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Monoclonal Antibodies to Molluskan Hemocyanin from *Concholepas concholepas* Demonstrate Common and Specific Epitopes among Subunits

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ABSTRACT

We studied the reactivity of mouse monoclonal antibodies (MAbs) against the hemocyanin from the Chilean marine gastropod *Concholepas concholepas* (CCH). This protein has been successfully used as a carrier to produce antibodies to haptens and peptides. All MAbs (13) belonging to IgG subclass exhibit dissociation constants (K_d) from 1×10^{-7} M to 1×10^{-9} M. MAbs were characterized by enzyme-linked immunosorbant assay (ELISA) using CCH treated with different procedures, including dissociation into CCH-A and CCH-B subunits, Western blot, enzymatic digestion, chemical deglycosylation, and thermal denaturation. MAbs were classified into three categories, according to subunit specificity by ELISA. The epitope distribution shows that CCH subunits display common epitopes (group I, 5 MAbs, 1H5, 2A8, 3A5, 3B3, and 3E3), as well as specific epitopes for CCH-A subunits (group II, 3 MAbs, 1B8, 4D8, and 8E5) and for CCH-B subunits (group III, 5 MAbs, 1A4, 1E4, 2H10, 3B7, and 7B4). The results can be summarized as follows: (1) six antibodies react with thermal denatured CCH, suggesting that they recognize linear epitopes, whereas seven recognize conformational epitopes; (2) oxidation of carbohydrate moieties does not affect the binding of the MAbs; (3) enzymatic digestion of CCH decreases the reactivity of all antibodies irrespective of the protease used (elastase or trypsin); (4) bringing together the above data, in addition to epitopic complementarity analysis, we identified 12 different epitopes on the CCH molecule recognized by these MAbs. The anti-CCH MAbs presented here can be useful tools to understand the subunit organization of the CCH and its complex structure, which can explain its immunogenic and immunostimulating properties in mammals.

INTRODUCTION

HEMOCYANINS ARE OXYGEN CARRIER GLYCOPROTEINS that are found in the hemolymph of many mollusks and arthropods. Hemocyanins are model proteins because they exhibit a complex three-dimensional structure, have multiple aggregation states, and coordinate O_2 through copper. Mollusk hemocyanins are organized as hollow cylindrical oligomers, with molecular masses from 3.5 to 4.0 MDa known as decamers, made by 10 subunits from 350 to 450 kDa in size. Each subunit consists of a string of seven or eight 45 to 50 kDa globular domains called functional units (FUs). In bivalves and gastropods, decamers can self-associate as stable di-decamers, displaying huge structures with molecular masses from 8.0 to 9.0 MDa.[1,2]

Hemocyanins cause strong immune responses in mammals due to their xenogenic nature and their big size, which support T and B lymphocyte multi-epitope recognition. Indeed, hemocyanins have been extensively used as carrier proteins for haptens and peptides, as standard antigens in the studies of the immune response, and as nonspecific immunostimulants.[3,4] The hemocyanin from the mollusk Keyhole Limpet (*Megathura crenulata*) known as KLH has been used for the above purposes.

Scarce information is available on the relationship among the structural and the immunotherapeutical properties of hemocyanins. After the studies of Olsson et al.,[5] who demonstrated a significant reduction in the recurrence rate of superficial bladder carcinoma in patients immunized with KLH, there is further evidence that support its biomedical applications.[6–10] KLH has a promising potential in the treatment of other carcinomas, including melanoma,[11,12] breast cancer, and ovarian cancer.[13–15] Besides cancer treatment, KLH has other bio-

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medical uses, for example, in the diagnosis and immunotherapy of *Shistosomiasis*,[16] in drug addiction,[17,18] and as a component of experimental synthetic minimal viral vaccines against AIDS,[19,20] and papilloma virus.[21]

The versatile properties of KLH has derived in a growing interest on the knowledge of hemocyanin structure, and encouraged us to seek other alternatives of mollusk hemocyanins with equivalent immunological and therapeutic properties.[3] Over the last decade, hemocyanin from *Concholepas concholepas* (CCH) (commonly known as Loco) has been successfully used as a carrier protein in the development of polyclonal[22–25] and monoclonal antibodies (MAbs) to peptides and haptens[26,27] and has proved to be a reliable alternative to KLH. The CCH structure is just begining to be known, and with a size about 8.0 MDa, it has two different subunits noncovalently linked named CCH-A (404 kDa) and CCH-B (351 kDa).[28]

Since the work of Köhler and Milstein that allowed the immortalization of B-lymphocytes secreting monospecific antibodies,[29] hybridomas have been widely used to study the epitope structure of many proteins.[30] Earlier model antigens such as lysozyme, cytochrome c and neuraminidase, through the property of antibody–protein interaction, provided epitope maps of these proteins.[31] More recently, x-ray-crystallographic studies have allowed a better definition of each epitope by identifying the amino acids that participate in the binding with the antibody.[32–38]

Polyclonal antibodies have been used extensively in the structural analysis of hemocyanin subunits, to determine the topological position of each subunit within the quaternary structure after protease digestion,[39–44] and to study the function[45] and evolution of hemocyanins.[46–48] However, MAbs have rarely been used to study the epitopic structure and organization of mollusk hemocyanins.

The present paper describes the development and characterization of specific MAbs to CCH. We identify some CCH epitopes and investigate its linear or conformational nature. Also, we demonstrate that CCH-A and CCH-B subunits of the protein contain common and specific epitopes.

MATERIALS AND METHODS

Chemicals and biochemicals

Bovine serum albumin (BSA), ethylenediaminetetra-acetic acid (EDTA), pancreatic elastase, trypsin type I from bovine pancreas, and pristane (2,6,10,14-tetramethyl-decanoic acid), were from Sigma-Aldrich Chemical (St. Louis, MO). Para-nitro phenyl phosphate, polyethylene glycol (PEG) 4000 and sodium periodate were from Merck Darmstadt, (Germany). Mono-Q 5/5 Column was from Pharmacia (Uppsala, Sweden). Bicinchoninic acid protein kit, complete and incomplete Freund's adjuvant, Coomassie Plus Protein Assay Reagent, Immunopure monoclonal antibody isotyping kit II, 5-bromo-4-chloro-indolyl-phosphate (BCIP), nitro-blue-tetrazolium (NBT), nitrocellulose membrane, polystyrene plates, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, SuperBlock in phosphate-buffered saline (PBS), Tween-20, and anti-mouse IgG-alkaline phosphatase conjugate were from Pierce-Endogen, (Rockford, IL). Dulbecco's minimum essential medium (DMEM) high glucose medium and fetal calf serum were purchased from HyClone (Logan, UT). Prestained markers, antibiotics and hypoxanthine, aminopterin, and thymidine (HAT) medium were obtained from Gibco (Gaithersburg, MD). All other chemicals were analytical-grade reagents, and all solutions were prepared using Mili-Q water.

Hemocyanin samples

Hemocyanin sources. Hemocyanin from *C. concholepas* (Blue Carrier), *Fisurella cumingi* (Lapa Frutilla), *Fisurella maxima* (Lapa Reina), and *Fisurella latimarginata* (Lapa Negra) were provided by Biosonda Corp. (Santiago, Chile). KLH was purchased from Pierce-Endogen (Rockford, IL) and *Limulus polyphemus* hemocyanin was from Sigma-Aldrich Chemical (St. Louis, MO).

Protease digestion of CCH. The general procedure described by Gebauer et al. was used.[43] Experiments were performed with samples of CCH (20 mg) dissolved in 3 mL 1.36 M glycine/NaOH buffer, pH 9.6 and enzymes (elastase or trypsin) were added to a final concentration of 2% w/w, previously dissolved in 1 mL of 0.1 M ammonium carbonate pH 8. Enzymatic digestion was performed at 37°C and samples were taken at 1, 3, and 5 h. To stop the enzyme reaction phenylmethysulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and samples were stored at −20°C until use. A CCH sample was incubated without the enzymes under the same conditions as control.

Chemical deglycosylation of CCH. The procedure described by Hermanson[49] was used. Samples of 10 mg/mL of CCH in PBS pH 7.5 were prepared, and 100 μL of a 100 mM sodium periodate solution was added. To stop the reaction, 100 μL of anhydrous glycerol was added and the samples were incubated for 30 min. Samples were deglycosylated for 3 and 24 h, and finally dialyzed against PBS.

Denatured CCH. Samples of 10 μg/mL of CCH dissolved in PBS were boiled for 5 min in a water bath and then cooled to 4°C.

Isolation of subunit CCH-A and CCH-B by high-performance liquid chromatography (HPLC). The general procedure described by Swerdlow et al.,[44] was applied. Briefly, CCH was dissociated by dialysis against glycine/NaOH pH 9.6, 10 mM EDTA at 4°C, and then applied to a Mono-Q 5/5 column at a flow rate of 1.0 mL/min. To elute the sample from the column, a linear gradient of 130 mM glycine/NaOH pH 9.6 10 mM EDTA and 1 M NaCl was used. Coomassie Plus Protein Assay Reagent was used to determine the protein concentration according to the supplier's instructions.

Development of MAbs to CCH

Mice immunization. Two-month-old BALB/c mice were immunized with CCH as follows: On Day 1, they received intraperitoneally 400 μg of antigen, emulsified in complete Freund's adjuvant; on Day 16, the same immunization was carried out, but using incomplete Freund's adjuvant. A booster was given 3 days prior to the somatic fusion to obtain hybridomas. Mice were bled prior to the immunizations to obtain pre-im-

mune control serum. Ten days after each injection of CCH, the mice were bled and a direct enzyme-linked immunosorbant assay (ELISA) was used to evaluate the humoral response.

Somatic fusion. The general procedure described by Köhler and Milstein[30] was used with minor modifications.[50] Prior to the somatic fusion, NS0/2 cells (provided by Dr. C. Milstein, from MRC Laboratory of Molecular Biology, Cambridge, England) were seeded on complete DMEM (Dulbecco's medium supplemented with 10% Fetal Calf Serum and antibiotics) and incubated at 37°C, 10% CO_2 atmosphere and 100% humidity. Three days before the fusion, an intravenous and intraperitoneal (i.p.) CCH booster was given to the immune mice. The spleen was disrupted mechanically and washed, then the splenic lymphocytes were mixed with NS0/2 cells, and 0.5 mL 50% PEG 4,000 was added for the fusion. The hybridomas were suspended in complete DMEM containing HAT, seeded on 96-well plates, and cultured under the same conditions described before. Seven days after fusion, 2 drops/well complete DMEM culture medium plus HT was added to the plates. Starting at Day 12, the supernatant of the wells with evident hybridoma growth were screened by ELISA, to determine antibodies to CCH. A plate coated with BSA was used as a primary specificity control.

Subclass antibody typing. The determination of MAb isotype was done using a commercial ELISA kit according to the supplier's instructions.

Immunochemical methods

Direct ELISA. The general procedure described by Crowther and Abu-Elzein[51] was used, with minor modifications. Microwell polystyrene plates were incubated overnight at 4°C with 100 μL/well of 10 μg/mL solution of CCH or isolated subunits, in PBS. The plates were blocked with 250 μL/well of Super Block or 1% PBS-Bovine Serum Albumin (PBS-BSA) for 2 to 3 h at room temperature. Then, serial half dilutions of the immune sera in blocking buffer or undiluted hybridoma supernatant were incubated for 2 to 3 h at room temperature. The plates were washed three times with 250 μL of 0.02% PBS-Tween, then 100 μL/well of goat anti-mouse IgG serum conjugated with alkaline phosphatase (ALP) diluted 1/1.000 in blocking buffer was added to the wells. After incubating for 30 min at room temperature, the plates were washed as described above and developed for 30 min at 37°C by adding 100 μL/well of 1 mg/mL para-nitrophenylphosphate (p-NPP) in ALP-buffer (Na_2CO_3/$NaHCO_3$ 0.2 M, pH 9.6). The reaction was stopped with 3N NaOH and was read spectrophotometrically at 405 nm.

For all MAbs, optic density (OD) values were obtained by ELISA against CCH-A and CCH-B subunits. Two criteria were applied for classifying the MAb panel into three groups: (1) group II or III comprised MAbs that gave OD values ~3-fold higher with one subunit in comparison to the other; (2) MAbs that gave a difference in OD values between subunits less than ~2-fold were assigned to group I.

Dissociation constant determination. The ELISA method developed by Friguett et al.[52] was used. Polypropylene tubes containing 200 μl of CCH in a range of 0 to 1×10^{-10} M and 200 μL of each hybridoma supernatant without dilutions, were incubated for 20 h at 4°C. Then, 100 μL from each tube was added in triplicate onto ELISA plates previously coated with 1 μg/well of CCH. To perform the competition, the plates were incubated for 4 h at 4°C, washed, and developed with a goat anti-mouse IgG serum, conjugated with ALP, as described above.

The K_d for each MAb was determined from a graphical analysis, where the ratio $A_0/(A_0\text{-}A)$ was plotted versus 1/c; A_0 represented the OD of the hybridoma supernatant containing the MAb alone, A was the OD of the hybridoma supernatant incubated with different concentrations of CCH and c was CCH concentration.

Determination of overlapping epitopes. A direct ELISA was used to determine the additivity index (AI) according to Friguet et al.(53,54) Briefly, 96-polystyrene plates coated with 100 mL/well of a 10 mg/mL CCH solution and blocked with SuperBlock, were incubated for 3 h at room temperature with a mixture that contained two Mab supernatants at the dilution that gave the highest OD. As control, a sample of the prediluted antibody alone, half diluted with the culture medium of the hybridomas, was included. Plates were developed as previously described. The additivity index was defined as:

$$AI = \left[\frac{2A_{1+2}}{A_1 + A_2} - 1\right] \times 100,$$

where A_1, A_2, and A_{1+2} are the OD reached in the direct ELISA with the first MAb, the second antibody, and the two antibodies together, respectively

Electrophoresis and Western blot. Samples of hemocyanin were subjected to SDS-PAGE in a 3 to 10% polyacrylamide gradient system[55] then transferred to 0.02-μm pore nitrocellulose membranes.[56] The membranes were incubated over night at 4°C with Super Block or 1% PBS-BSA, and then incubated for 3 h with anti-CCH mouse serum diuted 1:1,000 in SuperBlock or with undiluted hybridoma supernatants. After three washings with PBS-Tween 0.02%, the membranes were incubated for 1 h at room temperature with goat anti-mouse IgG serum, conjugated with ALP. The membranes were developed using NBT and BCIP. To stop the reaction, the membranes were washed with water.

RESULTS

Development of MAbs to CCH

To develop hybridomas secreting anti-CCH antibodies, BALB/c mice were immunized with *Concholepas concholepas* hemocyanin. Figure 1 shows a titer of around 1/8,000 against CCH after the second immunization, measured by ELISA. In this experiment, KLH was included to determine the epitopic relation between both hemocyanins and a poor cross reaction at low dilutions of the sera was observed. The immune response obtained after the immunizations were considered enough to develop hybridomas.

Somatic fusions were done, and finally 13 positive hybridomas were obtained and recloned, which showed a stable and

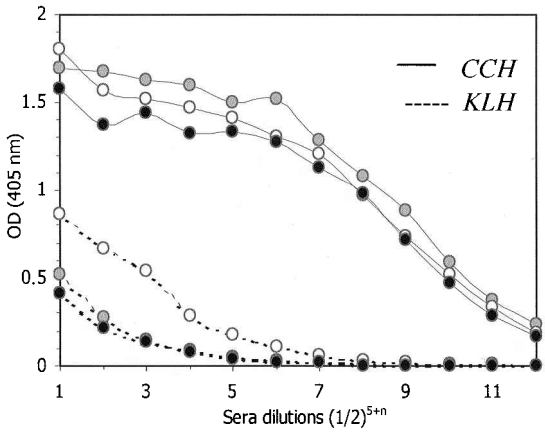

FIG. 1. Specificity of the humoral immune response of BALB/c mice to CCH. Titration by ELISA of sera from three mice immunized with CCH. The plates were coated with CCH or KLH, to determine the cross-reaction between both hemocyanins. Binding of the antibodies to the hemocyanins was determined using anti-mouse IgG labeled with ALP, and developed with a pNPP solution as described in the Materials and Methods section. The first dilution corresponds to 1:64.

rapid growth. To exclude sticky antibodies, a control using an ELISA with BSA was performed with negative results. The MAbs obtained were highly specific to CCH; they showed neither cross reaction by ELISA to KLH, nor to *L. polyphemus* hemocyanin, and nor against three Chilean limpet hemocyanins from the *Fisurella* genus: F. *cuming*, F. *maxima*, and F. *latimarginata* (data not shown).

Analysis of the epitope specificity of anti-CCH MAbs by ELISA and Western blot

Table 1 summarizes the general properties of anti-CCH monoclonals. All of them belong to the IgG subclass. We classified the antibodies under three categories, according to their subunit specificity by ELISA, as described in the Materials and Methods section. Group I included five antibodies that recognized common epitopes to both subunits (named 1H5, 2A8, 3A5, 3B3, and 3E3). Group II comprised three antibodies (named 1B8, 4D8, and 8E5) that reacted preferentially with the CCH-A subunit. Group III consisted of five antibodies (named 1A4, 1E4, 2H10, 3B7, and 7B4) that reacted preferentially with the CCH-B subunit. The K_d values were determined with native CCH, as shown in Fig. 2, for all antibodies except for MAb 1A4, because it did not bind to soluble CCH, so that competition was not observed. The lowest K_d values were from 3B3 and 4D8 antibodies: 4×10^{-9} M and 6×10^{-9} M, respectively.

Next, we investigated whether the antibodies were reactive by Western blot in CCH samples treated under SDS-PAGE denaturing conditions. Table 2 summarizes the results and shows that some monoclonals exhibited a different reactivity pattern than the one assigned by ELISA: MAbs 2A8 and 3A5 (group I) and 1E4 (group III) did not react by Western blot, suggesting that they recognized conformational epitopes on the CCH molecule. In group I, antibody 3E3 did not react with CCH-A, although by ELISA it reacted with both subunits. Interestingly, all MAbs of group II bound to epitopes displayed on both subunits. In the same way, monoclonal 3B7 from group III, bound to CCH-A and CCH-B subunits. The rest of the MAbs from the panel were positive by Western blot in keeping with the ELISA analysis.

TABLE 1. GENERAL PROPERTIES OF ANTI-CCH MABS DETERMINED BY ELISA

Group[a]	*MAb*	*Subclass*	*CCH*[b]	*CCH-A*[b,c]	*CCH-B*[b,c]	*Dissociation constant*[d]
I	1H5	IgG_1, κ	2.15	2.54	2.74	2×10^{-8} M
	2A8	IgG_1, κ	1.73	2.65	2.18	1×10^{-7} M
	3A5	IgG_{2a}, κ	1.56	2.80	2.54	3×10^{-8} M
	3B3	IgG_{2a}, κ	1.29	2.45	2.20	4×10^{-9} M
	3E3	IgG_1, κ	1.11	1.09	2.32	7×10^{-8} M
II	1B8	IgG_1, κ	0.66	1.07	0.08	3×10^{-7} M
	4D8	IgG_1, κ	1.01	2.76	0.49	6×10^{-9} M
	8E5	IgG_1, κ	1.66	2.15	0.17	1×10^{-8} M
III	1A4	IgG_1, κ	1.59	0.61	2.12	ND[e]
	1E4	IgG_1, κ	0.69	0.34	1.78	5×10^{-8} M
	2H10	IgG_{2a}, κ	0.92	0.69	1.83	4×10^{-8} M
	3B7	IgG_1, κ	0.74	0.61	1.98	8×10^{-8} M
	7B4	IgG_{2b}, κ	0.69	0.34	1.04	1×10^{-8} M

[a]Anti-CCH MAbs were classified in three categories, according to their reactivity with isolated CCH-A and CCH-B subunits of the protein.

[b]OD value to 405 nm, corresponding to undiluted supernatant of each hybridoma secreting anti-CCH MAbs. Experiments were run in triplicate with an experimental error below 10%.

[c]Subunits were obtained by CCH dissociation, by dialysis against Glycine-NaOH pH 9.6, and EDTA, then purified in a Mono-Q 5/5 column.

[d]MAb affinity was determined, as described in the Materials and Methods section. Figure 2 shows an example, where the slope of the curve is the dissociation constant.

[e]Undetermined. The anti-CCH 1A4 MAb did not bind to the protein in solution.

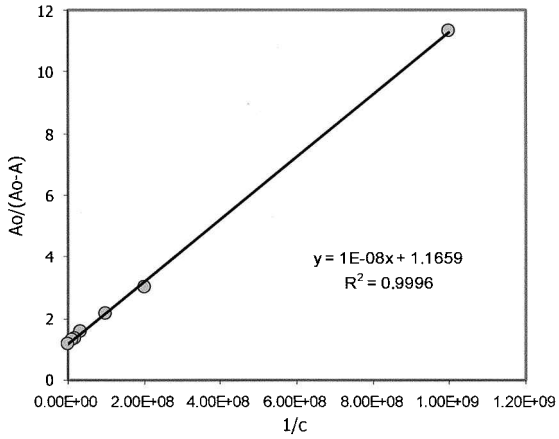

FIG. 2. Determination by ELISA, of the K_d for the anti-CCH Mab 7B4. Two hundred microliters of CCH in a range of 0 to 1×10^{-10} M were added to 200 μL of 7B4 hybridoma supernatant, and incubated for 20 h at 4°C. Then 100 μL of each sample was added onto plates previously coated with 1 μg/well CCH. Plates were incubated for 4 h at 4°C, washed, and developed as described in the Materials and Methods section. A_0 is the OD when antigen is not competing and A is the OD at each concentration of competing antigen. The plot gave a linear curve, where the slope is the dissociation constant. Experiments were run in triplicate with an experimental error lower than 10%.

Figure 3 shows some of the data summarized in Table 2. An anti-CCH mouse serum was used as positive control; it displayed a strong reactivity with the principal polypeptides of the molecule: subunits CCH-A and CCH-B. In turn, antibodies 1A4 and 2H10 exhibited specific and strong reactions to the epitopes on the CCH-B subunit.

TABLE 2. GENERAL PROPERTIES OF ANTI-CCH MABS DETERMINED BY WESTERN BLOT[a]

Group	*MAb*	*CCH-A*[b]	*CCH-B*[b]
I	1H5	+	+
	2A8	−	−
	3A5	−	−
	3B3	+	+
	3E3	−	±
II	1B8	+	+
	4D8	+	+
	8E5	+	±
III	1A4	−	+
	1E4	−	−
	2H10	−	+
	3B7	+	+
	7B4	−	+

[a]Samples of fresh purified CCH were subjected to denaturing SDS-PAGE, transferred to a nitrocellulose membrane, blocked, and incubated with hybridoma supernatants.

[b]Reactivity of MAbs against each subunit: (+) positive; (+/−) weakly positive; and (−) negative.

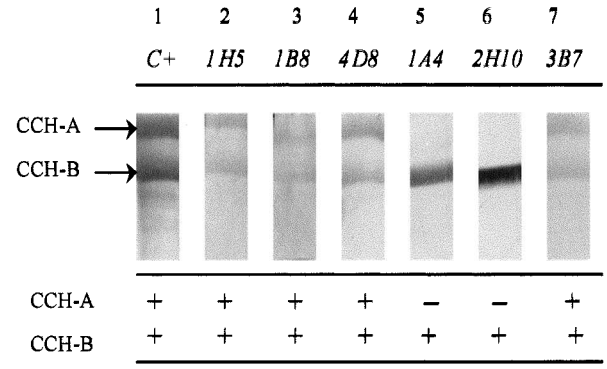

FIG. 3. Reactivity of MAbs against CCH by Western blot. Samples of CCH were run on a 3 to 8% polyacrylamide gel gradient system, transferred to nitrocellulose membrane and incubated with the undiluted hybridoma supernatant. Lane 1, positive control (C+), serum from a mouse used in a fusion, showing intense reactivity with polypeptides corresponding to CCH-A and CCH-B. Lane 2, MAbs 1H5 from group I, Lanes 3 and 4 were incubated with MAbs from group II, 1B8 and 4D8, respectively. Lanes 5 to 7 were incubated with MAbs from group III, 1A4, 2H10, and 3B7, respectively. Bottom of the figure, reactivity assigned to each MAb for subunit A and B: (+) positive; (±) weakly positive and (−) negative.

Characterization of the epitopes recognized by anti-CCH MAbs

To further characterize the CCH epitopes recognized by the antibodies, we evaluated by ELISA the effect of a limited proteolysis on CCH. We used either elastase or trypsin over CCH after different times of digestion (1, 3, and 5 h). As a control, CCH was incubated in reaction buffer but without enzyme. Figure 4 summarizes the following results: (1) The enzymes affected all the epitopes recognized by anti-CCH MAbs because the MAbs decreased their binding to the protein after 1 h of treatment; (2) the most destructive enzyme on CCH was trypsin and the less destructive was elastase; (3) the most resistant epitope to trypsin and to elastase was the one recognized by 3A5 MAb (group I), it maintained its reactivity by 50 and 75%, respectively, suggesting that this epitope would be protected from the proteolytic attack by steric hindrance; (4) in group II, the reactivity of monoclonals 1B8 and 8E5 was similar with the two enzymes. In contrast, the epitope recognized by antibody 4D8 from the same group is the most resistant to elastase degradation; and (5) the most labile epitope of CCH was recognized by the monoclonal 1E4 (group III); at 1 h it decreased its reactivity by 25% after the enzymatic treatment with elastase; suggesting that this epitope is greatly exposed on the molecule. On the other hand, epitopes recognized by antibodies 1A4 and 2H10 lost around 75% of reactivity after treatment of CCH with elastase for 1 h.

To approach the linear or conformational nature of the epitopes, we studied the effect of thermal denaturation on CCH and how that treatment affected the binding of MAbs. As shown in Table 3, monoclonals 3B3 (group I), 1B8 (group II), 1A4 and 2H10 (group III) improved their reactivity after denaturation of the protein; whereas, MAbs 8E5 (group II) and 3B7 (group III) decreased their binding by around 40% indicating

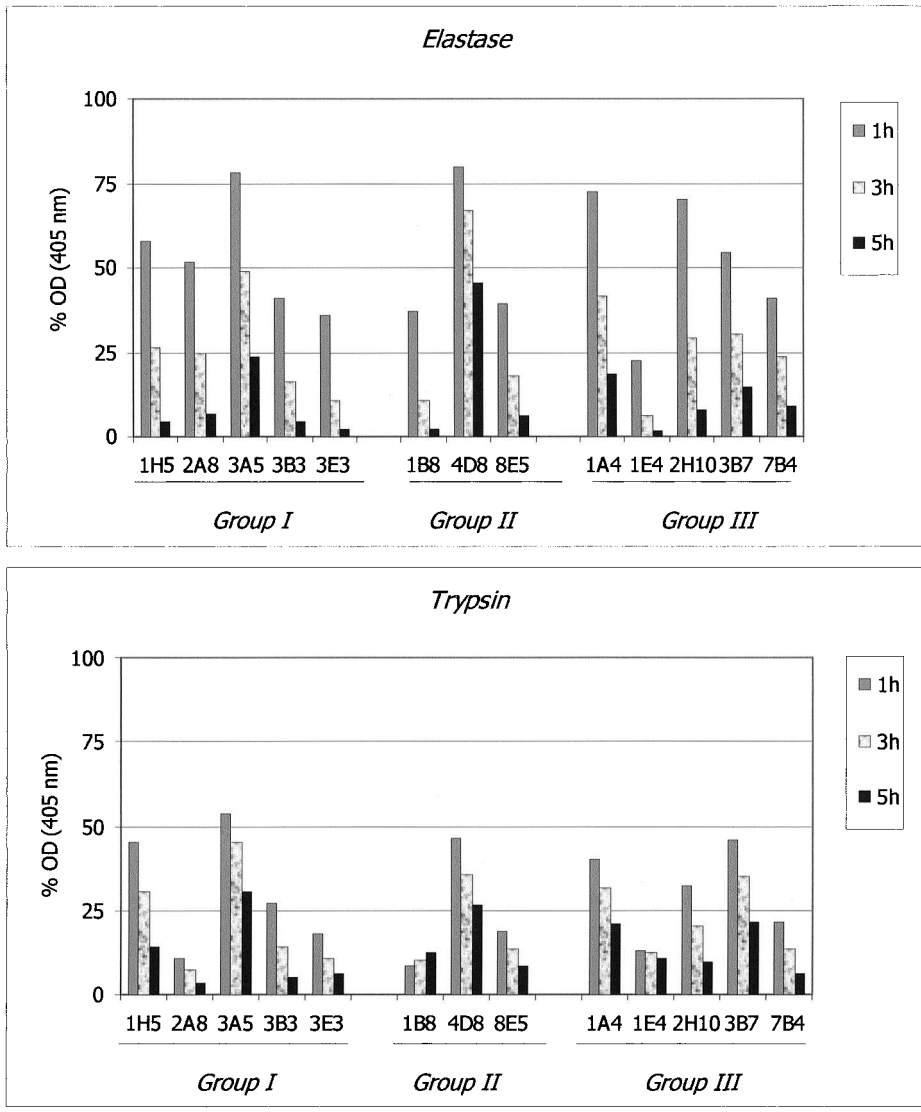

FIG. 4. Reactivity of anti-CCH MAbs with CCH digested enzymatically, using a direct ELISA. Samples of CCH were incubated with elastase or trypsin, as described in the Materials and Methods section. Samples were taken at 1, 3, and 5 h and the enzymatic digestion of CCH was stopped by adding PMSF and cooling. Plates were coated with digested CCH, incubated with the undiluted hybridoma supernatants, and developed with an anti-mouse IgG labeled with ALP. In the figure, the reactivity of each antibody with the different digested CCH samples is indicated in gray (1 h), in spotted grey bar (3 h), and in black (5 h). The percentage of binding was determined considering as 100% the OD of each MAb to CCH without enzyme treatments. The present experiment was run in duplicate and in parallel with an experimental error lower than 10%.

that the epitopes recognized by these 6 antibodies were linear, according to Western blot analysis. In contrast, the other seven MAbs of the panel did not react with denatured CCH, suggesting that they recognized conformational epitopes. However, four of them were reactive by Western blot: MAbs 1H5 and 3E3 (group I), 4D8 (group II), and Mab 7B4 (group III), suggesting renaturation of these epitopes during the transfer to the nitrocellulose membrane. This phenomenon has been reported for other proteins.[54]

Because hemocyanins are glycoproteins, it is worth studying whether any carbohydrate moiety was involved in the binding of the antibodies. We removed the oligosaccharide residues from CCH by chemical deglycosylation using a mild sodium periodate oxidation during 3 and 24 h. Table 3 shows that all anti-CCH MAbs improved their binding to CCH after periodate treatment for 24 h, indicating that carbohydrate residues would not be involved and that the epitopes are better exposed after the treatment.

Determination if anti-CCH MAbs are directed at overlapping or complementary epitopes

Finally, we investigated whether the MAbs recognized similar or different epitopes on the CCH molecule. We used direct competition assay between two unlabelled MAbs in an ELISA, to define the additivity index as described in the Materials and

TABLE 3. EFFECT OF DIFFFERENT TREATMENTS OF CCH ON THE BINDING OF MABS TO CCH, BY ELISA[a]

				Periodate treatment[c]	
Group	*MAb*	*CCH control*	*Denatured CCH*[b]	*3 h*	*24 h*
I	1H5	1.87	0.10	1.49	2.03
	2A8	1.33	0.10	1.32	1.68
	3A5	1.26	0.30	1.43	1.56
	3B3	0.98	1.28	1.55	1.60
	3E3	1.32	0.12	1.74	1.69
II	1B8	0.46	0.65	0.34	0.66
	4D8	1.30	0.16	1.05	1.60
	8E5	1.17	0.72	1.34	1.52
III	1A4	1.32	2.31	1.56	1.71
	1E4	0.70	0.07	1.29	1.17
	2H10	1.15	1.80	1.57	1.65
	3B7	0.60	0.41	0.86	1.21
	7B4	0.62	0.17	0.71	0.99

[a]OD value to 405 nm, corresponding to undiluted supernatant of each hybridoma secreting anti-CCH MAbs. Experiments were run in triplicate with an experimental error lower than 10%.
[b]Samples of CCH were dissolved in PBS and boiled for 5 min in a water bath, before coating the plates.
[c]Samples of CCH incubated with sodium periodate for 3 or 24 h and dialyzed against PBS, before coating the plates.

Methods section. The basis of the assay relies on an increase of OD when a combination of two MAbs binds to different epitopes (AI $\geq$ 50.1), but not when both react to the same epitope (AI $\leq$ 49.9). Table 4 summarize the results, where the MAbs were arranged in a Cartesian coordinate system; the intersection corresponds to the AI value for each pair of MAbs.

Table 4 shows that all MAbs from group I, were directed to the same or close epitopes, or that the binding of one antibody caused a conformational change in CCH so that the second antibody cannot bind, AI values below 40 (highlight gray). On the other hand, MAbs from groups II and group III were complementary, they recognized different epitopes in CCH-A and CCH-B subunits, with a majority of AI values higher than 60. Thus, group I MAbs recognize epitopes common for both subunits because they were not additive with almost all MAbs from groups II and III.

DISCUSSION

Hemocyanins have been used extensively in immunology as nonspecific immunostimulants and as carrier proteins to produce antibodies against haptens. The multiple use of KLH in basic and clinical research, for example, in cancer therapy, vaccine development, and diagnosis, suggests that the high demand for KLH will increase the search for alternative molecules. These candidate proteins must have comparative advantages such as abundance, easy obtention and purification, together with strong immunogenicity. In the same context, hemocyanin from *Concholepas concholepas* may be a potential alternative to KLH, based on its successful use as a carrier.[22–27] To conduct further studies, we considered it important to develop MAbs to CCH.

Our previous studies on the structure of CCH molecule,[28] using immunoelectrophoresis with specific polyclonal antibodies, showed that CCH subunits have partial identity, suggesting that they had common and specific epitopes. This observation has been confirmed in the present work, and supports our classification of the anti-CCH MAbs in 3 categories, according to the localization of the epitope on the CCH subunits. However, as mentioned before, when analyzed by Western blot, some MAbs exhibited a different specificity pattern than the one assigned by ELISA. MAbs from group II that are directed preferentially to the native CCH–A subunit, denote a remarkable feature of this protein: CCH big size and hollow cylinder structure favors "epitope masking" because by Western blot these antibodies bind to epitopes found in both subunits and probably under native conditions they would be buried in the native CCH-B subunit. An other possible explanation is that these masked epitopes of CCH-B would be similar but not identical to those from CCH-A, and they would appear as mimicry epitopes after denaturation, as seen by Western blot.

The criteria imposed by the classification of the MAbs, allowed us to identify *a priori* a minimum of three epitopes in CCH: one for each group. However, the differences in reactivity with CCH treated with diverse procedures, in addition to the epitopic complementarity analyses, indicated that at least 12 epitopes could be deduced.

In group I, we identified four epitopes: The first one was assigned to Mab 3B3, it bound to heated CCH and was complementary with certain MAbs from group III. The second was recognized by MAbs 2A8 and 3A5, which did not bind to heated CCH and were not complementary. A third epitope could be identified by 3E3 because it was negative by Western blot with CCH-A and displayed low reactivity with CCH-B subunit. Finally a fourth epitope was assigned to MAb 1H5; although it had the same additivity characteristics of the other MAbs from group I, this antibody was positive by Western blot with both subunits.

In group II, we identified three epitopes: The first one was

TABLE 4. DETERMINATION BY ELISA[a] OF THE EPITOPIC COMPLEMENTARITY FOR ANTI-CCH MABS

		I					*II*			*III*				
Group	*MAb*	*1H5*	*2A8*	*3A5*	*3B3*	*3 E3*	*1B8*	*4D8*	*8 E5*	*1A4*	*1 E4*	*2H10*	*3B7*	*7B4*
I	1H5	—												
	2A8	23.0	—											
	3A5	28.4	19.2	—										
	3B3	38.6	31.4	27.6	—									
	3-E3	32.9	21.4	21.7	−13.4	—								
II	1B8	89.0	66.4	64.7	49.3	67.2	—							
	4D8	35.6	31.2	29.2	26.2	47.7	61.8	—						
	8-e5	22.7	14.7	23.6	22.3	53.5	78.8	39.5	—					
III	1A4	32.0	17.6	34.9	43.6	59.4	94.4	56.1	53.8	—				
	1-E4	68.1	42.7	44.8	55.0	88.3	89.5	77.5	79.6	64.1	—			
	2H10	64.9	48.1	41.5	50.8	92.4	129.8	88.0	79.6	43.8	76.0	—		
	3B7	64.1	39.4	30.7	33.9	51.6	74.2	70.4	70.9	60.0	67.7	73.3	—	
	7B4	88.6	71.9	55.6	63.8	75.0	116.9	87.0	111.3	96.7	124.0	99.1	80.0	—

Non additivity Additivity

[a]To determine the complementary of the anti-CCH MAbs, we used the ELISA method developed by Friguet et al.,[52] who propose an additivity index, as described in the Materials and Methods section. Experiments were run in triplicate with an experimental error below 10%. The pairs of monoclonals that show a nonadditivity value are highlighted in grey, and the pairs that show an additivity value were distinguished in white.

assigned to 1B8 MAb because it was complementary with all MAbs from the panel. The second and third epitopes corresponded to the 4D8 and 8E5 MAbs. Although MAbs 4D8 and 8E5 were not complementary, they differed in their binding to heated CCH: antibody 4D8 maintained about 12% of its binding while antibody 8E5 showed 60%; the additivity index below 50 may only reflect a close proximity between both epitopes.

In group III, five epitopes were assigned: all antibodies were complementary except for MAbs 1A4 and 2H10, which may be explained by steric hindrance. Nevertheless, MAb 1A4 did not bind to CCH in solution (as shown in K_d determination experiments) so the nature of this epitope may be different from the one recognized by 2H10. The third epitope was defined by 3B7 antibody, which recognized both subunits by Western blot and improved its reactivity to heated CCH. The fourth epitope was assigned to 1E4 antibody, because it was negative by Western blot and had high sensitivity to elastase. Finally, 7B4 recognized a fifth epitope, it was complementary with all the antibodies from the panel.

It is worth noting that the presence of common epitopes on both subunits of CCH, as demonstrated by monoclonals from group I, suggests that subunits might have originated from a common mono-FU protein precursor, whose genes had undergone a series of duplication and fusion events.[1,2] This conception has been supported by recent cDNA sequence analysis of hemocyanin from the gastropod *Haliotis tuberculata*, where the subunit homology is about 65%.[57,58] Furthermore, these mechanisms, in addition to mutation events, would explain the generation of different isoforms for hemocyanin subunits, evidenced by the presence of specific epitopes, as demonstrated by monoclonals of groups II and III. In this respect, KLH subunits, KLH-1 and KLH-2, are a well-documented example of subunit divergence.[44]

Considering that oligosacharides can provide valuable targets for modification or conjugation reactions to proteins, away from critical points in the polypeptide chain,[49] we studied whether carbohydrate moieties were involved in the binding of anti-CCH MAbs. Periodate oxidation of CCH carbohydrate residues did not alter the binding of all MAbs to the protein. However, its participation in the epitopic structure cannot be fully discarded, unless specific enzymatic deglycosylation of CCH be performed, to determine the nature of the binding of carbohydrates to the CCH (*N*-linked or *O*-linked), and to reevaluate its effects on the binding of the MAbs. Nevertheless, our results agree with the nature of the subclass of the anti-CCH MAbs obtained; we did not find antibodies of the IgG_3 subclass, that are involved in carbohydrate response in mice.[54]

We also analyzed the effect of different enzymes to investigate the features of CCH epitopes. Elastase has been widely used in structural analysis of hemocyanins, for example, in KLH it cleaves the subunits into a series of single domains, without further damage; in contrast trypsin cleaves the molecule into fragments containing more than one domain.[42] Enzymatic treatments followed by immunochemical analysis of the fragments with polyclonal antibodies and MAbs[57] have been essential in identifying the number and organization of FUs in mollusk hemocyanins, data not available for CCH. Studies are in progress to identify the precise localization of epitopes recognized by the MAbs presented here within the subunits, for example, 4D8 MAb does not react by Western blot with CCH digested during 5 h with elastase, which means that the epitope recognized by this antibody is localized around the linker region of FUs. In contrast, 7B4 MAb treated under the same con-

ditions showed an intense reaction against the fragments, suggesting that the epitope was present on some FU (data not shown).

Finally, the anti-CCH MAbs presented here may be valuable tools in basic and applied studies of the protein, for instance, to explore the subunit organization of the molecule. The current data are not conclusive on whether the CCH didecamers are made of homogeneous or heterogeneous decamers. Also anti-CCH MAbs will be useful in certifying the quality of the protein under different treatments, especially those related to the chemical modification involved in vaccine development. Finally, these MAbs will be important for understanding the relationship between the structural and the immunostimulant properties of CCH. In this respect, Orlova et al.,[59] propose that immunogenicity of gastropod hemocyanins may be associated with the D5 point-group symmetry, analogous to some viral structures. This three-dimensional arrangement of repetitive epitopes, together with their big size and xenogenic amino acid sequence, can lead to an efficient T- and B-lymphocyte stimulation.

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REFERENCES

1. Van Holde KE, and Miller KI: Hemocyanins. Adv Protein Chem 1995;47:1–81.
2. Van Holde KE, Miller KI, and Decker H: Hemocyanins and invertebrate evolution. J Biol Chem 2001;276:15563–15566.
3. Harris JR, and Markl J: Keyhole limpet hemocyanin (KLH): a biomedical review. Micron 1999;30:597–623.
4. Markl J, Lieb B, Gebauer W, Altenhein B, Meissner U, and Harris JR: Marine tumor vaccine carriers: structure of the molluscan hemocyanins KLH and HtH. J. Cancer Res Clin Oncol 2001; 127:3–9.
5. Olsson CA, Chute R, and Rao CN: Immunologic reduction of bladder cancer recurrence rate. J Urol 1974;111:173–176.
6. Jurincic CD, Engelman U, Gash J, and Klippel KF: Immunotherapy in bladder cancer with keyhole-limpet hemocyanin: A randomized study. J Urol 1988;139:723–726.
7. Lamm DL, DeHaven JI, Riggs DL, and Ebert RF: Immunotherapy of murine bladder cancer with keyhole limpet hemocyanin (KLH). J Urol 1993;149:648–652.
8. Jurincic CD, Gallati H, Alvarez-Mon M, Sippel J, Carbarillo J, and Klippel KF: Urinary interleukin-1 alpha levels are increased by intravesical instillation with keyhole limpet hemocyanin in patients with superficial transitional cell carcinoma of the bladder. Eur Urol 1995;28:334–339.
9. Jurincic CD, Metz KA, Beuth J, Sippel J, and Klippel KP: Effect of keyhole limpet hemocyanin (KLH) and bacillus Calmete-Guérin (BCG) instillation on carcinoma in situ of the urinary bladder. Anticancer Res 1995;15:2771–2776.
10. Jurincic CD, Von der Kammer H, Beuth J, Scheit KH, and Klippel KP: Antibody response to keyhole limpet hemocyanin (KLH) treatment in patients with superficial bladder carcinoma. Anticancer Res 1996;16:2105–2110.
11. Helling F, Shang A, Calves M, Zhang S, Ren S, Yu RK, Oettgen HF, and Livingston PO: G_{D3} vaccines for melanoma: superior immunogenicity of keyhole limpet hemocyanin conjugated vaccines. Cancer Res 1994;54:197–203.
12. Livingston PO, Zhang S, Adluri S, Yao TJ, Graeber L, Ragupathi G, Helling F, and Fleisher M: Tumor cell reactivity mediated by IgM antibodies in sera from melanoma patients vaccinated with GM2 ganglioside covalently linked to KLH is increased by IgG Antibodies. Cancer Immunol Immunother 1997;43:324–330.
13. Sandmaier BM, Oparin DV, Holmberg LA, Reddish MA, MacLean GD, and Longenecker BM: Evidence of a cellular immune response against sialyl-Tn in breast and ovarian cancer patients after high-dose chemotherapy, stem cells rescue, and immunization with Theratope STn-KLH cancer vaccine. J Immunother 1999;22:54–66.
14. Bowen-Yacyshyn MB, Poppema S, Berg A, MacLean GD, Reddish MA, Meikle A, and Longenecker BM: $CD69^+$ and $HLA\text{-}DR^+$ activation antigens on peripheral blood lymphocyte populations in metastatic breast and ovarian cancer patients: correlation with survival following active specific immunotherapy. Int J Cancer 1995;61:470–474.
15. Adluri S, Gilewski T, Zhang S, Ramnath V, Ragupathi G, and Livingston PO: Specificity analysis of sera from breast cancer patients vaccinated with MUC1-KLH plus QS-21. Br J Cancer 1999;7: 1806–1812.
16. Li .Y, Rabello ALT, Simpson AJG, and Kats N: The serological differentiation of acute and chronic *Schistosoma japonicum* infection by ELISA using keyhole limpet hemocyanin as antigen. Trans R Soc Trop Med Hyg 1994;88:249–251.
17. Ettinger RH, Ettinger WF, and Harless WE: Active immunization with cocaine-protein conjugate attenuates cocaine effects. Pharmacol Biochem Behav 1997;58:215–220.
18. Beike J, Köhler H, and Blaschke G: Antibody-mediated clean-up of blood for simultaneous HPLC determination of morphine and morphine glucuronides. Int J Legal Med 1997;110:226–229.
19. Naylor PH, Sztein MB, Wada S, Maurer S, Holterman D, Kirkley JE, Naylor CW, Zook BC, Hitzelberg RA, Gibbs CJ, Zagury D, Achour A, O'Toole C, Gazzard B, Youle M, Rios A, Sarin PS, and Goldstein AL: Preclinical and clinical studies on immunogenicity and safety of the HIV-1 p17-based synthetic peptide AIDS vaccine -HPG-30- KLH. Int J Immunopharmacol 1991;13:117–127.
20. Meyer D, Anderson DE, Gardner MB, and Torres JV: Hypervariable epitope constructs representing variability in envelope glycoprotein of SIV induce a broad humoral immune response in rabbits and Rhesus macaques. AIDS Res Human Retroviruses 1998;14:751–760.
21. Campo MS, O'Neil BW, Grindlay GJ, Curtis F, Knowles G, and Chandrachud L: A peptide encoding a B-cell epitope from the N-terminus of the capsid protein L2 of bovine papillomavirus-4 prevents disease. Virology 1997;234:261–266.
22. Tan IP, Roy C, Sáez JC, Sáez CG, Paul DL, and Risley MS: Regulated assembly of connexin33 and connexin 43 into rat Sertoli cell gap junctions. Biol Reprod 1996;54:1300–1310.
23. Torres M, Monosalva H, Carrasco I, De Ioannes A, and Becker MI: Procedure for radiolabeling Gizzerosine and basis for a radioimmunoassay. J Agric Food Chem 1999;47:4231–4236.
24. Arredondo M, Muñoz P, Mura C, and Núñez MT: HFE inhibits apical iron uptake by intestinal epithelial (Caco-2) cells. FASEB J 2002;15:1276–1278.
25. Mura CV, Becker MI, Orellana A, and Wolff D: Immunopurification of Golgi vesicles by magnetic sorting. J Immunol Methods 2002;260:263–271.
26. Becker MI, Carrasco I, Beltrán C, Torres M, Jaureguiberry B, and

De Ioannes AE: Development of monoclonal antibodies to Gizzerosine, a toxic component present in fish meal. Hybridoma 1998;17:373–381.
27. Córdova JL, Jamett A, Aguayo J, Fauré MT, Villaroel O, and Cárdenas L: An *in vitro* assay to detect paralytic shellfish poison. J Shell Aquatic Toxicol 2001;20:55–61.
28. Becker MI, De Ioannes P, Moltedo B, Oliva H, Pacheco R, and De Ioannes AE: Physico and immunochemical study of a new immunogenic protein of the hemocyanin family from the mollusk *Concholepas concholepas* (CCH). Scan J Immunol 2001;54(Suppl.1):107.
29. Köhler G, and Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) 1975;256:495-497.
30. Becker MI, and De Ioannes AE: Anticuerpos Monoclonales. In: *Fundamentos de Inmunología.* Palomo I, Ferreira, A, Sepulveda C, Rosenblatt S., and Vergara U (Eds.). Universidad de Talca, Chile, 1998, pp. 607–630.
31. Benjamin C, Berzofsky JA, East IJ, Gurd FRN, Hannum C, Leach SJ, Margoliash E, Michael JG, Miller A, Prager EM, Reichlin M, Sercarz EE, Smith-Gill SJ, Todd PE, and Wilson A: The antigenic structure of proteins: A reappraisal. Annu Rev Immunol 1984;2: 67–101.
32. Amit AG, Mariuzza RA, Phillips SEV, and Poljak RJ: Three-dimensional structure of an antigen–antibody complex at 2.8 A° resolution. Science 1986;233:747–753.
33. Colman PM, Laver WG, Varghese JN, Baker AT, Tulloch PA, Air GM, and Webster RG: Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. Nature 1987;326: 358–363.
34. Davies DR, and Padlan EA: Antibody–antigen complexes. Annu Rev Biochem 1990;59:439-473.
35. Tulip WR, Varghese JN, Laver WG, Webster RG, and Colman PM: Refined crystal structure of the influenza virus N9 neuraminidase-NC41 Fab complex. J Mol Biol 1992;227:122–148.
36. Braden BC, Souchon H, Eiselé JL, Bentley GA, Bhat TN, Navaza J, and Poljak RJ: Three-dimensional structures of the free and the antigen-complexed Fab from monoclonal anti-lysozyme antibody D44.1. J Molec Biol 1994;243:767–781.
37. Lescar J, Pellegrini M, Souchon H, Tello D, Poljak RJ, Peterson N, Greene N, and Alzari PM: Crystal structure of a cross-reaction complex between Fab F9.13.7 and Guinea.fowl lyzozyme. J Biol Chem 1995; 270:18067–18076.
38. Saul FA, and Alzari PM: Crystallographic Studies of antigen-antibody interactions. Methods Molec Biol 1996;66:11–23.
39. Lamy J, Leclerc M, Sizaret PY, Lamy J, Miller KI, McParland R, and Van Holde KE: Octopus doflenini hemocyanin: structure of the seven-domain polypeptide chain. Biochemistry 1987;26: 3509–3518.
40. Lamy J, You V, Taveau JC, Boisset N, and Lamy JN: Intramolecular localization of the functional units of *Sepia officinalis* hemocyanin by immunoelectron microscopy. J Mol Biol 1998;284: 1051–1074.
41. Gebauer W, Harris JR, Heid H, Süling M, Hillenbrand R, Söhngen S, Wegener-Strake A, and Markl J: Quaternary structure, subunits and domain patterns of two discrete forms of keyhole limpet hemocyanin: KLH1 and KLH2. Zoology 1994;98:51-68.
42. Gebauer W, Harris JR, Geisthardt G, and Markl J: Keyhole limpet hemocyanin type 2 (KLH2): detection and immunolocalization of a labile functional unit. J Structural Biol 1999;128;280–286.
43. Gebauer W, Stoeva S, Voelter W, Dainese E, Salvato B, Beltramini M, and Markl J: Hemocyanin subunit organization of the gastropod *Rapana thomasiana.* Archiv Biochem Biophys 1999;372:128–134.
44. Swerdlow RD, Ebert RF, Lee P, Bonaventura C, and Miller KI: Keyhole limpet hemocyanin: structural and functional characterization of two different subunits and multimers. Comp Biochem Physiol 1996;113B:537-548.
45. Söhngen SM, Stahlman A, Harris JB, Müller SA, Engel A, and Markl J: Mass determination, subunit organization and control of oligomerization states of keyhole limpet hemocyanin (KLH). Eur J Biochem 1997;248:602-614.
46. Keller H, Lieb B, Altenhein B, Gebauer D, Richter S, Striker S, and Markl J: Abalone (*Haliotis tuberculata*) hemocyanin type 1 (HtH1). Organization of the ≈400kDa subunit, and amino acid sequence of its functional units f, g, and h. Eur J Biochem 1999;264: 27–38.
47. Markl J, and Winter S: Subunit-sepecific monoclonal antibodies to tarantula hemocyanin. J Comp Physiol B 1989;159;139–151.
48. Lieb B, Altenhein B, Lehnert R, Gebauer W, and Markl J: Subunit organization of the abalone *Haliotis tuberculata* hemocyanin type 2 (HtH2), and the cDNA sequence encoding its functional units d, e, f, g, and h. Eur J Biochem 1999;265:134–144.
49. Hermanson GT: Introduction of aldehyde residues. In: Bioconjugate Techniques. Academic Press, Inc, New York, 1996, pp. 114–118.
50. Becker MI, Aguayo J, Jamett A, Juica F, Yudelevich A, Foradori A, and De Ioannes AE: An alternative ELISA for T4 determination based on idiotype anti-idiotype interaction and a latex method for anti-idiotype monoclonal antibody selection. J Immunol Methods 1995;192:73-85.
51. Crowther J, and Abu-Elzeein E: Detection of antibodies against foot -and mouth- disease virus using purified *Staphylococcus-A* protein with alkaline phosphatase. J Immunol Methods 1980;34: 261–267.
52. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, and Goldberg M: Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Methods 1985;77:305–319.
53. Friguet B, Djavadi-Ohaniance L, Pages J, Bussard A, and Goldberg M: A convenient enzyme-linked immunosorbent assay for testing whether monoclonal antibodies recognize the same antigenic site. Application to hybridoma specific for the β_2–subunit of *Escherichia coli* tryptophan synthase. J Immunol Methods 1983;60: 351–358.
54. Campbell AM: Monoclonal antibody and immunosensor technology. Lab Techniques Biochem Mol Biol 1991;23:310–323.
55. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
56. Towbin H, Stahelin T, and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. PNAS 1979;76:4350-4354.
57. Lieb B, Altenhein B, and Markl J: The sequence of a gastropod hemocyanin (HtH1) from *Haliotis tuberculata.* J Biol Chem 2000;275:5675–5681.
58. Lieb B, Altenhein B, Markl J, Vincent A, Van Olden E, Van Holde KE, and Miller KI: Structures of two molluscan hemocyanin genes: significance for gene evolution. PNAS 2001;98:4546–4551.
59. Orlova EV, Dube P, Harris JR, Beckman E, Zemlin F, Markl J, and Van Heel M: Structure of keyhole limpet hemocyanin type 1 (KLH1) at 15A° resolution by electron cryomicroscopy and angular reconstitution. J Mol Biol 1997;271:417-437.

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