Immunotherapeutic Effect of Concholepas Hemocyanin in the Murine Bladder Cancer Model: Evidence for Conserved Antitumor Properties Among Hemocyanins

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Purpose: We determined the antitumor properties of a newly available hemocyanin obtained from the Chilean gastropod Concholepas concholepas (Biosonda Corp., Santiago, Chile) in a syngeneic heterotopic mouse bladder carcinoma model. Since keyhole limpet hemocyanin (Pierce, Rockford, Illinois) is used increasingly in biomedicine as a carrier for vaccines and an immunotherapeutic agent for bladder transitional cell carcinoma, there is a growing interest in finding new substances that share its potent immunomodulatory properties. Considering that keyhole limpet hemocyanin and Concholepas concholepas hemocyanin differ significantly, it was not possible to predict a priori the antitumor properties of Concholepas concholepas hemocyanin.

Materials and Methods: C3H/He mice were primed with Concholepas concholepas hemocyanin before subcutaneous implantation of mouse bladder tumor-2 cells. Treatment consisted of a subcutaneous dose of Concholepas concholepas hemocyanin (1 mg or 100 μg) at different intervals after implantation. Keyhole limpet hemocyanin and phosphate buffered saline served as positive and negative controls, respectively. In addition, experiments were designed to determine which elements of the immune response were involved in its adjuvant immunostimulatory effect.

Results: Mice treated with Concholepas concholepas hemocyanin showed a significant antitumor effect, as demonstrated by decreased tumor growth and incidence, prolonged survival and lack of toxic effects. These effects were similar to those achieved with keyhole limpet hemocyanin. We found that each hemocyanin increased natural killer cell activity but the effect of Concholepas concholepas hemocyanin was stronger. Analysis of serum from treated mice showed an increased interferon-γ and low interleukin-4, which correlated with antibody isotypes, confirming that hemocyanins induce a T helper type 1 cytokine profile.

Conclusions: To our knowledge our results are the first demonstration of the antitumor effect of a hemocyanin other than keyhole limpet hemocyanin. They suggest that this is an ancient conserved immunogenic mechanism shared by those hemocyanins that is able to enhance T helper type 1 immunity and lead to antitumor activity. Therefore, Concholepas concholepas hemocyanin may be an alternative candidate for providing safe and effective immunotherapy for human superficial bladder cancer.

Key Words: bladder, bladder neoplasms, hemocyanin, immunotherapy, Gastropoda

It is known that various nonspecific compounds enhance the immune response and act as effective antitumor agents. One of the best examples of this phenomenon is found in the treatment of superficial TCC of the bladder with bacillus Calmette-Guerin. However, despite successful results their negative side effects led to the investigation of other substances.[1] In the early 1970s Olsson et al reported that in patients with bladder TCC subcutaneous stimulation with KLH significantly decreased the frequency of tumor recurrence.[2] This provided a promising opportunity for the use of KLH in tumor therapy and prompted experimental studies in murine models together with several other studies that also revealed its effectiveness in humans. Jurincic et al reported that KLH was effective for decreasing TCC recurrence and it lacked side effects.[3] Flamm et al compared KLH and ethoglucid in patients with resected TCC and found no significant difference.[4] Wishahi et al reported that KLH decreased tumor recurrence in patients with schistosomal associated TCC.[5] Thus, the effectiveness of the antitumor activity of KLH has been long established.

Hemocyanins are widely used as hapten carrier proteins to produce antibodies and to stimulate major histocompatibility complex class I CD8 and 2nd class II CD4 T-cell responses. Hemocyanins have a large molecular size (4 to 8 MDa) and due to xenogenic nature they are strongly immunogenic. Today most applications use KLH as a carrier of vaccines against infectious diseases, as a carrier for mucin-like and ganglioside epitopes used to treat cancer and as an adjuvant in patients with melanoma vaccinated with antigen pulsed dendritic cells.[6] Curiously although biomedical interest in KLH goes back more than 30 years, to our knowl-

Submitted for publication October 25, 2005.

Supported by FONTEC-CORFO 2001-2930 and 2003-3816 to Biosonda Corp., and FONDECYT 199-0258 and 105-0150 (MIB).

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DOI:10.1016/j.juro.2006.07.136

edge it is not known whether hemocyanins from other mollusks might be equally or more efficient as an immunomodulatory agent.

We investigated the antitumor properties of hemocyanin obtained from the Chilean gastropod Concholepas concholepas. Its structure has been elucidated and it was found to differ significantly from that of KLH. Although the 2 hemocyanins have 2 isoforms or subunits, the structure of CCH shows a heterodecameric array of subunits that contain common and specific epitopes. In contrast, the KLH subunit forms homodecamers and does not show shared epitopes. Despite these structural differences CCH is a potent immunogen and as such it has been successfully used as a hapten carrier protein.[7]

We determined if CCH has immunotherapeutic value as an antitumor agent. In addition, experiments were designed to investigate which elements of the immune response were involved in its adjuvant immunostimulatory effect.

MATERIALS AND METHODS

Immunotherapeutic Reagents

CCH and KLH were prepared using lipopolysaccharide-free water (Baxter Healthcare, Irvine, California).

Animals

C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) were bred at Biosonda Corporation.

Tumor Cells

MBT-2 murine bladder cells were cultured according to Riggs et al[8] with modifications. Cells were cultured at 37C in a 10% CO_2 atmosphere in Dulbecco's medium containing 10% fetal bovine serum (HyClone, Logan, Utah), 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, Grand Island, New York). Confluent cell cultures were treated with trypsin-ethylenediaminetetraacetic acid (Gibco). To set the subcutaneous MBT-2 cell number for challenge experiments different numbers of cells (10,000 to 500,000) were implanted into groups of 5 mice and the tumor incidence was evaluated. Tumor dimensions were calculated (fig. 1). Survival in the mice was recorded for 70 days.

Immunotherapy Experiments

The mice were challenged with MBT-2 cells according to Riggs et al[8] with modifications. Two weeks before tumor implantation the mice were randomized and primed subcutaneously with 200 to 400 μg CCH in 100 μl PBS. At time zero the mice were challenged by subcutaneous injection of 150,000 to 200,000 MBT-2 cells per 100 μl in the right thigh or flank. Immunotherapy was performed according to Lau[9] and Swerdlow[10] et al with modifications. Briefly, after the challenge with tumor cells 1 mg protein in 100 μl PBS per dose was administered intratumorally at 6 hours and 1 to 6 days (treatment 1 with a cumulative dose of 7 mg), and 100 μg protein in 100 μl PBS per dose were administered intratu-

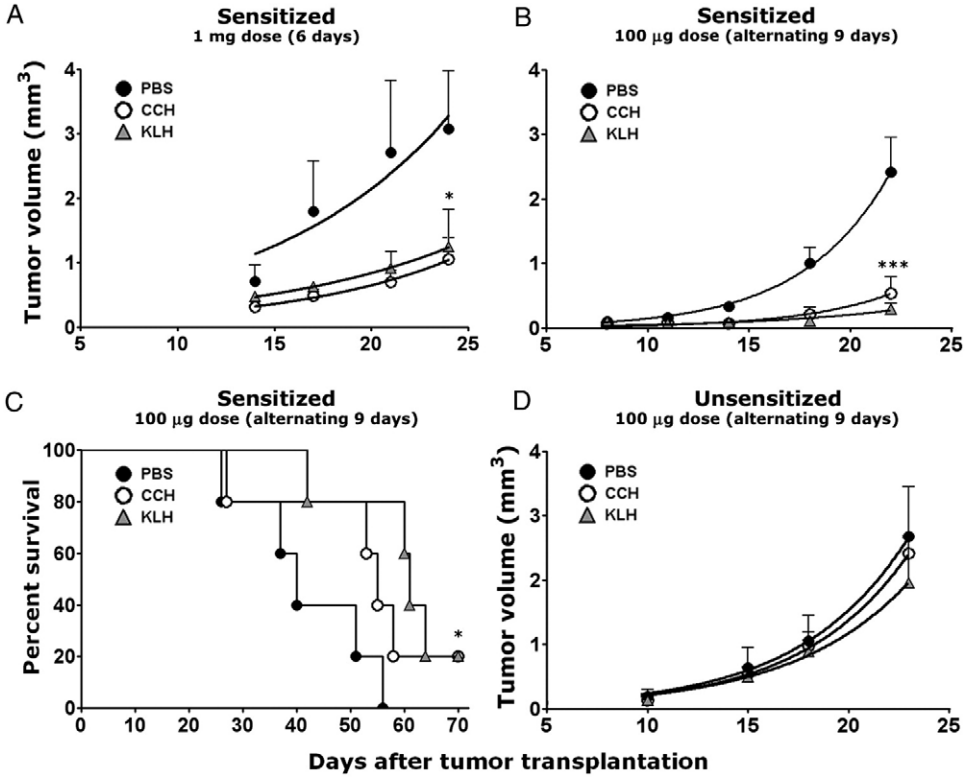

FIG. 1. CCH antitumor activity. Groups of 5 to 7 mice were subcutaneously immunized with CCH or KLH in PBS and challenged subcutaneously with 150,000 to 200,000 MBT-2 cells. Tumor size was measured every 3 to 5 days and tumor volume was calculated using ellipsoid formula, volume = $0.52 \times L \times W^2$. *A*, immunotherapy schedule 1 with 1 mg hemocyanin per dose daily during 6 days. Tumor growth decreased as consequence of CCH treatment, similar to that in KLH group vs PBS group. Asterisk indicates at day 24 PBS vs CCH or KLH group 2-way ANOVA $p < 0.05$. *B*, immunotherapy schedule 2 with 100 μg hemocyanin per dose on alternate days during 9 days caused decreased tumor growth compared with that in PBS group. Asterisks indicate 2-way ANOVA $p < 0.001$. *C*, animal survival was higher in CCH and KLH groups than in PBS group. CCH and KLH Kaplan-Meier $p = 0.0503$ and < 0.05, respectively. *D*, priming omitted. At day 23 PBS vs CCH or KLH 2-way ANOVA p not significant.

morally at 1, 3, 5, 7 and 9 days (treatment 2 with a cumulative dose of 500 μg). A similar schedule was used in controls. In some experiments primary sensitization with hemocyanins was omitted. The presence of secondary tumors in the animals was determined by necropsy at the end of the experiment.

Cytotoxicity Assay

The procedure of Puente et al[11] was performed. The YAC-1 cell line sensitive to NK cells was used. YAC-1 cells were cultured as MBT-2 cells. They were labeled by incubating 3×10^6/ml cells with 100 μCi $Na_2{}^{51}Cr$ (Perkin-Elmer, Foster City, California) for 1.5 hours at 37C. As effector cells, we used splenic mononuclear cells purified by Ficoll™-Hypaque™ and suspended (10^7 cells per ml) in Dulbecco's medium. Medium (100 μl) containing 5×10^3 target cells was then mixed with 100 μl effector cells diluted according to the desired effector-to-target ratio of 25:1 to 200:1. Plates were incubated for 4 hours at 37C in 10% CO_2 and centrifuged. Each supernatant (100 μl) was measured in a γ radiation counter (Packard Instrument Co., Meriden, Connecticut). As a positive control, mononuclear cells obtained from Poly I:C (Sigma, St. Louis, Missouri) treated mice were used. Assays were performed in triplicate and a specific lysis value was calculated (fig. 2).

Cytokine Detection

IL-4 and IFN-γ levels were measured in mice serum using ELISA kits (Pierce).

Antibody Isotype Determination

The procedure of Jutel et al was used[12] with modifications. Briefly, 96-well plates (Pierce) were coated with a solution containing 10 μg/ml CCH or KLH in PBS. Two-fold serial dilutions of mice serum were made and incubated for 2 to 3 hours at 37C, and the plates were washed. Alkaline phosphatase conjugated anti-mouse IgG or anti-subclass specific rabbit anti-mouse isotyping serum (Santa Cruz Biotechnology, Santa Cruz, California) was added and incubated for 1 hour at 37C. The plates were washed and developed at 37C with p-nitrophenyl phosphate (Merck, Darmstadt, Germany).

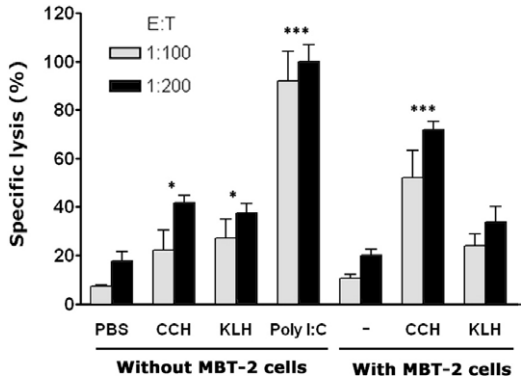

FIG. 2. Cytotoxicity of hemocyanin stimulated mononuclear cells from mice on immunotherapy. Groups of 4 mice previously primed with or without tumor challenge were treated with 1 mg hemocyanin per dose daily for 6 days. Results of 100 and 200 cells effector-to-target ratio (*E:T*) on day 22 of bioassay and next day to last therapeutic dose. Specific lysis in percent was calculated using formula, lysis = 100 (experimental release – spontaneous release)/(total release – spontaneous release). In controls target cells were incubated with complete medium (spontaneous release) or detergent (total release). Dose-response increase in NK cell activity was noted in mice treated with CCH and KLH. Poly I:C served as positive control. Without tumor cells single asterisk indicates PBS vs CCH $p < 0.05$. Without tumor cells CCH vs KLH p not significant. Without tumor cells triple asterisks indicate PBS vs Poly I:C $p < 0.001$. With tumor cells and hemocyanin triple asterisks indicate only MBT-2 vs MBT-2 and CCH 2-way ANOVA $p < 0.001$.

MBT-2 Microscopy Analysis

For indirect immunofluorescence and transmission electron microscopy cells were adequately processed[13] and observed elsewhere.

Western Blot

The procedure of De Ioannes et al[7] was used. For polyacrylamide gel electrophoresis sodium dodecyl sulfate gels samples were denatured by heating in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. For native gel electrophoresis hemocyanin samples were run in alkaline dissociating conditions with ethylenediaminetetraacetic acid. The proteins were then transferred to nitrocellulose membranes, blocked and incubated with anti-CCH or anti-KLH mouse serum. After washing the membranes were incubated with goat anti-mouse IgG serum alkaline phosphatase conjugate and developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoxyl-phosphate (Pierce).

Statistical Analyses

Results are expressed as the mean ± SE. Comparison between groups was done using 1-way ANOVA, the Turkey test or 2-way ANOVA with the Bonferroni posttest. The survival rate was estimated by the Kaplan-Meier method using the log rank test. GraphPad® Prism® software was used.

RESULTS

Experimental Setting

Many different protocols have been used to study the antitumor activity of potential pharmacological substances in murine bladder cancer models.[14,15] Therefore, we first determined the appropriate subcutaneous MBT-2 cell dose required to develop solid tumors in C3H/HeJ mice. A tumor incidence dose-response curve was created after subcutaneous injection in a range of 10,000 to 500,000 MBT-2 cells. Thus, it was concluded that around 200,000 cells per mouse were adequate since approximately 70% of mice showed palpable tumors within 2 weeks and the animals started dying beginning week 5 and thereafter (data not shown).

Antitumor Activity of CCH

The dose and schedule of CCH administration were investigated. In the initial experiments the effect of CCH was assessed in primed mice challenged subcutaneously with 200,000 MBT-2 cells and intratumorally injected with a 1 mg dose of CCH for 6 days (treatment 1). Figure 1 shows that after CCH treatment the tumor growth rate decreased significantly ($p < 0.05$), similar to that in the KLH group when compared with the PBS group (fig. 1, *A*). Animal survival was also prolonged in the hemocyanin treated groups compared to the PBS group (data not shown). The incidence of tumors up to day 70 was 83.4% and 75% in the CCH and

KLH groups, respectively, and 100% in the PBS group. By day 55, 100% of the mice in the PBS group had died.

To further confirm the antitumor activity of CCH we decreased the dose and frequency of treatment. CCH primed mice were challenged with 150,000 MBT-2 cells injected into the flank and then treated with 100 μg CCH intratumor doses on alternate days for 9 days (treatment 2). Results showed a significant decrease in tumor growth, similar to that observed for KLH ($p < 0.001$, fig. 1, *B*). Survival rates in the hemocyanin groups at day 70 were higher than the rate in the PBS group (fig. 1, *C*). The tumor incidence on day 70 was 80% in the 2 hemocyanin groups compared with 100% in the PBS group. Throughout the assays we did not observe allergic reactions, any toxic effects of CCH, visible metastases or organ lesions.

Furthermore, we analyzed whether priming with CCH was needed to induce antitumor activity. Figure 1, *B* and *D* confirm that pre-immunization was essential to delay tumor growth.

Activation of the Immune Response by Hemocyanins and its Contribution to Antitumor Activity

To study the consequence of CCH treatment on the innate immune response we assessed NK cell activity at day 22 of the bioassay, ie the day next to the last therapeutic dose (treatment 1). We included controls to determine the NK cell activity induced by hemocyanins in the absence of tumor cells. Figure 2 shows the results of a representative experiment using 1:100 and 1:200 effector-to-target cell ratios, ie spleen mononuclear cells containing NK cells and YAC-1 cells, respectively. A sharp increase in NK cell activity was observed in mice treated with hemocyanins and this result contrasted with that in the PBS group. The control with Poly I:C showed 99.9% NK cell activity. Furthermore, the CCH injected group showed a higher increase in NK cell activity than the KLH group ($p < 0.001$), suggesting that the reported structural differences among hemocyanins might influence innate immunity stimulation.

To investigate the mechanism underlying the adaptive immune response involved in the antitumor effects of hemocyanins we determined by ELISA the effect of the hemocyanin dose on the specific humoral response in mice challenged with tumor cells. Results indicated a significant dose-response effect in mice injected with 100 μg or 1 mg therapeutic doses of hemocyanin ($p < 0.05$, fig. 3, *A*). Mice immunized with the same doses of hemocyanin but not challenged with MBT-2 cells showed similar antibodies titers and serum demonstrated cross-reactions between the hemocyanins (data not shown).

We then determined by Western blot whether hemocyanin cross-reactivity was directed to the whole molecule or whether it was subunit specific. Interestingly this analysis showed that when using denaturant gels, the result was negative. In contrast, when using native gels, a clear cross-reaction was observed. Anti-CCH mouse serum reacted with the 2 KLH subunits and anti-KLH mouse serum reacted

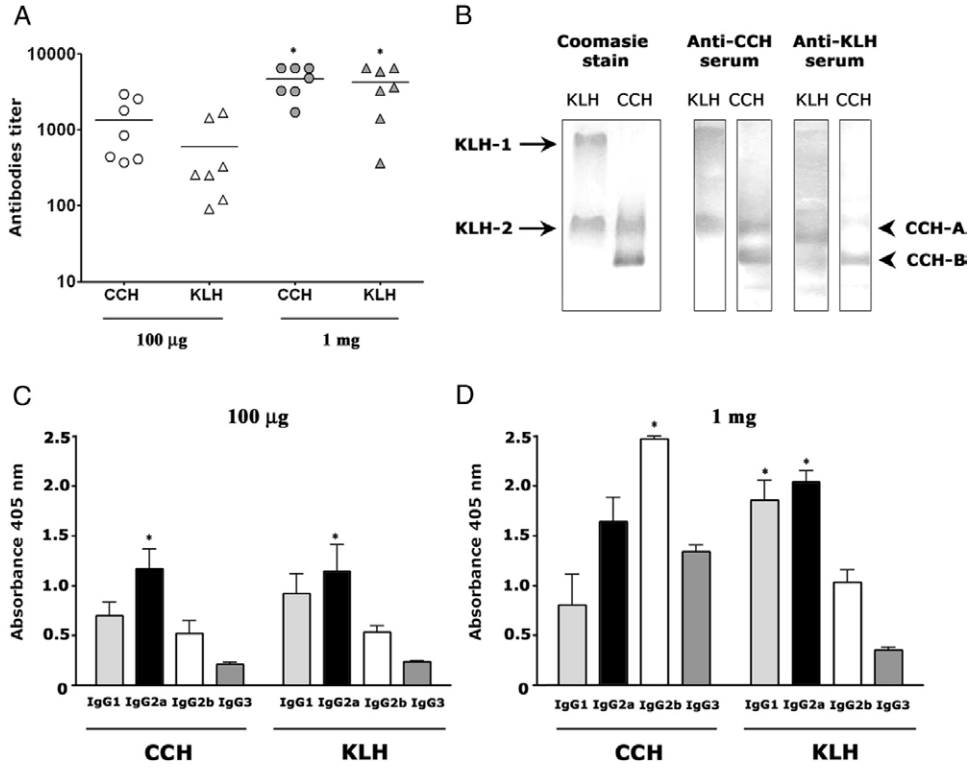

FIG. 3. Effect of CCH on humoral immune response. *A*, dose-response of anti-hemocyanin antibody titer determined by ELISA. Dots represent serum of individual mouse on day 27 of bioassay with 100 μg or 1 mg CCH or KLH treatment. Asterisk indicates CCH or KLH 100 μg vs CCH or KLH 1 mg 1-way ANOVA $p < 0.05$. *B*, Western blots in native gels show cross-reactivity between CCH and KLH. Coomassie blue stained gel shows KLH and CCH subunits KLH-1 and KLH-2, and CCH-A and CCH-B, respectively. *C*, hemocyanin specific IgG subclass production in tumor challenged mice on immunotherapy, determined by isotype specific ELISA. On schedule 2 treatment with 100 μg serum dilutions were 80-fold for IgG1 and IgG2a, and 20-fold for IgG2b and IgG3. Asterisk indicates CCH and KLH IgG2a vs IgG2b and IgG3 $p < 0.05$. *D*, on schedule 1 with 1 mg serum dilutions were 400-fold for IgG1 and IgG2a, and 200-fold for IgG2b and IgG3. Asterisk indicates CCH IgG2b vs IgG1 and IgG3, and KLH IgG1 and IG2a vs IgG2b and IgG3 1-way ANOVA $p < 0.05$.

with the 2 CCH subunits (fig. 3, *B*). These results suggest that cross-reactivity between the 2 hemocyanins is related to conformational rather than lineal epitopes. Nevertheless, we could not preclude the possibility that sugar moieties might be also involved.

We found that anti-CCH and anti-KLH serum from immunized mice but without a tumor challenge did not react by indirect immunofluorescence with tumor cells. Hence, there were no related or mimic epitopes between hemocyanins shared with tumor cell surface components.

To determine whether the anti-hemocyanin response corresponded to the Th1 or Th2 type of immune response we first studied the IgG subclass profile of hemocyanin specific antibodies. Figure 3, *C* and *D* shows that at a dose of 100 μg CCH and KLH induced high levels of specific IgG1 and IgG2a subclasses. In contrast, at the 1 mg dose KLH kept the IgG subclass profile invariant but CCH induced high levels of IgG2b and IgG3 antibodies.

With the same purpose we compared the serum levels of IL-4 and IFN-γ that characterized Th1 and Th2 responses on day 13 before tumor injection and on day 22 of treatment, that is the day after the last therapeutic dose. A background level of IL-4 was observed in all groups at days 13 and 22 (fig. 4). A moderate increase in IFN-γ in mice treated with KLH was observed at day 22. Furthermore, a major increase in serum IFN-γ in CCH treated mice was observed ($p < 0.05$).

DISCUSSION

We determined whether CCH is an effective antitumor agent in the syngeneic heterotopic murine bladder model and whether it could be an alternative treatment for TCC. This model has been widely used to evaluate potential pharmaceutical substances, given the great technical difficulties of the orthotopic model.[14] Therefore, first we had to assess if CCH behaved in the same way as KLH in an accepted and sensitive model.[8–10] There are differences in the literature concerning the antitumor effect of KLH on mice but this may be explained either by differences in the experimental designs or by the presence of lipopolysaccharide in the KLH preparations.[1] The latter can be overcome by using C3H/HeJ mice, which bear a mutation that affects the Toll-like receptor-4 gene.[16]

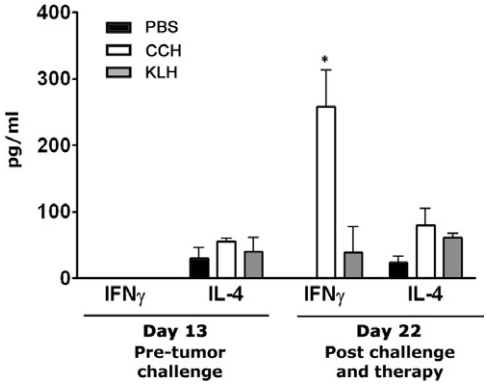

FIG. 4. IFN-γ and IL-4 determination in groups of 5 mice on immunotherapy primed subcutaneously with CCH, KLH or PBS. On day 15 they were challenged in right flank with 150,000 MBT-2 cells and received intralesionally 100 μg PBS with 100 μl CCH or KLH, or 100 μl PBS only as control. Results show mouse serum IFN-γ and IL-4 on day 13 after priming with hemocyanin, 1 day before tumor injection, and on day 22, 1 day after last therapeutic dose, as determined by ELISA. Asterisk indicates day 22 IFN-γ for PBS vs CCH 1-way ANOVA $p < 0.05$. On days 13 and 22 IL-4 for PBS vs CCH and KLH p not significant.

We confirmed the ability of MBT-2 cells to induce a specific humoral immune response. According to the observations of De Boer et al[13] and as confirmed in our study (data not shown), it is likely that the cell surface reactivity found in the serum of mice bearing tumors could be due in part to antibodies directed to C-retrovirus antigens or to a tumor associated cell membrane antigen expressed by C-retrovirus infected MBT-2 cells. However, we assume that the presence of C-retrovirus in MBT-2 cells could not contribute to or interfere with the antitumor effect of hemocyanins observed in mice.

Our results confirm that priming with hemocyanins before tumor inoculation is crucial to the buildup of antitumor activity.[1] In patients with bladder TCC who are on intravesical KLH therapy a delayed-type hypersensitivity reaction occurs. Jurincic-Winkler et al noted a considerable increase in CD4+ T lymphocytes in the submucosa and urothelial cells, in contrast to a slight increase in CD8+ T lymphocytes.[17] Currently we know that such responses are mediated by a population of antigen specific T-helper lymphocytes secreting IFN-γ, which is a characteristic of Th1-type responses. Despite this, KLH conjugate vaccines against cancer have been shown to induce tumor specific antibodies of the IgG1 and IgG3 isotypes, which are typical of the Th1 response. Also, in mice immunized with a T-cell receptor-KLH conjugate IgG2c antibodies developed that provided protection against T-cell lymphoma through an FcR dependent, antibody dependent cellular cytotoxicity mechanism.[18] We observed that, like KLH, CCH promoted an increase in IFN-γ and a change from IgG1 to IgG2a. However, at a higher CCH dose high levels of IgG2b and IgG3 were found (fig. 3, *D*), unlike in KLH treated mice. These results may reflect the structural differences between the hemocyanins.[7]

The fact that the immunotherapeutic effects of hemocyanins are not due to any super antigen like-activity, but rather rely on adequate priming suggests that the therapeutic properties could be attributable to a bystander effect on the antitumor response. This would occur through indirect stimulation of latent specific responses by breaking tolerance or suppressing the immune reaction against the tumor. This is supported by the local secretion of cytokines such as IFN-γ and IL-2 in the regional lymph nodes. In this respect NK cells are strongly stimulated by IL-2 from T lymphocytes, leading to differentiation into lymphokine-activated killer cells, thus, increasing the destructive elements against tumor cells. Furthermore, it was reported that MBT-2 cells do not grow when they are injected into the bladder of mice treated with IL-2 and cyclophosphamide combined.[19] Moreover, it was reported that KLH enhances NK cell activity and stimulates IFN-γ secretion in patients with superficial TCC of the bladder.[1,6] The results presented confirm these observations since mice treated with hemocyanins, particularly CCH, had increased NK cell activity as well as increased serum IFN-γ (figs. 2 and 4). NK cell depletion abolished the immunotherapeutic effect of bacillus Calmette-Guerin on mice bladder cancer, confirming that these cells are key actors in the removal of primary tumors.[20] In addition to the antitumor effect provided by IFN-γ secretion, NK cells can delay tumor growth by antibody dependent cellular cytotoxicity effector cells, which then kill bladder tumor target cells.

Finally, there is the question of how hemocyanins such as KLH and CCH, which broadly differ in their origin and subunit organization, can show similar immunomodulatory properties. We believe that the answer may be in the complex organization and the conserved molecular architecture of these molecules.[7] In this context gastropod hemocyanins have regions of high sequence homology, as demonstrated by the presence of common conformational or mimic epitopes and revealed by cross-reaction analysis (fig. 3, *B*). Moreover, gastropod hemocyanins have D5 point group symmetry, analogous to some viral structures, in addition to an extremely xenogenic nature. Thus, after hemocyanin processing they would generate numerous peptides that would fit with high affinity with most major histocompatibility antigen haplotypes involved in T-lymphocyte stimulation. It is worth noting that an additional adjuvant is not required for their antitumor effect. Thus, they would incorporate inflammatory signals into their structure that would stimulate the innate immune system.

CONCLUSIONS

CCH was as effective as KLH for preventing tumor growth in a murine bladder cancer model. Each hemocyanin increased the activity of NK cells. Immunity against hemocyanins is a polarized Th1 response that leads to enhanced antitumor activity. Data suggest a common immunostimulatory mechanism shared by hemocyanins that greatly differ in their origin and subunit organization.

ACKNOWLEDGMENTS

Dr. Dale Riggs, West Virginia University School of Medicine provided MBT-2 cells. Dr. John Ortaldo, University of the Health Sciences provided YAC-1 cells. Dr. Sergio Quezada, Memorial Sloan-Kettering Cancer Center, and Dr. Augusto Manubens, Alejandra Fuentes and Daniel Laporte, Biosonda Corp., critically read the manuscript. Dr. Carolina López, Mount Sinai School of Medicine, Dr. Jorge Ferreira, Universidad de Chile, Ignacia Aguirre, Alejandro Munizaga and José Peña, and René Barrios, Fundación Quintay provided assistance. MBT-2 microscopy analysis was done at the Servicio de Microsocopía Electrónica, Universidad Católica de Chile.

Abbreviations and Acronyms

CCH	=	Concholepas concholepas hemocyanin
ELISA	=	enzyme-linked immunosorbent assay
IFN-γ	=	interferon-γ
IL	=	interleukin
KLH	=	keyhole limpet hemocyanin
MBT-2	=	mouse bladder tumor-2
NK	=	natural killer
PBS	=	phosphate buffered saline
TCC	=	transitional cell carcinoma
Th1	=	T helper type 1
Th2	=	T helper type 2

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