 4:1 molar ratio.

***In vitro* stimulations:** *In vitro* stimulations were initiated in 96 well plates with $1x10^4$ responder cells plus $2x10^5$ irradiated peptide pulsed (5 μg ml^{-1}) DCs per well in 200 μl T cell medium: RPMI 1640 containing HEPES and L-glutamine (Gibco, Paisley, UK) supplemented with 10% heat-inactivated human serum (PAA, Cölbe, Germany), 50 μ ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 20 μg ml^{-1} gentamycin (BioWhittaker) in the presence of 10 ng ml^{-1} human IL-12 (Promocell, Heidelberg, Germany) and 20 ng ml^{-1} of KLH. After 3-4 days coincubation at 37°C, fresh medium and 80 μ ml^{-1} IL-2 (Proleukin, Chiron Corporation, Emeryville, CA, USA) was added and cells were incubated for 3-4 days. This stimulation cycle was repeated twice.

Tetramer staining: 2×10^5 stimulated cells were stained with fluorescent MHC tetramers and incubated at 4°C for 20 min in the dark, followed by 30 min incubation with Okt8-FITC antibody at 4°C in the dark. After washing, cells were resuspended in 1% paraformaldehyde in PFEA (PBS supplemented with 2% heat inactivated FCS (PAN Biotech, Aidenbach, Germany), 2 mM EDTA (Roth) and 0.01% sodium azide (Merck, Darmstadt, Germany)). Cells were analysed by flow cytometry on a four-color FACSCalibur cytometer (Becton Dickinson).

RESULTS

The effect of Hcs on the proliferation activity of spleen lymphocytes to T and B cell mitogens: The effect of the immunization of healthy BDF1 mice with hemocyanins and fragments of *Rapana venosa, Helix vulgaris, Megathura crenulata* and *Carcinus aestuarii* hemocyanins and the proliferation activity of mouse spleen lymphocytes to T and B cell mitogens were studied. As shown in Fig. 1, the lymphocyte response to the T-cell mitogen phytohemagglutinin (PHA) is most pronounced in mice immunized with native hemocyanin from *Carcinus aestuarii* as well as by its fragment and Stimulation Indices (SI) of 3.3 and 1.66, respectively, were determined. The fragment of *Carcinus* hemocyanin induced highest mitogen response to the B-cell mitogen *E. coli* LPS (SI = 2.3). SI of the lymphocytes from healthy control mice were 1.57 and 1.23 for PHA and LPS respectively and the lymphocytes from animals injected with CFA only showed lower stimulation indexes (1.05 and 0.97 for PHA and LPS respectively) (Fig. 1).

The stimulation indices of lymphocytes from mice immunized with *Rapana* hemocyanin and its fragment to the PHA and LPS are lower compared to the controls (SI = 0.3 and 0.48 and SI = 0.96 and 0.67 for PHA and LPS respectively) (Fig. 1). The mitogen reactivity of lymphocytes from KLH (whole molecule and fragment)-injected mice to the two mitogens is near to the control values. The immunization with KLH did not increase lymphocyte mitogen reactivity and the stimulation indices are approximately the same (1.28 and 1.15, respectively) for PHA and LPS (Fig. 1). Stimulation indices of lymphocytes from mice immunized with KLH fragment are near the control values (SI = 0.98 and 0.93 for PHA and LPS, respectively).

The specific proliferation response of the lymphocytes to the hemocyanins used for immunization was most pronounced (SI = 3.45 and 3.24, respectively) in animals injected with both forms of *Carcinus*

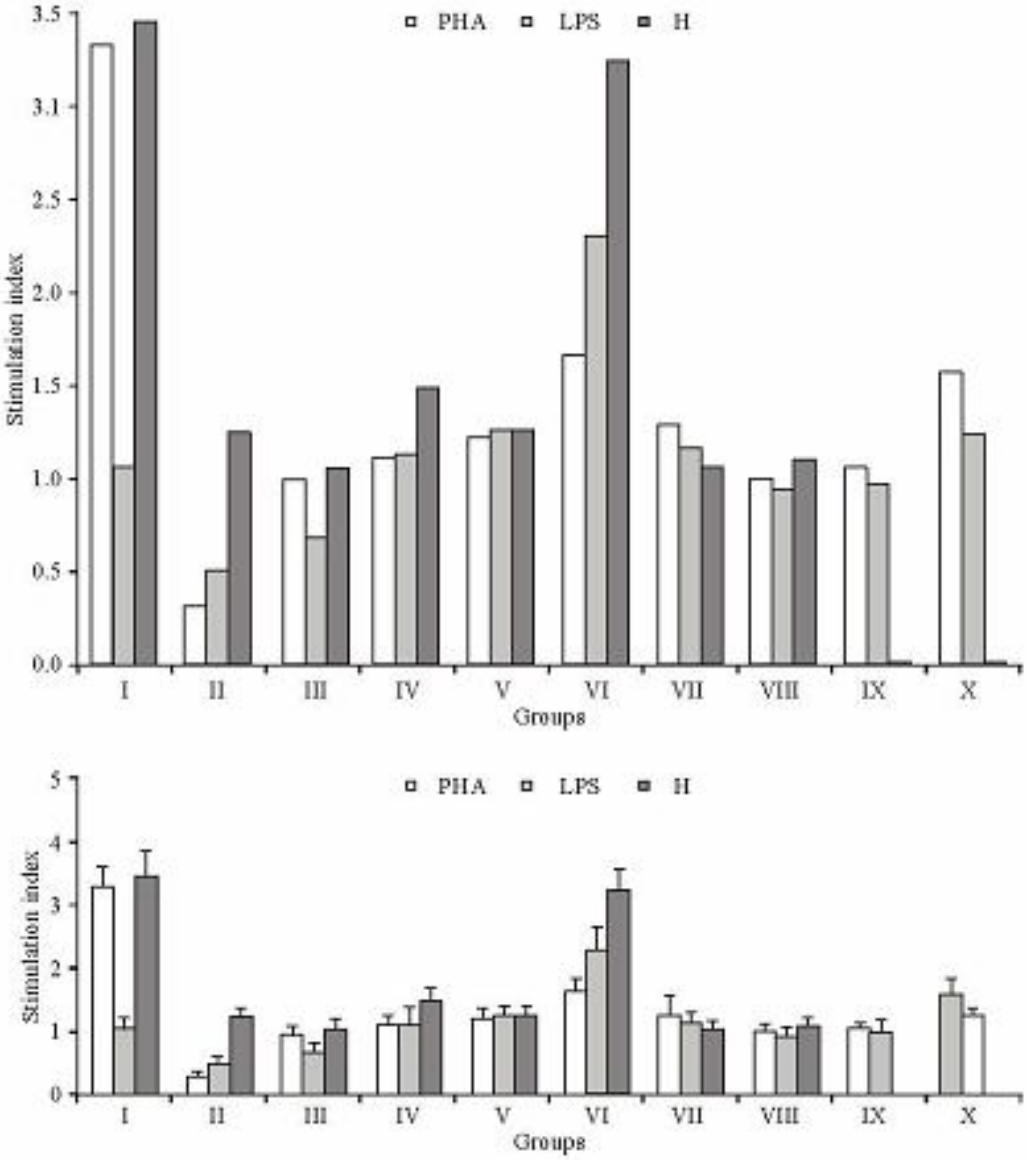

Fig. 1: Stimulation Indices (SI) of spleen lymphocytes of BDF1 mice after immunization with hemocyanins. Cell proliferation was assayed by determining [^{3}H]thymidine incorporation in the presence of PHA, LPS and respective hemocyanins and their fragments. Experimental groups, mice immunized with: 1. *Carcinus aestuarii* (native) hemocyanin; 2. *Rapana venosa* (native) hemocyanin; 3. *Rapana venosa* hemocyanin (fragment); 4. *Helix vulgaris* (native) hemocyanin; 5. *Helix vulgaris* hemocyanin (fragment); 6. *Carcinus aestuarii* hemocyanin (fragment); 7. KLH (native); 8. KLH (fragment); 9. Complete Freund s adjuvant; 10. No immunization

hemocyanin. The specific immune response to KLH hemocyanin (whole molecule and fragment) was not connected with any increase of the lymphocyte proliferation (SI = 1.04 and 1.08, respectively) (Fig. 1). An increase of the lymphocyte SI to the immunization hemocyanin was established in animals injected with whole molecules of *Helix vulgaris* and *Rapana venosa* Hc (1.49 and 1.24, respectively) and fragment of *Helix vulgaris* hemocyanin (SI = 1.25) (Fig. 1).

Determination of interleukin 2 in supernatants of *in vitro* cultivated spleen lymphocytes: Freshly isolated spleen cells from immunized mice were cultured *in vitro* in presence of hemocyanins and their fragments and the culture supernatants was then analyzed by ELISA for levels of IL-2. Data are presented in Fig. 2. The highest levels of IL-2 were established in the supernatants of lymphocytes cultivated in the presence of fragments of CaH (5.5 pg ml^{-1}), *Helix vulgaris*

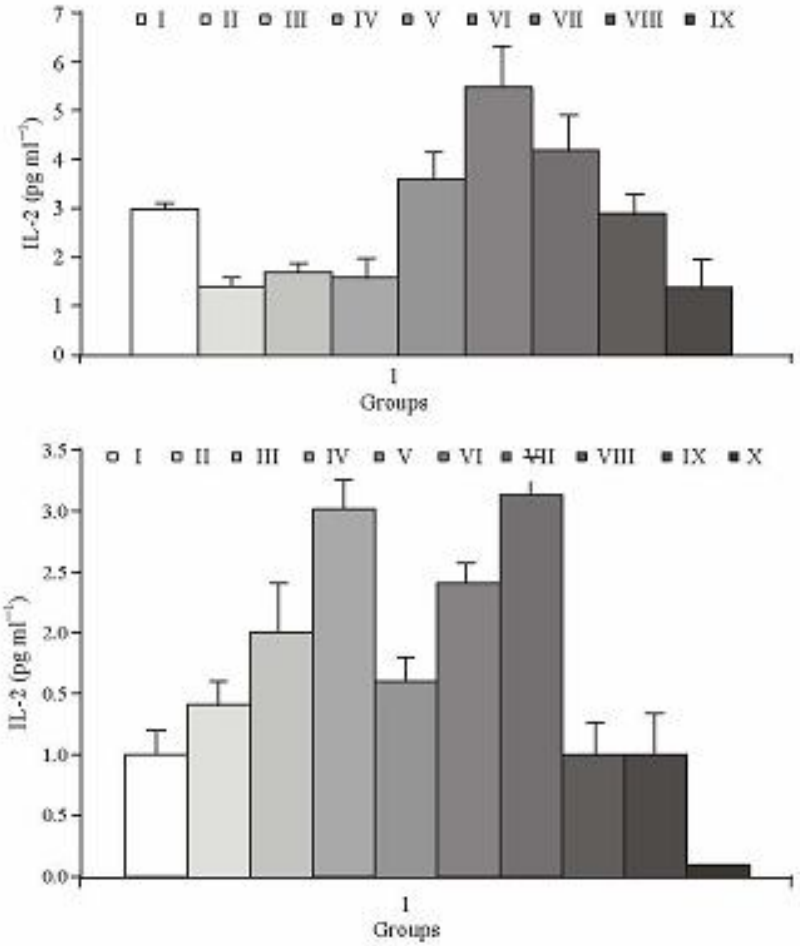

Fig. 2: IL-2 content (pg ml^{-1}) in supernatants of *in vitro* cultivated spleen cells. Splenocytes (5x10^5/well) obtained from different groups of immunized mice were cocultured in presence of respective hemocyanins in 96-well flat-bottomed microtiter plates in a final volume of 200 µL. Cell-free supernatants were harvested after 72 h of culture and IL-2 level was measured by standard ELISA kit. Data represent means ± SE of triplicate cultures. Experimental groups, mice immunized with: 1. *Carcinus aestuarii* (native) hemocyanin; 2. *Rapana venosa* (native) hemocyanin; 3. *Rapana venosa* hemocyanin (fragment); 4. *Helix vulgaris* (native) hemocyanin; 5. *Helix vulgaris* hemocyanin (fragment); 6. *Carcinus aestuarii* hemocyanin (fragment); 7. KLH (native); 8. KLH (fragment); 9. Complete Freund's adjuvant; 10. no immunization

hemocyanin (3.6 pg ml^{-1}) and native KLH (4.2 pg ml^{-1}). Comparatively high IL-2 levels in presence of native molecules of CaH (3.0 pg ml^{-1}) and fragment of KLH (2.9 pg ml^{-1}) were determined. RvH (native form and fragment) and HvH (native form) induced IL-2 production similar to that determined in supernatants of control lymphocytes (Fig. 2).

Determination of interleukin 2 levels in sera of immunized animals: The same ten groups were used for analysis of the IL-2 levels in sera of the immunized animals and the results are presented in Fig. 3. The highest serum levels of IL-2 were found in the group of animals injected with native molecules of *Helix vulgaris* He and KLH (3.0 pg ml^{-1} and 3.12 pg ml^{-1}, respectively, control animals 1.0 pg ml^{-1}). Comparatively high IL-2 serum levels in the groups of mice injected with fragments of *Carcinus* hemocyanin (2.4 pg ml^{-1}), *Rapana* hemocyanin (2.0 pg ml^{-1}) and of *Helix vulgaris* (1.6 pg ml^{-1}) were established (Fig. 3). Native RvH and *Carcinus* Hcs induced IL-2 production similar to that induced by KLH fragment and a little higher than determined in healthy non-treated mice (Fig. 3).

***In vitro* stimulation of CD8+T cells with autologous dendritic cells loaded either with peptide ELAGIGILTV**

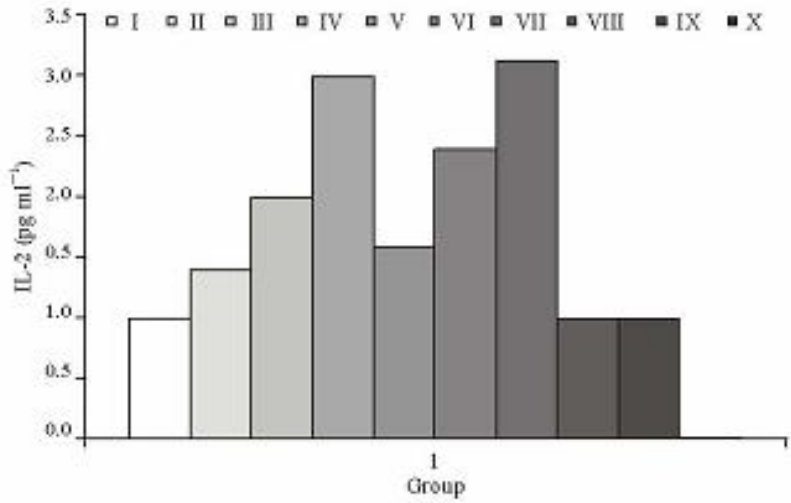

Fig. 3: Il-2 content (pg ml^{-1}) in sera from different groups of mice immunized with hemocyanins or fragments of hemocyanins. Experimental groups: 1. *Carcinus aestuarii* (native) hemocyanin; 2. *Rapana venosa* (native) hemocyanin; 3. *Rapana venosa* hemocyanin (fragment); 4. *Helix vulgaris* (native) hemocyanin; 5. *Helix vulgaris* hemocyanin (fragment); 6. *Carcinus aestuarii* hemocyanin (fragment); 7. KLH (native); 8. KLH (fragment); 9. Complete Freund's adjuvant; 10. no immunization

from Melan-A 26-35 or with NLVPMVATV from HCMV pp65 495-503: Monocyte derived dendritic cells of two donors (B299, B302 which of them is shown in Fig. 4), activated with 10 ng ml^{-1} human TNF-α and 20 μg ml^{-1} poly (IC) were used as antigen presenting cells. T cell priming and restimulation was performed by incubation of autologous PBMCs (HLA-A*0201+and HCMV+) 3 times with peptide-loaded activated dendritic cells. *In vitro* stimulations were carried out in 96 well plates with 1×10^6 responder cells per well. For tetrameric analyses, cells were stained with fluorescent Melan A tetramer-PE, HCMV tetramer-APC and CD8-FITC. Cells were gated on CD8+T cells within the lymphocyte population. Percentages refer to tetramer+T cells within the CD8+population. As can be seen in Fig. 4A, T cells specific for ELAGIGILTV (Melan A_{26-35}) were detected in all samples which were stimulated with ELAGIGILTV-peptide. Nevertheless, no significant differences were obtained between the different Hc samples. Stimulation with NLVPMVATV (HCMV $pp65_{495-503}$) worked only with cells of donor B302, but without significant differences between Hc variants (Fig. 4B).

DISCUSSION

In this study we present the results obtained from testing the immune response of BDF1 mice immunized by several hemocyanins and selected fragments. The parameters such a mitogen induced lymphocyte proliferation and the IL-2 cytokine level in serum and supernatants were assayed. It was established that CaH (native form) stimulate the T-cell mitogen response (SI = 3.3) and CaH (fragment) increase the B-cell mitogen response (SI = 2.3) of spleen lymphocytes (Fig. 1).

Assuming that antigen presenting cells prime naive T cells toward Ag-specific T cells and that these cells predominantly proliferate when exposed to antigen, we assessed the proliferation of spleen cells from immunized mice in presence of different hemocyanins as stimulants. For positive control Con A was used in these experiments. Significant proliferation (SI = 3.45 and 3.24) was obtained in presence of CaH-native form and fragment respectively (Fig. 1). Spleen cells from the mice immunized with other hemocyanins showed negligible stimulation. The proliferation of T cells from KLH-immunized mice in the presence of respective KLH (native form and fragment), was significantly lower than the values obtained with CaH as the stimulant. However the splenocytes harvested from CaH-immunized mice and placed in secondary culture *in vitro* in presence of CaH resulted in CaH-specific proliferative response.

We also analyzed the level of IL-2 released *in vitro* by stimulated T cells of immunized mice. Th1 subsets of CD4+T-helper cells secrets IL-2. A Th1-associated response was observed, with a significant enhancement of IL-2 production in the group of mice immunized with CaH (5.5 pg ml^{-1} and 3.0 pg ml^{-1} for native form and

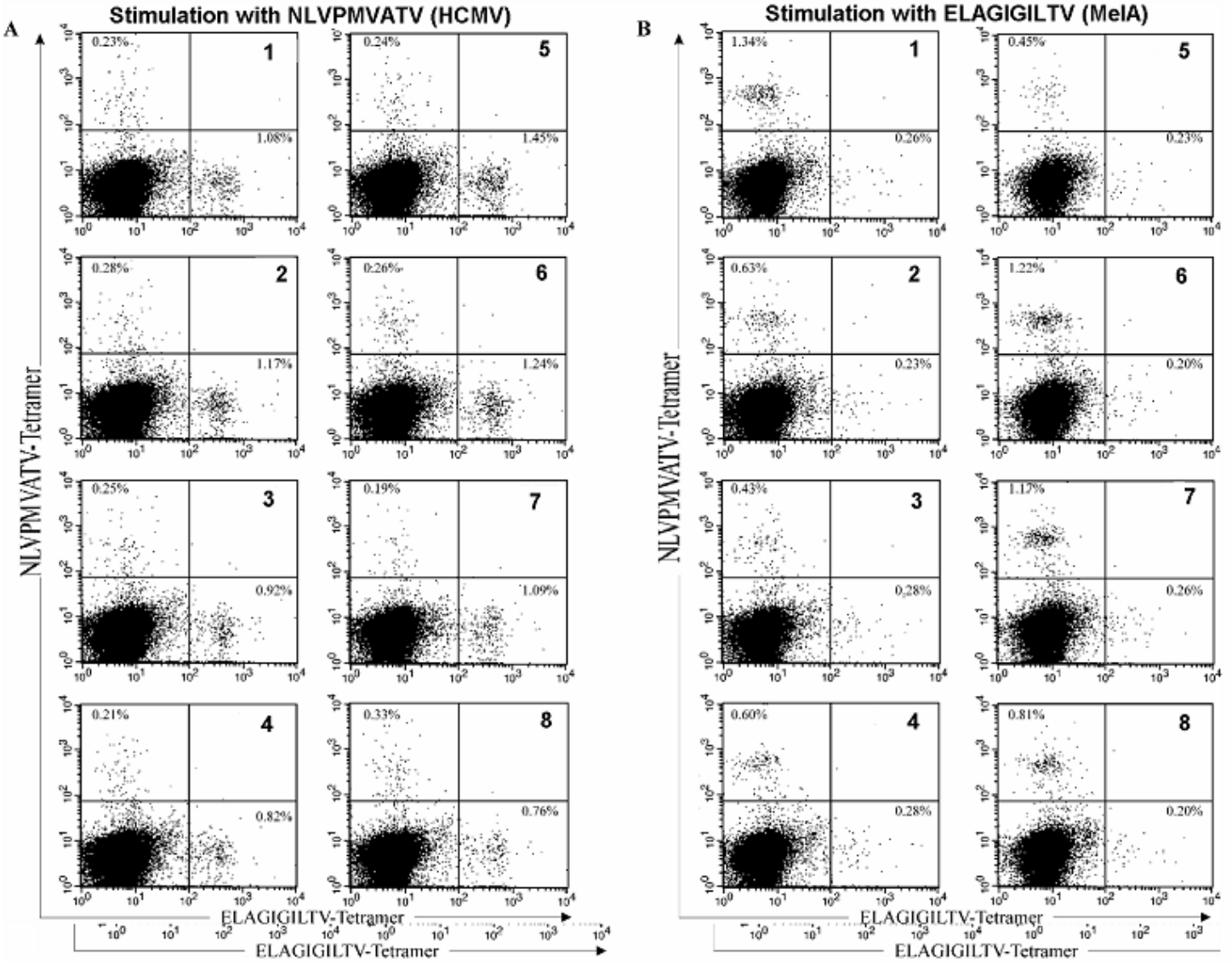

Fig. 4: HLA tetramer staining of PBMCs stimulated with (A) MelA $_{26-35}$ peptide and (B) HCMV pp65$_{495-503}$ peptide pulsed on DC, plus different Hcs: 1. *Rapana venosa* (native) hemocyanin; 2. *Rapana venosa* hemocyanin (fragment); 3. KLH (native); 4. *Carcinus aestuarii* (native) hemocyanin; 5. *Carcinus aestuarii* hemocyanin (fragment); 6. *Helix vulgaris* (native) hemocyanin; 7. *Helix vulgaris* hemocyanin (fragment) and 8. No Hc. Cells were stained with MelA-tetramer-PE, HCMVtetramer- APC and CD8-FITC. Cells were gated on CD8+T cells within the lymphocyte population. Percentages refer to tetramer+T cells within the CD8+population

fragment respectively), KLH (4.2 and 2.9 pg ml^{-1} for native form and fragment respectively) and HvH-fragment (3.6 pg ml^{-1}). The established IL-2 levels in the other group of immunized mice were lower (Fig. 2).

The cytokine profile of sera from mice immunized with Hcs indicated the induction of a Th1 immune response with high level of IL-2 in the group of mice immunized with KLH (3.12 pg ml^{-1}), CaH-fragment (2.4 pg ml^{-1}) and HvH-fragment (3.0 pg ml^{-1}) (Fig. 3).

According to the present investigation *Carcinus* hemocyanin (native form and fragment) shows the most potent immunogenicity compared to several hemocyanins and fragments isolated from different mollusks.

It is known that KLH is a natural substance for *in vivo* use in human pathology as a direct antigenic stimulus and immunotherapeutic agent. The immunological response to KLH has been attributed to the carbohydrate moiety than protein alone. KLH exhibits several carbohydrate determinants, some of which are immunogeneic [31]. KLH can be used also as a carrier molecule for conjugation with tumor specific peptides and oligosaccharides and with anti-idiotypic antibodies [12, 29, 32, 33]. Pandha *et al.* used KLH as an adjuvant in dendritic cell immunotherapy for urological cancers using allogenic tumor-lysate pulsed cells [34].

Metastatic melanoma patients have been vaccinated with DCs pulsed with autologous tumor lysates and KLH. CD8+T-cell responses to melanoma peptides as well as IFN-γ releasing KLH-specific T cells were found [35]. Using ddendritic cells of two donors (B299, B302) as antigen presenting cells, T cells specific for ELAGIGILTV ($MelA_{26-35}$) were detected in all samples which were stimulated with ELAGIGILTV-peptide. Nevertheless, no significant differences were observed between the different hemocyanins. Stimulation with NLVPMVATV (HCMV $pp65_{495-503}$) worked only with cells of donor B302, but also without significant differences between hemocyanins.

Kedding *et al.* reported a totally synthetic pentameric vaccine based on carbohydrate antigens, which mimic prostate tumor associated antigens. Two different immunogenic carriers including KLH were used for the vaccine construct [29].

In summary, our results demonstrate the priority of *Carcinus* hemocyanin as specific and non specific stimulator of mice spleen lymphocytes, as a carrier of higher antigen potential compared to tested hemocyanins. The successful application of KLH in the immunotherapy of cancer [23, 33, 36-38] is a reason to consider the present results with potentially applied significance.

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