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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

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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 al8 with modifications. Cells were cultured at 37°C in a 10% CO2 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 al8 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 Lau9 and Swerdlow10 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-
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 al11 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 with 100 μCi Na_2^51Cr (Perkin-Elmer, Foster City, California) for 1.5 hours at 37°C. 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 37°C in 10% CO2 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 used12 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 37°C, 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 37°C. The plates were washed and developed at 37°C with p-nitrophenyl phosphate (Merck, Darmstadt, Germany).

MBT-2 Microscopy Analysis
For indirect immunofluorescence and transmission electron microscopy cells were adequately processed13 and observed elsewhere.

Western Blot
The procedure of De Ioannes et al7 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...
with the 2 CCH subunits (fig. 3, B). These results suggest that cross-reactivity between the 2 hemocyanins is related to conformational rather than linear 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. Therefore, first we had to assess if CCH behaved in the same way as KLH in an accepted and sensitive model. 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. The latter can be overcome by using C3H/HeJ mice, which bear a mutation that affects the Toll-like receptor-4 gene.

We confirmed the ability of MBT-2 cells to induce a specific humoral immune response. According to the observations of De Boer et al 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. 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. 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.

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.

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. Moreover, it was reported that KLH enhances NK cell activity and stimulates IFN-γ secretion in patients with superficial TCC of the bladder. 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.

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.

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Abbreviations and Acronyms

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