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Research paper

Antitumor activity and carrier properties of novel hemocyanins coupled to a mimotope of GD2 ganglioside

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ABSTRACT

Conjugation to carrier proteins is a way to improve the immunogenicity of peptides. Such is the case for peptides mimicking carbohydrate tumor-associated antigens in cancer vaccine development. The most used protein for this purpose is the keyhole limpet hemocyanin (KLH) from *Megathura crenulata*. Its limited bioavailability has prompted interest in finding new candidates; nevertheless, it is not known whether other hemocyanins might be equally efficient as carrier of carbohydrate peptide mimotopes to promotes anti-tumor responses. Here, we evaluated the carrier and antitumor activity of novel hemocyanins with documented immunogenicity obtained from *Concholepas concholepas* (CCH) and *Fissurella latimarginata* (FLH), coupled through sulfo-SMCC to P10, a mimetic peptide of GD2, the major ganglioside constituent of neuroectodermal tumors, and incorporating AddaVax as an adjuvant. The humoral immune responses of mice showed that CCH-P10 and FLH-P10 conjugates elicited specific IgM and IgG antibodies against P10 mimotope, similar to those obtained with KLH-P10, which was used as a positive control. The CCH-P10 and FLH-P10 antisera, exhibited cross-reactivity with murine and human melanoma cells, like anti-CCH and anti-FLH sera suggesting a cross-reaction of CCH and FLH glycosylations with carbohydrate epitopes on the tumor cell surfaces, similar to the KLH antisera. When mice were primed with each hemocyanin-P10 and challenged with melanoma cells, better antitumor effects were observed for FLH-P10 than for CCH-P10 and, as for KLH-P10, irrespective of conjugation. These data demonstrate that CCH and FLH are useful carriers of carbohydrate mimotopes; however, the best antitumor activity of FLH preparations, indicate that is a suitable candidate for further cancer vaccines research.

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1. Introduction

In recent decades, there has been increased interest in developing immunotherapeutic strategies against cancer, which would be a less invasive and equally effective alternative to surgical and chemotherapeutic treatments [1,2]. In this respect, therapeutic vaccines against tumor-associated antigens (TAAs) have been developed to induce a specific immune response against tumors, because their over-expression or aberrant expression on the cell surface of tumor cells, prevents damage in normal tissues [3,4]. One of the widely recognized TAAs is GD2 (molecular formula $C_{74}H_{134}N_4O_{32}$), the major ganglioside constituent of neuroectodermal tumors, whose aberrant expression in human neuroblastomas, soft tissue osteosarcomas and melanomas makes it an ideal target for immune attack by antibodies to induce antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity [5–8]. Certainly, monoclonal antibodies anti-GD2 have an inhibitory effect at the level of formation of tumor metastasis in murine models and in humans. Indeed, the US FDA recently has approved the use of Dinutuximab, a chimeric anti-GD2 therapeutic antibody, which is used in the treatment of high-risk

Abbreviations: CCH, *Concholepas concholepas* hemocyanin; DTH, Delayed-type hypersensitivity; FLH, *Fissurella latimarginata* hemocyanin; KLH, Keyhole limpet hemocyanin; PBS, Phosphate-buffered saline; TAA, Tumor-associated antigen.

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neuroblastoma [9]. Because of the glycolipidic nature of GD2, which is normally embedded in the outer surface of the plasma membrane, with its ceramide tail and sugar moiety exposed to the extracellular milieu [10], active immunization with this molecule has presented low immunogenicity, with subsequent induction of poor immunologic memory, a short half-life and low IgM antibody titers [11,12]. More importantly, GD2 vaccines generate a poor response of specific $CD8^+$ T lymphocytes to kill tumor cells, in part because they are T-cell-independent self-antigens, generating central and peripheral immune tolerance against them, even after initial production of high numbers of $CD8^+$ [13]. Therefore, diverse strategies have been designed to stimulate an effective immune response against the TAAs [14,15]. The coupling of these TAAs to highly immunogenic carrier molecules, such as mollusk hemocyanin, a classic T cell-dependent protein antigen, has been well documented [16,17]. In fact, a bivalent vaccine comprising the ganglioside GM2, a ganglioside that is not expressed by all melanomas, and GD2 both coupled to keyhole limpet hemocyanin (KLH), obtained from the marine gastropod *Megathura crenulata*, exposed the poor immunogenicity of GD2 [18]. To increase GD2 immunogenicity and to involve T lymphocytes in the immune response stimulating the production of IgG isotype antibodies, the GD2 epitope was introduced into a molecule with a proteic nature. This GD2 epitope is called a mimetic peptide because it is capable of inducing specific antibodies against this ganglioside [19–22]. Currently, several peptides have been shown to induce antibodies that bind to pure GD2 and to GD2 on tumor cells; these peptides have been isolated with specific anti-GD2 antibodies, such as mouse 14G2a antibody [20], 14G2a [23] and ME361 [18]. The ME361 antibody was used to isolate the P10 peptide, a mimotope of GD2 that was coupled to KLH to promote a specific anti-GD2 immune response. The KLH-P10 conjugate inhibited the tumor growth rate in a mouse melanoma model, in contrast to KLH-S266, which was used as a control [18]. In summary, the advantages of using peptide mimetics as xenoantigens of TAAs is related to their chemical design [24], easier mass production, increased stability and safety, prevention of undesired epitope formation and their less complex coupling to carrier molecules [8,20,25].

Increasing interest in KLH and its limited bioavailability, as its supply depends on natural resources, has prompted interest in finding new carrier candidates with better biochemical and immunological properties [26]. The KLH genes have been cloned and sequenced; however, until now, it has not been possible to express a heterologous KLH. Therefore, new gastropod hemocyanins from *Concholepas concholepas* (CCH) [27] and *Fissurella latimarginata* (FLH) [28], including *Haliotis tuberculate* [29], *Helix pomatia* [30] and *Rapana venosa* [31], have been biochemically characterized and immunologically evaluated according to their immunomodulatory properties [32].

Hemocyanins are enormous oligomers whit a basic structure of a decamer composed of 10 subunits, ranging from 350 to 550 KDa, that are self-assembled into a cylinder approximately 35 nm in diameter and 18 nm in height [33]. In the hemocyanins of gastropods, such as CCH, FLH and KLH used herein, the decamers are ensembled in pairs forming mostly didecamers. These hemocyanins present clear structural differences at the quaternary level: KLH preparation is made up of two independent isoforms that coexist in the hemolymph of animals, each composed of one type of subunit, without shared epitopes [26]. In contrast, although CCH also has two subunits (CCHA and CCHB) with shared epitopes, they are intermingled in the molecule, forming heterodidecamers, and unlike FLH and KLH, it does not require divalent cations to stabilize its structure [27,34]. FLH, is composed of a single type of subunit that forms homodidecamers [28]. Currently, no information is available regarding the amino acid sequence of the CCH and FLH subunits. Concerning their immunological properties, FLH is highly immunogenic and has been showed to be a better antitumor agent in a melanoma model than CCH or KLH [28]. Currently, CCH is used as an adjuvant in a vaccine based on DCs loaded with prostate tumor cell lysates, which has been shown to be safe and effective to induce the T cell memory response in prostate cancer patients [35,36].

Regarding the mechanisms of action of these large glycoproteins, CCH, FLH and KLH are internalized by antigen-presenting cells through the participation of C-type lectin receptors such as mannose receptors [37,38]. Moreover, hemocyanins are processed slowly, resulting in prolonged antigen presentation to either T or B cells and in the modulation of the immune response to the Th1 profile, which is characterized by the production of $CD4^+$ T lymphocytes and IFN-γ secretion [39]. We have demonstrated that macrophages undergo activation in response to these structurally diverse hemocyanins. In fact, macrophages display a different temporal pattern of proinflammatory cytokine gene expression, along with protein secretion, which leads to an M1-polarized proinflammatory milieu [38].

In the present study, due to the promising immunomodulatory capacities demonstrated by CCH and particularly by FLH, we investigated the use of these hemocyanins as P10 peptide carriers. It is not known whether hemocyanins from other species might be equally or more efficient than KLH as carriers of carbohydrate mimotopes. We incorporated AddaVax, an MF59-like nanoemulsion as a standard adjuvant [40], to compare the humoral immune responses elicited by CCH- and FLH-P0 peptide conjugates and their antitumor effects in the B16F10 murine melanoma model. The principal results showed that CCH and FLH are safe and useful carriers of P10 mimotope of GD2 ganglioside, inducing specific IgM and IgG antibodies against this peptide without toxic effects in the mice, similar to KLH-P10, which was used as positive control. However, like KLH peptide conjugates, FLH-peptide conjugates showed better antitumor activity than CCH-conjugates, as evidenced by a significantly lower tumor growth rate and tumor incidence in C57Bl/6 mice challenged with murine B16F10 melanoma cells.

2. Results

2.1. CCH and FLH exhibit differences during the process of coupling to the P10 peptide mimotope of the GD2 ganglioside

To assess immunogenicity of CCH- and FLH-P10 peptide conjugates, prior to inoculation of experimental animals, the P10 mimotope of GD2 ganglioside and the peptide S266 used as a control were conjugated to each hemocyanin and subsequently analyzed to confirm peptide binding. The cross-linking efficiency was determined by Ellman's test because it allows the measurement of cysteine residues that bind to the maleimide group in sulfo-SMCC. Thus, we determined the difference in the amount of cysteine that binds to non-activated hemocyanin and hemocyanin treated with the crosslinker. The results showed that 45% of the initially added sulfo-SMCC was bound to CCH, while 85% was bound to FLH (Fig. 1A). Accordingly, these values indicate whether the P10 mimotope or S266 peptide was coupled, because these peptides may bind proportionally to CCH and FLH by the bond between the terminal cysteine residue on each peptide and maleimide groups on activated hemocyanins. Fig. 2B shows the statistically significant differences between CCH and FLH nonactivated and activated with sulfo-SMCC. KLH, which was used as a positive control, presented a different result. Untreated KLH showed high-binding of cysteine, similar to that of maleimide-activated KLH (Fig. 2B), and this phenomenon was attributed to the unknown formulation of KLH.

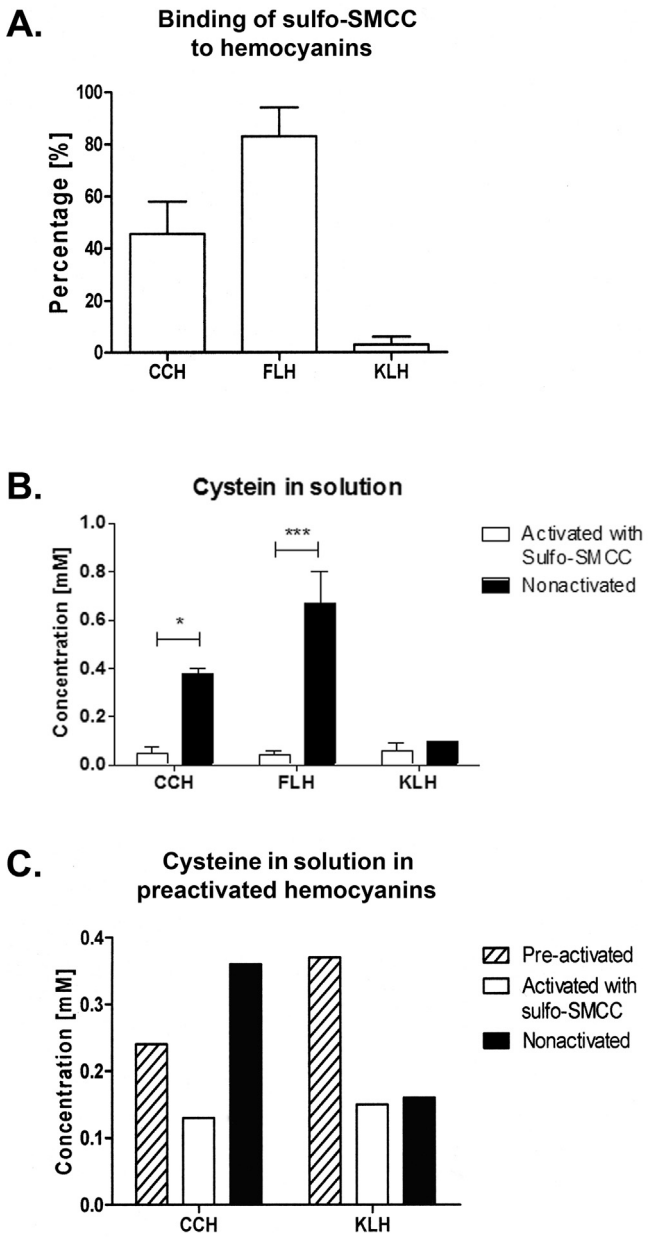


Fig. 1. Activation of hemocyanins with maleimide groups on sulfo-SMCC. The cysteine in solution was measured using Ellman's reagent, which react with the thiol group of cysteine, using spectrophotometry (lectures at 405 nm). Differences in cysteine concentrations in solution between activated and non-activated hemocyanins correspond to the maleimide group bonded to cysteine. The data correspond to two independent experiments. A) Binding of sulfo-SMCC to hemocyanins. Graphical representation of the percentage of sulfo-SMCCs bound to CCH, FLH and KLH relative to the amount that was initially added. B) Concentration of cysteine in solution. Graphical presentation of the cysteine concentration (mM) in activated (white bars) and non-activated (black bars) CCH, FLH and KLH samples. $^{*}P < 0.05$; $^{***}p \leq 0.001$. C) Comparison between cysteine in solution in commercial pre-activated CCH and KLH and CCH and KLH activated with sulfo-SMCC. Graphical presentation of the results of the evaluation of cysteine concentration in both, commercial pre-activated KLH (Imject Maleimide Activated mcKLH) and commercial CCH (Imject Maleimide Activated Blue Carrier Protein) (grated bars), with CCH and KLH activated with sulfo-SMCC by our laboratory (white bars) and nonactivated hemocyanins (black bars).

Furthermore, comparable results were obtained when we measured cysteine binding to commercial maleimide-preactivated KLH, in contrast to commercial maleimide-preactivated CCH (Fig. 1C).

Overall, our data showed that CCH and FLH have different grades of pre-activation during the process of peptide coupling, which could be explained in part by their structural differences, as previously described [27,28], which are also reflected in the immune response that they induce in different experimental settings [32,38].

2.2. CCH and FLH conjugates to the P10 peptide mimotope induce specific IgG antibodies in mice

To evaluate the immunogenicity of hemocyanin-peptide conjugates, three groups of mice were immunized two times with either CCH, FLH and KLH (the positive control) coupled to the P10 peptide mimotope, incorporating AddaVax as adjuvant. Additionally, the other three groups of mice were immunized in the same way with each hemocyanin conjugated to S266 peptide as a negative control. To enable a comparison between the different experimental groups of mice, using an indirect ELISA we evaluated the immunogenicity of each carrier protein. Fig. 2 shows comparable antibody titers against each hemocyanin, with approximately 1:3500 dilutions in the sera of mice immunized with CCH-P10, FLH-P10 and KLH-P10, approximately 1:2800 dilutions for CCH-S266 and KLH-S266, and slightly higher dilutions for FLH-S266 of approximately 1:4000 (Fig. 2A), To determine the specific antibodies titers against each peptide in the sera of immunized mice, we modified the indirect ELISA using plates that were pre-activated with maleimide to bind P10 and S266 peptides directly. In this way, the antibodies that effectively bound to the peptide were assessed, without the physicochemical influence of the carrier proteins. As shown in Fig. 2B and C, specific antibodies for the P10 peptide mimotope (1:230 dilutions) and S266 peptide (1:150 dilutions), respectively, were obtained. With regards to the isotypes, as expected, the CCH-P10 and FLH-P10 conjugates induced higher specific IgG antibodies titers in mice than the IgM specific antibodies titers, similar to the KLH-P10 conjugate (Fig. 2D). In the immunization with each hemocyanin-S266 peptide, the relationship of specific IgG antibodies titers with respect to specific IgM titers was slightly lower (Fig. 2E). As shown, the specificity of the reactivity of each antiserum with the respective peptide, was confirmed based on its negligible reactivity with the opposite peptide.

Collectively, these data indicate that CCH and FLH conjugates to P10 peptide induced a specific adaptive humoral immune response for this GD2 ganglioside mimotope, mostly of the IgG isotype, like the KLH conjugated to the P10 peptide, which was used as a positive control. It was not possible to test the reactivity of these sera with pure GD2 ganglioside, which would have been suitable. The ELISA results with GD2 were negative probably because of the demonstrated instability of this molecule [41]. As an alternative, the reactivity of the anti-P10 sera with mouse and human melanoma cell lines was evaluated.

2.3. Anti-hemocyanin peptide sera have antigenic cross-reactions with epitopes on the cell surface of mouse and human melanoma cell lines

The mimesis from P10 has been previously described, indicating that antibodies that were induced for KLH-P10 are also capable of recognizing epitopes on other gangliosides, such as GD1b, GD1a, GD3 and GM2, which are expressed by melanoma cells [20]. In this context, to investigate whether the anti-P10 mimotope antibodies present in the sera of mice immunized with CCH- and FLH-P10 could react with gangliosides expressed on the tumor cell surfaces, we performed an indirect immunofluorescence analysis with B16F10 mouse melanoma cells. As shown in Fig. 3, at low dilutions of sera (1:50), a positive reaction with both anti-CCH-P10 (Fig. 3a)

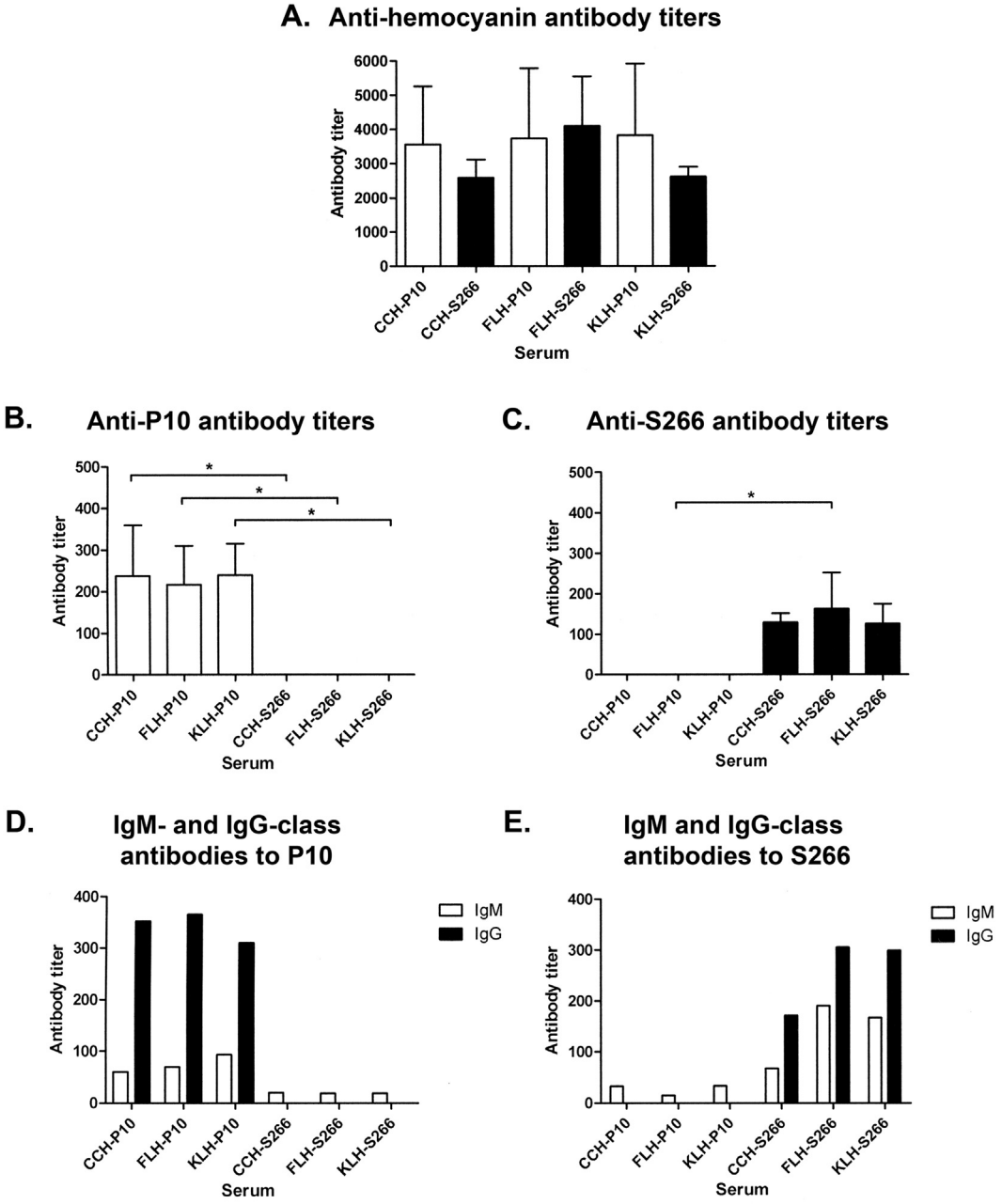
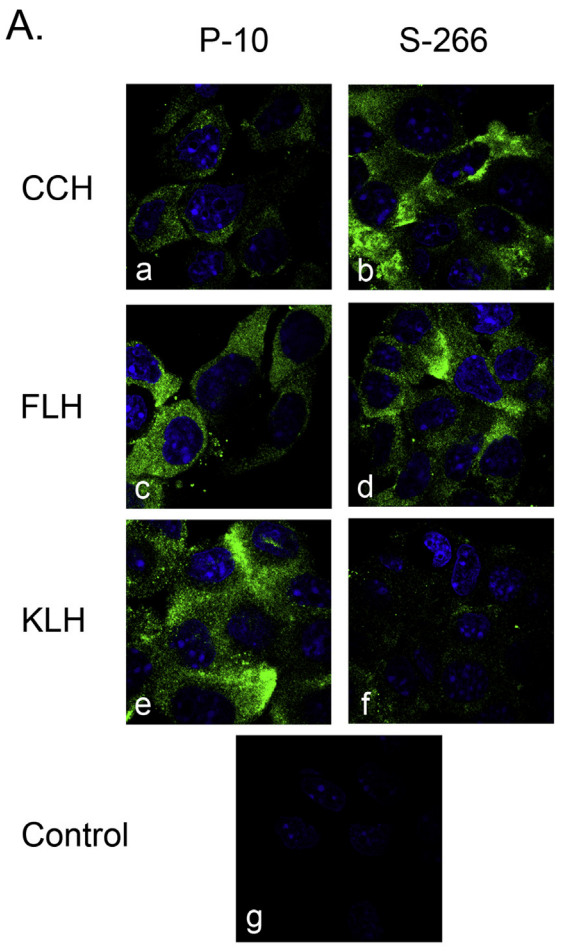


Fig. 2. Humoral immune response of C57BL/6 mice immunized with two doses of hemocyanin-peptide conjugates. The results in A to C are shown as the mean ± SEM of two independent ELISA assays. A) The serum antibody titers of mice immunized with each hemocyanin peptide conjugated (P10 and S266) to each hemocyanin alone (CCH, FLH and KLH). No significant differences were found between them. B) The serum antibody titers of mice immunized with each hemocyanin-P10 against P10 and S266 peptides. Significant differences were found between them * $P < 0.05$. C) The serum antibody titers of mice immunized with each hemocyanin-S266 peptide against P10 and S266 peptides. Significant differences were found between them * $P < 0.05$. D) IgM or IgG antibody class titers of mice immunized with each hemocyanin-P10 against P10 and S266 peptide as control. n = 1. E) IgM or IgG antibody class titers immunized with each hemocyanin-S266 against S266 and P10 peptide as control. n = 1. In D and E, a pool of sera from three mice was used as the primary antibody. Goat antiserum anti-IgG (H + L) from a mouse coupled to ALP was used in A), B) and C), and rabbit antisera anti-IgM (μ) and anti-IgG (γ) of a mouse coupled to ALP were used in D) and E). The absorbance was measured at 405 nm. The titer was calculated as the serum dilution that corresponded to the point of inflection in the ELISA reaction dose-response curve.

and anti-FLH-P10 (Fig. 3c) sera was observed; surprisingly, with anti-CCH-S266 (Fig. 3b) and anti-FLH-S266 sera (Fig. 3d), we also observed a reactivity similar to that found in the respective controls with KLH-P10 (Fig. 3e) and KLH-S266 (Fig. 3f). The immunofluorescent staining was revealed green fluorescence that was variable in intensity on the cell surfaces, similar to the reactivity observed by Riemer et al. [21] with GD2 mimotope antisera tested with the human melanoma cell line M21. Our results were attributed to a cross-reaction between epitopes exposed on the cell surface from B16F10 melanoma cells and hemocyanins, which was confirmed by the high reactivity observed by flow cytometry with sera from animals immunized with CCH, FLH and KLH alone (Fig. 3Bd, 3Be and 3Bf, respectively), compared with sera of Sham, PBS and PBS-AddaVax mice used as controls (Fig. 3Ba, 3Bb and 3Bc, respectively).

Taken together, these results led us to evaluate three human melanoma cell lines derived from metastatic lesions of patients [36]. These human cell lines (designed as RQ, AK and BE) also showed a specific positive immunofluorescence staining with a



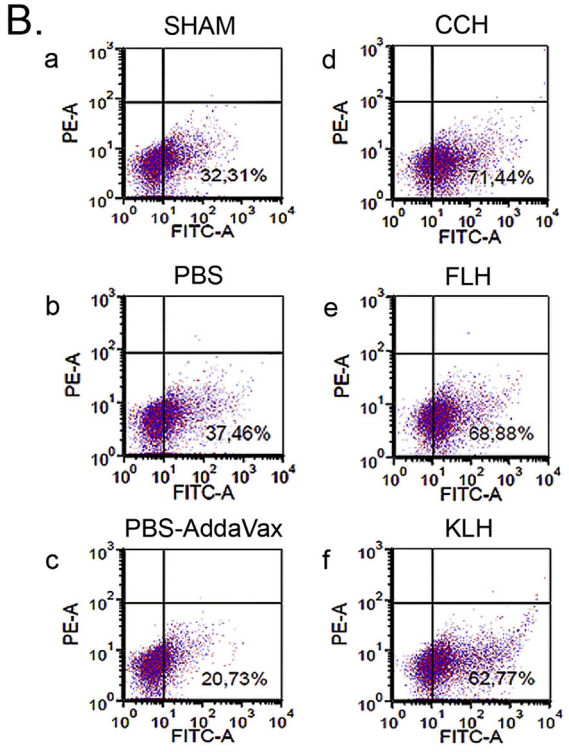


Fig. 3. Analysis of murine B16F10 melanoma cells with sera from mice immunized with hemocyanin-peptide conjugates. A) Indirect immunofluorescence analysis. The cells were fixed with paraformaldehyde and incubated with a pool of sera from three twice-immunized animals at a 1:50 dilution. The results were revealed with fluorescein-conjugated anti-IgG (H + L) mouse antibody. The nuclei were stained with DAPI. The images correspond to immune sera obtained from mice immunized with a) CCH-P10, b) CCH-S266, c) FLH-P10, d) FLH-S266, e) KLH-P10, f) KLH-S266 and g) Control, in which only secondary antibody was used. Magnification 100×. B) Flow cytometry analysis. The cells were incubated with a pool of sera from five animals per group. The pool was diluted 1:25, incubated with serum containing FITC-conjugated goat anti-mouse IgG, and then fixed in paraformaldehyde prior to analysis using the flow cytometer. a) Sham, b) PBS, c) PBS-AddaVax, d) CCH, e) FLH and h) KLH. The images correspond to the dot graphic intensity of the fluorochrome PE (R-phycoerythrin) in comparison to FITC for the cell population. The percentages correspond to the percentage of cells that showed fluorescence intensity for FITC relative to the negative controls. The criterion applied for positive results was a minimum of 60% of immunofluorescent cells over the threshold.

variable intensity depending on the anti-CCH-P10 (Fig. 4Aa, 4Bb and 4Cc, respectively), anti-FLH-P10 (Fig. 4Ag, 4Bh and 4Ci, respectively) and anti-KLH-p10 sera (Fig. 4Am, 4Bn and 4Co, respectively), or the respective anti-CCH-S266, anti-FLH-S266 and anti-KLH-S266 peptide sera, in contrast to the absence of green fluorescence in the control of the secondary antibody (Fig. 4As, 4Bt and 4Cu, respectively).

Collectively, these data indicate that CCH and FLH alone, like KLH, emulsified with AddaVax adjuvant, induce antibodies that cross-react with epitopes on murine and human melanoma cells. In this respect, the antigenic cross-reactivity of KLH with the oligosaccharide structures expressed in tumor cells, as Thomsen Friedenreich antigen, has been previously documented [42,43]. In this context, FLH has a similar carbohydrate composition to KLH; moreover, both CCH and FLH have high-mannose oligosaccharides, as well as fucosylated oligosaccharides, such as KLH [28].

2.4. Prophylactic vaccination with FLH-P10 mimotope induces a better anti-tumor effect in a melanoma model than CCH-P10

The CCH-P10 and FLH-P10 conjugates mixed with AddaVax as adjuvant, were evaluated in a prophylactic setting for their capacity to inhibit cell growth in the B16F10 mouse melanoma model, using the KLH-P10 conjugate as positive a control. The experimental design is shown in Fig. 5. Because the antitumor effects of hemocyanins are mediated by adaptive responses [32], mice were primed with a dose of the hemocyanin-P10 and hemocyanin-S266 conjugates 14 days before challenge with B16F10 melanoma cells. In the same way, we used a group of mice treated with each native hemocyanin alone mixed with AddaVax as positive control because the antitumor activity of the three hemocyanins alone has been previously reported [28,39,44]. As a negative control, we used PBS mixed with AddaVax and AddaVax alone. In addition, we included a Sham control. Tumor growth was periodically monitored by visual inspection and palpation.

As shown in Fig. 5A, the AddaVax control group showed a significantly greater tumor growth rate than both the PBS-AddaVax and Sham control groups at day 22, reaching a volume of 2000 mm^3. Thus, these mice were euthanized for ethical reasons. CCH-P10- and CCH-S266-treated mice showed a larger tumor volume (Fig. 5B) than FLH- and KLH-treated mice (Fig. 5C and D, respectively). A significantly ($P < 0.01$) lower tumor growth rate was found in the FLH, FLH-P10 and FLH-S266 treated mice (Fig. 5C), similar to the KLH, KLH-P10 and KLH-S266 groups (Fig. 5D), than in the PBS-AddaVax control group (Fig. 5A). Regarding the tumor incidence, on day 29 after challenge, CCH-P10- and CCH-S266-treated mice, showed a higher tumor incidence (Fig. 5F) than the FLH and KLH groups of mice that were treated with both, coupled and not coupling peptides to hemocyanins, displaying lower tumor incidences (Fig. 5G and H, respectively) than the control groups of

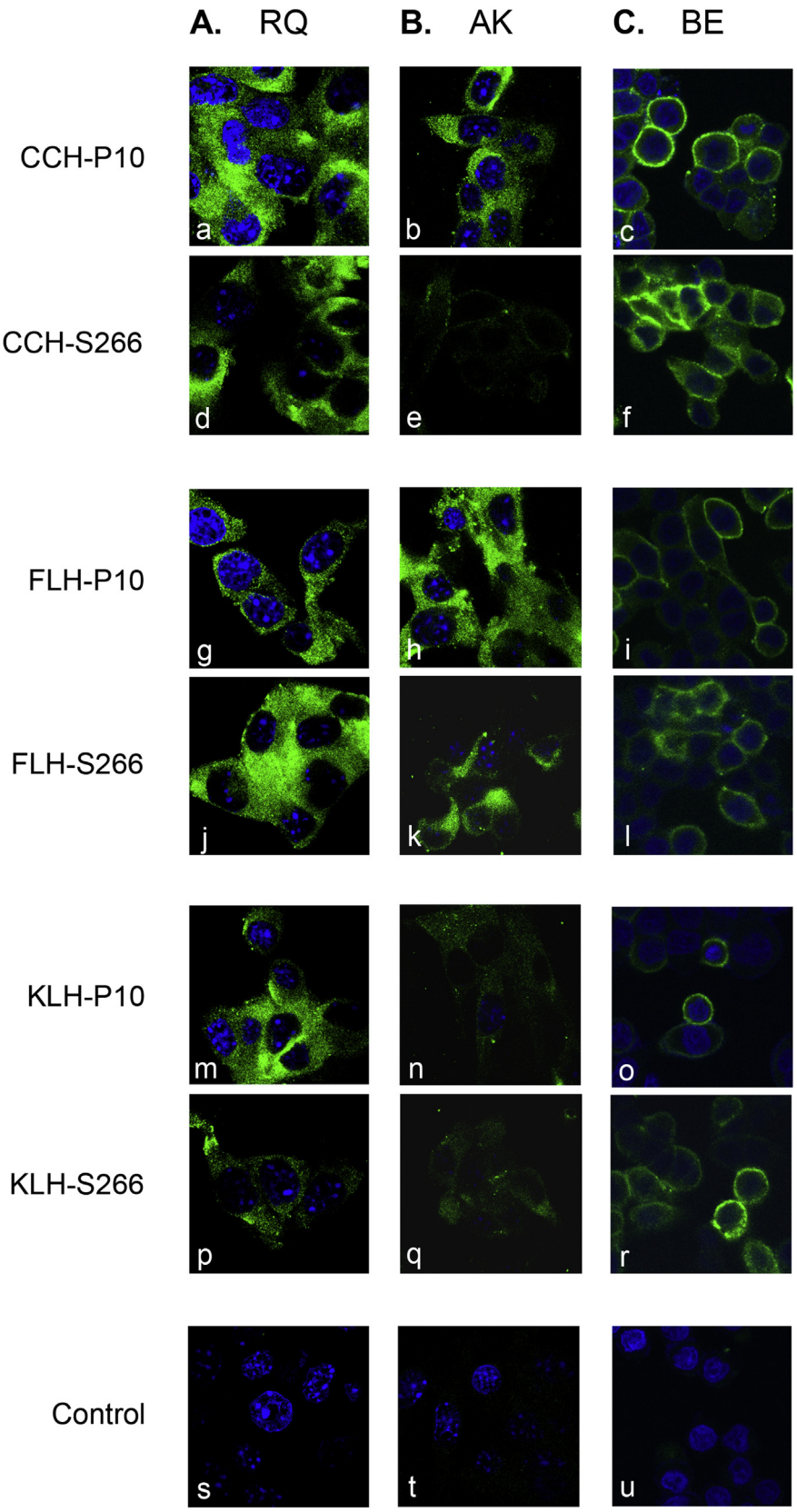


Fig. 4. Indirect immunofluorescence analysis in human melanoma cells with sera from mice immunized with hemocyanin-peptide conjugates. The cells were fixed with paraformaldehyde and incubated with a pool of sera from three twice-immunized animals at a 1:50 dilution. The results were revealed with FITC-conjugated goat anti-mouse IgG. The nuclei were stained with DAPI. The images in the columns correspond to A) RQ human melanoma cells, B) AK human melanoma cells and C) BE human melanoma cells. The images from the rows correspond to immune sera obtained from mice immunized with a, b and c) CCH-P10; d, e and f) CCH-S266; g, h and i) FLH-P10; j, k and l) FLH-S266; m, n and o) KLH-P10; p, q and r) KLH-S266; s, t and u) control, only the secondary antibody was used. Magnification 100×.

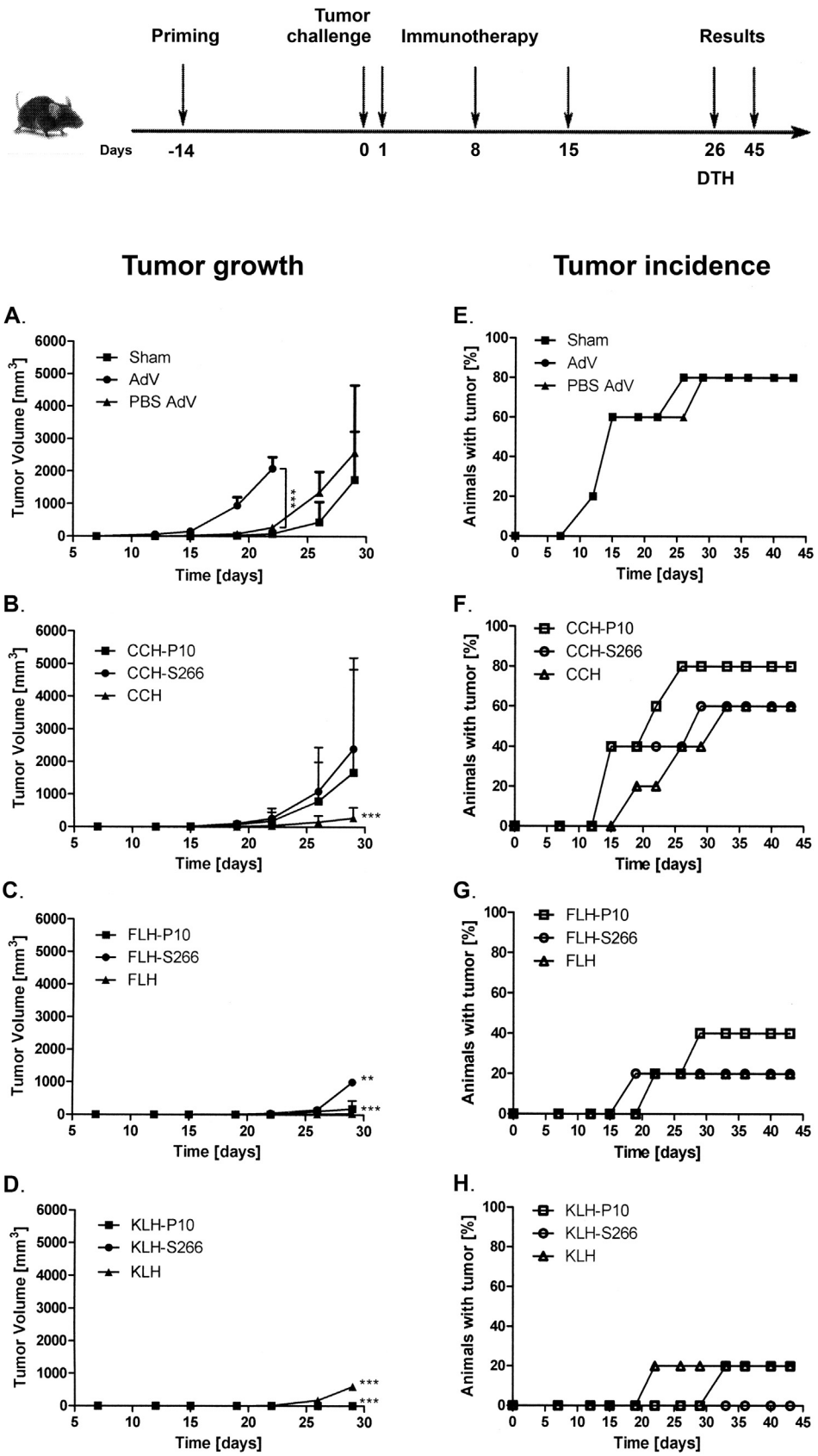


Fig. 5. Antitumor effects of hemocyanin-peptide conjugates in the B16F10 mouse melanoma model. The schedule of the experimental therapeutic design is shown. Five C57BL/6 mice were primed prior to tumor inoculation with each treatment 14 days before challenge and then, received sc 1.5×10^5 B16F10 melanoma cells on the day denoted as 0. The animals were therapeutically treated on days 1, 8 and 15 with 50 μg of hemocyanin alone or hemocyanin conjugated to P10 and S266 peptides, in 80 μL of PBS and AddaVax 1:1 vol by sc injection. Effects of hemocyanin peptide conjugates on tumor growth: A) Controls, Sham, AddaVax and PBS-AddaVax groups of mice. B) CCH, CCH-P10 and CCH-S266, $^{***}P < 0.001$; C) FLH, FLH-P10 and FLH-S2666, $^{**}P < 0.01$ and $^{***}P < 0.001$. D) KLH, KLH-P10 and KLH-S266, $^{***}P < 0.001$. Effects of hemocyanin peptide conjugates on tumor incidence: E) Controls, Sham, AddaVax and PBS-AddaVax groups of mice. F) CCH, CCH-P10 and CCH-S266; G) FLH, FLH-P10 and FLH-S2666. H) KLH, KLH-P10 and KLH-S266.

mice (Fig. 5E). The tumor incidence was approximately 80% for the control groups at day 45, in contrast to 40% or less for FLH-, FLH-P10- and FLH-S266-treated mice (Fig. 5G), similar to the KLH, KLH-P10 and KLH-S266 mice (Fig. 5H). It is important to note that none of the treatments in this study generated toxic or side effects in the mice. The cross-reactivity between anti-sera that were obtained from animals in the bioassay and B16F10 cells was evaluated by indirect immunofluorescence analysis, and the results were similar to those previously described in Fig. 3 (data not shown).

Additionally, to evaluate whether the hemocyanin-peptide conjugates generated a specific cellular immune response against the peptides, we conducted DTH tests against P10 or S266 peptides alone on day 26 in the mice using this bioassay. The results showed that mice immunized with the respective hemocyanin-peptide conjugate, presented swelling kinetics characteristic of the DTH reaction within 24 and 48 h post-injection against S266 peptide (Fig. 6A) and P10 peptide (Fig. 6B), which was not observed in the PBS-AddaVax control group.

Collectively, these data showed that in this experimental setting, the induction of specific humoral and cellular immune responses against the P10 mimotope coupled to CCH, FLH and KLH did not improve significantly the antitumor response in the B16F10 melanoma model, as similar results were achieved with S266 peptide coupled to these hemocyanins, and the three hemocyanin alone. However, the superior antitumor performance of FLH than CCH using AddaVax as adjuvant for the first time, indicates that it may be a suitable alternative or complement to KLH for potential applications in cancer vaccine research.

3. Discussion

The beneficial immune response in a mouse melanoma model using P10, a mimetic peptide of GD2 ganglioside, coupled to mollusk hemocyanin KLH as a xenoantigens in experimental therapeutic vaccines against cancer, has been previously reported [18]. Hemocyanins have the ability to bias the immune response towards a Th1 phenotype [32], activating the immune system to abandon the propose state of equilibrium in which cancer cells resist immune-mediated cell death [45,46]. Furthermore, hemocyanins alone can be used as nonspecific immunomodulators during antitumor therapies. The effectiveness of KLH has been demonstrated in diverse human cancer cell lines, through the inhibition of cellular growth in an apoptotic-dependent or independent manner [47,48]. Additionally, it has been reported that hemocyanins from *Helix aspersa* [49] and *Rapana venosa* [50] have cytotoxic and antiproliferative effects on tumor cell lines, such as bladder cancer, ovarian cancer, prostate cancer and glioma cancer cell lines. In addition, the use of CCH and FLH during therapy for recurrent superficial bladder cancer after transurethral surgical resection has been reported with negligible toxic side effects, making them ideal for long-term ongoing treatments [51]. Despite this, there is no current evidence to demonstrate whether hemocyanins from other species than KLH might be equally or more efficient to prevent tumor growth as a carrier of carbohydrate mimotopes. Thus, in this study we evaluated the effectiveness of two novel hemocyanins, CCH and FLH, as carriers of the P10 mimotope and the proficiency of these hemocyanin-peptide conjugates to exert an antitumor immune response in a mouse melanoma model, since they have documented immunomodulatory properties and structural differences compared with KLH [27,28].

We considered the importance of the conjugation method in a manner that would not interfere with the antigenic epitope of the mimotope, and would achieve a high antigen/carrier ratio [8]. Thus, to prepare the hemocyanin-peptide conjugates, sulfo-SMCC was used to overcome the problems caused by classic agents such as glutaraldehyde. These agents tend to modify carrier molecules and peptides extensively and lead to complications during standardization and validation processes, and the goal is to ensure the reproducibility of the conjugates. Sulfo-SMCC is a heterobifunctional cross-linker that binds specifically to lysine residues on carrier proteins by limiting modified epitopes, preserving the immunogenicity of conjugates and preventing the agglomeration and precipitation of molecules [52].

Additionally, we considered a potent adjuvant to be another necessary component, given its ability to induce a memory immune response and to improve antigenic presentation [40]. The titers against CCH and KLH obtained herein were significantly higher than the titers reported by Arancibia et al. [28], in which mice were immunized with 200 μg, of CCH and KLH alone, in contrast to the present experimental setting in which the mice were immunized with 50 μg of each hemocyanin. The determining factor explaining the improved humoral immune response is the AddaVax adjuvant, which promotes a balance in cellular Th1 and Th2 immune responses, and a robust humoral immune response against the antigen of interest, to induce IgG-class antibody production with improved titers and avidity [53]. Thus, AddaVax enhances the immunogenicity of proteins, and its efficacy has been evaluated in anti-pathogen vaccines such as the flu virus vaccine [54,55], among others. In the experimental setting of Wondimu et al. [18], these authors use KLH-P10 mimotope mixed with QS-21 as an adjuvant with better results, but it is not possible to compare our results since the immunization protocol for the experimental animals is different. In fact, the authors mention the amount of peptide used to immunize mice (15 μg), but they do not mention the amount of hemocyanin used and the percentage of peptide bound to KLH.

Our results strongly suggest that the reactivity of anti-hemocyanin antisera, alone and conjugated to P10 and S266 peptides, was related to a common factor between the carrier proteins, which was even greater than P10 reactivity and could be explained by a cross-reaction between the epitopes on the tumor cell surface and hemocyanins. As previously mentioned, this idea is based on the observation that hemocyanins possess diverse glycosylation patterns, some of which may be shared with glycans on tumor cell surfaces [56]. Indeed, the Thomsen-Friedenreich antigen that is over-expressed in some types of cancer has been described in KLH [42]. FLH has a similar carbohydrate composition in comparison to KLH, including oligosaccharides containing sialic acid [28], a tumor marker that is present in a variety of cancerous conditions [57]. Furthermore, a study by *Rapana thomasiana* and *Helix pomatia* hemocyanins has shown the cross-reactivity of immune sera and C-26 mouse colon carcinoma cells, suggesting the production of polyspecific antibodies that recognize hemocyanin molecules, or the existence of cross-reactive epitopes on the tumor cell surface [56]. Studies of hemocyanin glycosylations in KLH [26,42,43] and hemocyanins of *Helix lucorum* [58,59] and *Rapana venosa* [60,61] have described these sugars as a mixture of complex and heterogeneous glycans, which are mannose-rich structures, with unusual acidic terminal structures and hexuronic acids. These structures can also be found as mono- and bi-antennary glycans, as well as hybrid type structures, and most of them are highly modified by xylose, fucose, and methylation. This complexity and diversity of hemocyanin glycosylations can be related to their immunogenicity and to possible shared epitopes with tumor cells.

In the comparison of the hemocyanins used herein, FLH showed the greatest immunological effects. In the search for other structural reasons that might underlie these differences, apart from different glycosylation patterns, information is lacking. As mentioned, the KLH structure is well described, and its amino acid sequence is available; however, we currently we do not know the sequence of CCH and FLH, thus hampering a structural analysis that

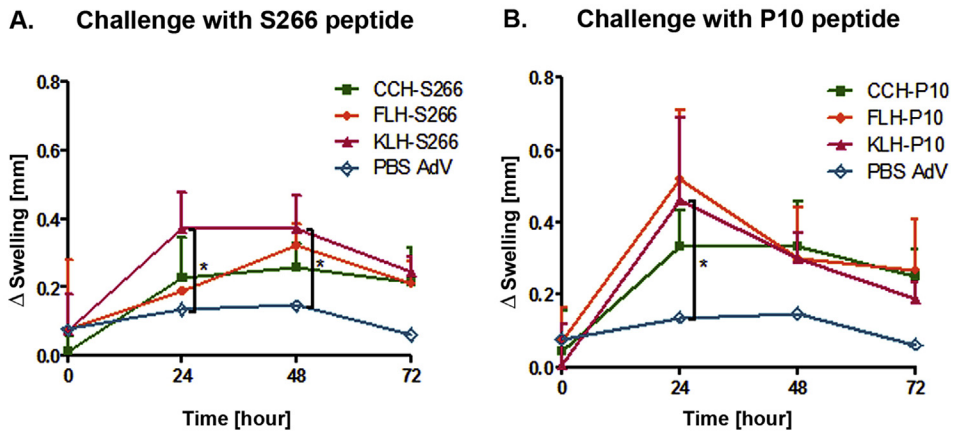


Fig. 6. DTH reaction induced by antigenic peptides P10 and S266 in C57BL/6 mice challenged with B16F10 melanoma cells and therapeutically treated with hemocyanin-peptide conjugates. For the DTH experiment, 75 μg of peptide in 20 μL of PBS was injected sc into the left footpads of mice treated with the respective conjugates (hemocyanin-P10 or S266). The thicknesses of the bearings for the left and right feet were measured at 0, 24, 48 and 72 h after injection. The PBS-AddaVax group was used as a negative control. A) DTH induced by S266 in groups treated with hemocyanin-S266 conjugates. B) DTH induced by P10 in groups treated with hemocyanin-P10 conjugates. The results show a significant increase in swelling ($^*P < 0.05$) in the hemocyanin treated groups in comparison to the PBS-AddaVax mice used as a control.

could explain the differences in immunological effects. However, considering that didecameric hemocyanins have, an approximate D5 symmetry, analogous to some viral structures [62], we hypothesize that since FLH has only one type of subunit, the molecule has more repetitive domains that contain potential immunodominant epitopes that are more representative than in the case of KLH and CCH, which have two different subunits. Therefore, high dosage of the same epitope occurs in FLH (on a molar ratio basis). Consequently, the three-dimensional arrangement of repetitive epitopes arranged in the repetitive subunit of FLH may involve a more efficient antigen presentation by T- and B-lymphocyte stimulation. In this respect, the family of variable surface lipoproteins of *Mycoplasma bovis*, which are highly immunogenic, show the presence of epitopes in tandemly arranged repetitive peptide units [63].

Because we did not detect immunofluorescence reactions for two commercials anti-GD2 monoclonal antibodies (clones 11H3 and 2Q59 from Abcam, UK) in the murine and human melanoma cell lines used herein, we could not confirm the presence of GD2. However, these monoclonal antibodies were induced by purified GD2 from bovine cerebral mass, which can express other conformational epitopes in addition to those resulting from embedding on the plasmatic membrane in melanoma. In this respect, a change in the hydrophobic portion of the GD3 ganglioside can provoke a change in the angle or orientation of the sugar chain [64], and therefore, its antigenic expression on melanoma can differ from its expression in the brain. Additionally, the B16F10 cell line primarily expresses GM3 [65] and GM4 [8,66] gangliosides, which can be equally recognized by antibodies induced by P10 [21]. Additionally, GD2 shares some chemical groups with other gangliosides, all of which are lipids containing a sphingoid base and sialic acid [67].

In melanoma patients who were vaccinated with dendritic cells pulsed with melanoma peptides, a challenge with the antigenic peptide was used to assess the DTH response. An increase in $CD4^+$ and $CD8^+$ T cells occurred, and thus, antigen presentation led to an anti-tumor immune response [68]. In this regard, the DTH reaction to KLH-P10 depends on $CD4^+$ T lymphocytes, and consequently, DTH stimulation by the P10 mimotope coupled to CCH and FLH would imply a similar mechanism involving anti-hemocyanin cross-reactivity with tumor $CD4^+$ T lymphocytes via antigenic presentation in MHCII. In addition, the induction of IgM and IgG isotype antibodies obtained with mimetic peptides, which target shared carbohydrate residues on melanoma cells, are known to be responsible for the effector mechanisms of the humoral immune response against tumor cells, inducing antibody-dependent cellular cytotoxicity and complement dependent cytotoxicity [14,69]. In this respect, we demonstrated that CCH, FLH and KLH elicited the complement activation mediated by C1 binding to human natural antibodies that cross-react with these glycoproteins [70].

Based on the results described herein, it can be inferred that hemocyanins have an important influence on the anti-tumor effects of different therapeutic strategies, not only to stimulate a modulated immune response towards a Th1 type but also to generate a bystander effect, in addition to a strong antibody response that possesses cross-reactivity with glycans expressed on tumor cell sufaces. This result could generate a cytotoxic T cell response (CTL) that is desirable for eradicating micrometastases, as in anti-tumor therapy based on dendritic cells in which hemocyanins have been used as adjuvants [35,71,72]. Considering that systemic therapy is the mainstay treatment for metastatic melanoma in patients with stage IV melanoma [73], we suggest that FLH would be effective for a systemic treatment as an anti-cancer immunomodulator.

In conclusion, our study demonstrated that CCH and FLH are safe and useful carriers of carbohydrate mimotopes. The induction of specific humoral and cellular immune responses against the P10 mimotope of the GD2 ganglioside through its coupling to CCH and FLH did not significantly improve the antitumor immune response in the B16F10 melanoma model than these hemocyanins alone, which was a result also observed for KLH. However, the best performance of immunogens based on FLH relative to CCH in evaluations of their antitumor effects, indicating that this protein is the most promising hemocyanin as an alternative or complementary candidate to KLH, for potential applications in cancer vaccine research.

4. Experimental section

4.1. Hemocyanin sources

The hemocyanins from *Concholepas concholepas* [27] and *Fissurella latimarginata* [28] isolated and purified under sterile and pyrogen-free conditions and suspended in PBS (0.1 M sodium phosphate, 0.15 M NaCl; pH 7.2); were provided by Biosonda

Corporation (Santiago, Chile). Lyophilized KLH in PBS (Imject Maleimide Activated mcKLH Kit) with a proprietary stabilizer was purchased from Thermo Scientific (Waltham, MA, USA). Lyophilized CCH (Maleimide-Activated Blue Carrier Protein Kit from Thermo Scientific) was also used. Lyophilized KLH and CCH were reconstituted with ultrapure deionized water, as recommended by the manufacturer. All the reagents were of analytical grade, and the solutions were prepared with water from Baxter Healthcare Corp. (Charlotte, USA) and filtered through a 0.2 μm membrane filter (Millipore, Billerica, MA, USA).

4.2. Adjuvant

AddaVax, a preclinical-grade adjuvant that promotes a balance in Th1/Th2 immune responses [40], was provided by InvivoGen (San Diego, CA, USA). It consists of a 2% squalene-based oil-in-water sterile nanoemulsion. This adjuvant is based on the formulation of MF59, which has been licensed in Europe as an adjuvant for flu vaccines [54].

4.3. Peptides

P10 peptide (C-GVVWRYTAPVHLGDG), a mimotope of GD2 ganglioside, and S266 peptide (C-IRIQRGPGRAFVTIGKI), a peptide derived from HIV, as described Wondimu et al. [18], were synthesized by GenScript (Piscataway, NY, USA). The terminal cysteine residue was added to each peptide to obtain an effective method of conjugating the peptides to CCH, FLH and KLH.

4.4. Animals

C57BL/6 mice were obtained from ADG (Santiago, Chile) and GrupoBios S.A. (Santiago, Chile). The experimental mice (8–12 weeks old) were maintained according to institutional guidelines for the care of animals. The study was performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals (National Commission for Scientific and Technological Research of Chile). The National Commission for Scientific and Technological Research Committee on Animal Welfare approved all the animal protocols used in this study.

4.5. Cell lines

The B16F10 mouse melanoma cells and the BE, AK and RQ human melanoma cell lines that were derived from metastatic lesions of patients and established at the Millennium Institute on Immunology and Immunotherapy, University of Chile, were grown at 37 °C in a humidified atmosphere with 5% CO_2 in air in RPMI medium (Thermo Scientific) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 mM non-essential amino acids (Gibco, BRL, Rockville, MD, USA), as described previously [28]. Confluent cell cultures were harvested using 0.25% trypsin/EDTA (Thermo Scientific).

4.6. Coupling peptides to hemocyanins

Coupling was performed with a sulfo-SMCC cross-linker (Thermo Scientific), and the protocol was established according to Kafi et al. [52] and Hermanson et al. [74] with modifications. During the activation and desalting reactions of hemocyanins plus sulfo-SMCC, we observed differences in their solubilities in the coupling reaction buffer. CCH was noted for its great solubility in comparison with FLH and KLH, which tended to form precipitates. Therefore, to avoid this problem, EDTA was removed from the conjugation buffer, and the temperature was maintained at 4 °C. CCH, FLH and KLH aliquots (10 mg/mL each) were incubated for 1 h with sulfo-SMCC (Thermo Scientific) at room temperature in the dark. The excess salt was removed with desalting columns (Thermo Scientific) that were previously equilibrated with PBS. Finally, maleimide-activated hemocyanins were incubated with P10 or S266 at a 1:1 ratio by mass for 30 min at room temperature in the dark. The protein concentration was determined using a Coomassie Blue kit (Thermo Scientific). Ellman's test was applied to determine the sulfhydryl group concentration in maleimide-activated hemocyanins [75]. Cysteine residues (Thermo Scientific) in reaction buffer (0.1 M sodium phosphate and 1 mM EDTA, pH 8.0) at the same molarity as the sulfo-SMCC in the reaction above were added to 100-μL maleimide-activated and untreated hemocyanin samples at the same mass concentration as the native hemocyanins. Cysteine and hemocyanins were incubated for 30 min at room temperature in the dark. A standard curve with cysteine between 0 and 1.5 mM was prepared. Aliquots of all the samples (250 μL) were then added to 2.5 mL of reaction buffer and 50 μL of Ellman's reagent solution (4 mg/mL Ellman's reagent in reaction buffer). After the samples reacted for 15 min, their absorbance was measured at 405 nm [76]. The cysteine concentration in the hemocyanin samples was calculated as the quotient between the absorbance and the standard cysteine curve slope. The percentage of SMCC that binds to maleimide-activated proteins was estimated with respect to the difference between the cysteine concentrations of maleimide-activated and native hemocyanin samples.

4.7. Immunization schedule and humoral immune response assessment

Three mice per group were immunized with CCH-P10, FLH-P10, KLH-P10, or their respective control conjugates CCH-S266, FLH-S266 and KLH-S266. On day 1, the animals received an intraperitoneal (ip) injection of 50 μg of conjugate in 100 μL of PBS and AddaVax at a 1:1 (vol/vol); the immunization was repeated on day 15 and fifteen days later, and the animal sera were obtained. The presence of specific antibodies against hemocyanins and peptides in the sera was detected by indirect ELISA, as previously described [77]. In brief, 96-well polystyrene plates (Thermo Scientific) were incubated overnight at 4 °C with 100 μL/well of a 10 μg/mL solution for each hemocyanin in PBS. The plates were blocked with 1% casein-PBS for 3 h at room temperature. Serial 2-fold dilutions of the sera in blocking buffer were then incubated for 1.5 h at 37 °C. The plates were washed with 0.02% Tween-20- PBS and 100 μL/well of goat anti-mouse IgG serum conjugated to alkaline phosphatase (ALP) (Thermo Scientific) diluted 1:2500 in blocking buffer was added to the wells and incubated for 1 h at room temperature. After washing, the plates were developed for 15 min at 37 °C by adding 100 μL/well of 1 mg/μL pNPP in ALP buffer (Na_2CO_3/$NaHCO_3$ 0.2 M, pH 9.6). The reaction was stopped, and the absorbance was measured at 405 nm. For the P10 and S266 peptides ELISAs, 96-well maleimide-activated plates (Thermo Scientific) were incubated overnight at 4 °C with 50 μL/well of a 35 μg/mL solution of each peptide dissolved in reaction buffer (0.01 M PBS-EDTA, pH 7.2). The plates were then washed and blocked with 100 μL/well of a 10 μg/mL solution of cysteine-HCl in reaction buffer for 1 h at room temperature. The plates were again washed, and the protocol was followed as described above without the addition of 1% PBS-casein blocking agent. For IgM and IgG class determination, goat anti-mouse IgM (μ) and anti-mouse IgG (γ) sera were used, respectively, and they were conjugated to ALP (Thermo Scientific) and diluted 1:10,000 in blocking buffer.

4.8. Indirect immunofluorescence assay

The procedure developed by Riemer et al. was used [21]. First, murine or human melanoma cell lines were cultivated in 8-well plates (Thermo Scientific) for 2–3 days. The cells were then fixed with 300 μL/well of 4% PBS-paraformaldehyde for 30 min at room temperature, washed with 0.02% PBS-Tween-20 and incubated overnight at 4 °C with 1:50 dilutions of immune sera in blocking buffer. The plates were washed and 200 μL/well that was FITC conjugated goat anti-mouse IgG serum (Thermo Scientific) diluted 1:100 in blocking buffer was added and then incubated for 1 h at room temperature. After the plates were washed again, DAPI (4′,6-diamino-2-fenilindol, Sigma-Aldrich) solution was added, and the plates were finally covered with DABCO mounting medium (1,4-diazabicyclo [2.2.2]octane, Sigma-Aldrich). The samples were analyzed under a Nikon microscope with epifluorescence at the Electronic Microscopy Facility (Pontificia Universidad Católica de Chile), and the resulting images were processed with NIS-Elements Microscope Imaging software.

4.9. Immunotherapy experiments to assess antitumor activity

Two-month-old C57BL/6 female mice were primed with each hemocyanin-peptide conjugate, native hemocyanins, PBS-AddaVax and AddaVax alone; they were then challenged with B16F10 according to Arancibia et al. [28,44] with modifications. A Sham group was also included. In brief, 2 weeks prior to tumor implantation, groups containing 5 mice each were randomized and primed subcutaneously (sc) with 50 μg/80 μL of PBS containing each hemocyanin-peptide-conjugated or hemocyanin-alone emulsified with AddaVax at a 1:1 vol/vol, and PBS AddaVax at 1:1 vol/vol as the vehicle control or with 40 μL of AddaVax as the adjuvant control. After day 14, the mice were challenged with an intralesional sc injection of 1.5×10^5 melanoma cells in 100 μL of DMEM into the right flank. Immunotherapy was administered 3 times every 7 days after the challenge with tumor cells. The tumor dimensions (lengths and widths) were measured every 3–4 days with an electronic caliper through day 29 (prior to the exponential growth of tumor cells), and the tumor volume was calculated using the ellipsoid formula (Volume = 0.52 × Length x Width^2). The animals were also bled on day 21 after being challenged with the tumor cells.

4.10. Serum flow cytometry analysis

The procedure developed by Arancibia et al. [28] and Monzabi-Karbassi et al. [20] was used. In brief, the cells were harvested and resuspended in 300 μL/tube immune sera solutions at 1:25 dilutions in 2% SFB-PBS, and they were incubated for 1.5 h at 4 °C. After being washed with 2% SFB-PBS, the cell pellets were resuspended in 300 μL/tube of goat anti-mouse IgG (H + L) serum conjugated to FITC and diluted 1:100 in 2% SFB-PBS. They were then incubated for 20 min at room temperature in the dark. After being centrifuged and washed in the dark, the cells were resuspended at 300 μL/tube and analyzed in a FACScan flow cytometer (Flow Cytometry Facility, Faculty of Science, Universidad de Chile). The results were analyzed using FCS Express 4 Flow Cytometry computer software (De Novo, USA).

4.11. DTH assay

The Luo and Dorf procedure was used [78]. In brief, on day 26 after tumor challenge, the mice that were treated with the hemocyanin-peptide conjugates were anesthetized and injected intradermally with 20 μL containing 75 μg of P10 or S266 in PBS into the left foot, depending on whether the mice were treated with hemocyanin-P10 conjugates or with hemocyanin-S266 conjugates, respectively. The PBS-AddaVax control group was injected with 20 μL of PBS. The increases in foot thickness were measured using a digimatic caliper (Mitutoyo, Japan) at 0, 24, 48 and 72 h post-challenge. The specificity of the response was determined according to the differences in thickness increases between the left and right feet.

4.12. Statistical analyses

The results of the experiments are expressed as the mean ± SEM. Comparisons between groups were performed using t-tests or one-way ANOVAs and the Bonferroni post-test. Statistical significance was defined as a P value smaller than 0.05. The analyses were performed using GraphPad Prism software (La Jolla, CA, USA).

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