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Mollusk Hemocyanins as Natural Immunostimulants in Biomedical Applications

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http://dx.doi.org/10.5772/57552

1. Introduction

Hemocyanins, the massive oxygen-transporting glycoproteins found freely dissolved in the hemolymph of some mollusks, are potent natural immunostimulants when inoculated in mammals, enhance the innate and adaptive immune response with beneficial clinical outcomes. Hemocyanins are easily purified and molecularly correspond to large multi-subunit structures, some over 10^7 Da (van Holde and Miller, 1995). Currently, hemocyanins are commonly used as carriers/adjuvants for producing antibodies against different antigens. These antigens include tumor-associated antigens (TAAs), such as glycolipid and glycoprotein (mucin-like) antigens in cutting-edge therapeutic vaccines for cancer, along with idiotypes, the most commonly used tumor antigen to prepare vaccines for nearly all non-Hodgkin lymphomas. Other therapeutic strategies using hemocyanins include their use as adjuvants to disrupt self-tolerance to tumor antigens in the generation of *ex vivo* autologous tumor cell lysate-loaded dendritic cells (DCs) to induce T-cell responses in cancer patients (Del Campo et al., 2011). Furthermore, hemocyanins can be used as non-specific immunostimulants during therapy for recurrent superficial bladder cancer after transurethral surgical resection with negligible toxic side effects, thus making them ideal for long-term on going treatments (Arancibia et al., 2012b).

Biomedical interest in hemocyanins arose in the 1960s when their remarkable immunogenicity and immunostimulatory properties in experimental animals, and in human beings, were discovered. The most popular hemocyanin, due to being the earliest discovered and the first used in biomedical studies, is purified from the giant keyhole limpet gastropod *Megathura crenulata* and is commonly known as **KLH** (keyhole limpet hemocyanin) (Harris

and Markl, 1999). However, the limited resource availability and the growing demand for KLH, in addition to the biodiversity of hemocyanins, have led to a growing interest in identifying new hemocyanin candidates with either similar or improved immunological and biochemical properties. Therefore, today, there are a variety of new gastropod hemocyanins that have been purified and studied biochemically and immunologically, including *Concholepas concholepas* (**CCH**) (De Ioannes et al., 2004; Moltedo et al., 2006), *Fissurella latimarginata* (**FLH**) (Arancibia et al., 2014), *Haliotis tuberculata* (**HtH**) (Keller et al., 1999; Markl et al., 2001) and *Rapana thomasiana* (**RtH**) hemocyanins (Idakieva et al., 1993; Tchorbanov et al., 2008).

In this chapter, we reviewed what is presently known about the uses of mollusk hemocyanins as carriers/adjuvants and non-specific immunostimulants in biomedicine, with a general description of hemocyanin structure and, finally, the current knowledge on the innate and adaptive mechanisms involved in the biomedical efficacy of these colossal proteins. Various authors explain the causes of immunogenicity and immunostimulatory effects of hemocyanins in mammals as due to their extremely xenogenic complex structures, which have abundant carbohydrates, and their symmetry, which resembles an icosahedral virus capsid (D5 point group symmetry, i.e., consisting of 10 equivalent subunits). However, this explanation does not account for the mechanisms involved in the effects of hemocyanin on the mammalian immune system; our investigations have attempted to elucidate such mechanisms.

2. Structure of the mollusk hemocyanins

Mollusk hemocyanins are enormous glycoproteins (4–8 MDa) formed by an intricate arrange-ment of 10 subunits, approximately 350-450 kDa each, that are self-assembled into hollow cylinders 35 nm in diameter and are referred to as **decamers**. This structure is easily observed by negative staining using transmission electron microscopy, as shown in Figure 1. Each subunit consists of 7 or 8 globular folded domains known as functional units (FUs), connected by linker peptide strands of 10–15 amino acid residues. FUs vary in size from 45–55 kDa, and each of them is capable of reversibly binding one oxygen molecule through a pair of copper atoms. In gastropod hemocyanins, the hemocyanins being described in this review, the decamers may associate in pairs to form truly immense molecules called **didecamers** with molecular masses of approximately 9×10^6 Da (Decker et al., 2007; van Holde and Miller, 1995; Markl, 2013).

An additional feature of hemocyanin structures are their carbohydrate contents, which play fundamental roles in their organization and their immunological efficacy (Paccagnella et al., 2004). Gastropod hemocyanins contain variable, heterogeneous N-and O-glycosylation sites, with as high as 9% (w/w) mannose as the most abundant oligosaccharide (Hall and Wood, 1976). Some hemocyanins possess the Thomsen-Friedenreich antigen disaccharide (T antigen disaccharide Galβ1-3GalNAcα1-Ser/Thr), which, together with mannose, may play a role in the immune-related properties of hemocyanins (Geyer et al., 2005; Harris and Markl, 1999), as discussed later.
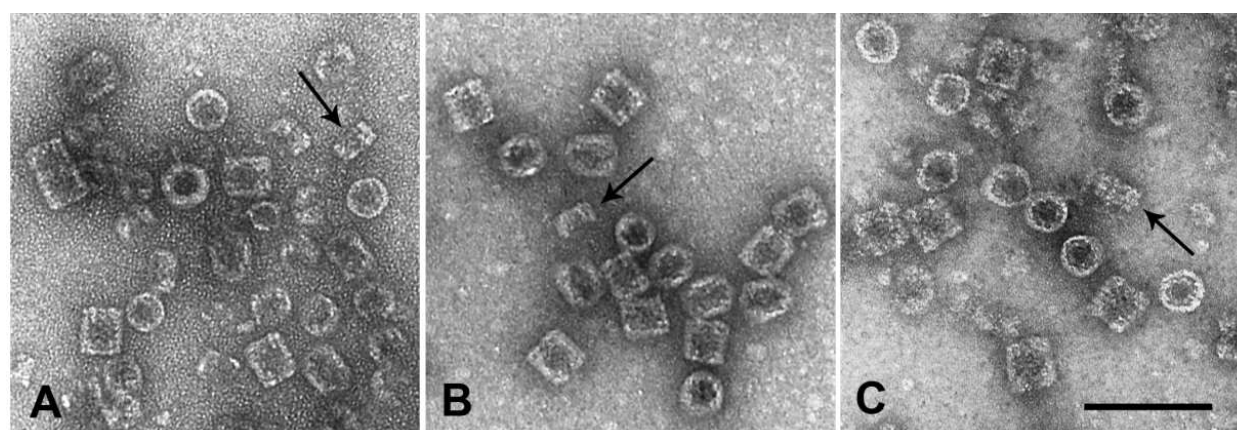
Figure 1. Electron microscopy of negatively stained purified mollusk hemocyanin molecules showing their characteristic hollow cylinder form. A. Hemocyanin from the Keyhole limpet (*Megathura crenulata*), known as KLH. B. Hemocyanin from *Concholepas concholepas,* known as CCH. C. Hemocyanin from *Fissurella latimarginata,* known as FLH. The images show the top (circles) and lateral (rectangles) views of the molecules. The side views show the characteristic didecameric forms of the protein, with subunits arranged in layers. The arrow shows decamers. The scale bar represents 100 nm.

3. Hemocyanins used in biomedicine

KLH from *Megathura crenulata* has been used for more than 40 years for biomedical and biotechnological applications. In the search for new hemocyanins that might act with similar immunostimulatory properties, the hemocyanins from *Concholepas concholepas* (CCH), *Rapana thomasiana* (RtH) and *Haliotis tuberculata* (HtH) emerge as the most promising. Surprisingly, in this search, we identified a new hemocyanin from *Fissurella latimarginata* (FLH) with superior immunogenicity compared to any other hemocyanin known to date (Arancibia et al., 2014). Background on these hemocyanins and the experimental data that support their potential use as immunostimulants is provided below and summarized in Table 1.

3.1. *Megathura crenulata* hemocyanin (KLH)

The hemocyanin from the giant keyhole limpet *Megathura crenulata*, which naturally lives on the coast of Southern California to Baja California, is a mixture of two immunologically different didecamers, named KLH1 and KLH2. In fact, diverse electrophoretic analyses have revealed that the KLH preparation is made up of two independent oligomeric isoforms that coexist in variable proportions in the animal's circulation, each composed of one type of subunit, KLH1 (350 KDa) and KLH2 (350 KDa) (Gebauer et al., 1999a; Swerdlow et al., 1996). Immunoelectrophoretic analyses using polyclonal antibodies provided insight into the antigenic individuality of these KLH isoforms, indicating that they are very different structurally (i.e., they do not display shared epitopes) (Harris and Markl, 1999). Although both KLH subunits are glycosylated, they differ in their oligosaccharide patterns: KLH1 (3.0% carbohydrate, w/w) has higher mannose content than KLH2 (3.4% carbohydrate, w/w), which has more N-acetylgalactosamine than KLH1, in addition to the presence of an O-glycosylation site

(Idakieva et al., 2004). Whether or not KLH1 and KLH2 have similar effects on the immune response has not been comprehensively analyzed. Currently, the variable proportions of KLH subunits in different preparations of the agent, in addition to its propensity to precipitate (Gathuru et al., 2005), can be problematic for some biomedical applications in which the components should be invariant and precisely defined. In addition, the KLH genes have been cloned and sequenced, and the complete amino acid sequences of its subunits are now known; however, until now, it has not been possible to express a heterologous KLH (Altenhein et al., 2002; Gatsogiannis and Markl, 2009; Markl et al., 2001).

In the 1970s, KLH was introduced in the clinic as part of a test to evaluate immunocompetence in immunosuppressed patients (Curtis et al., 1971; Curtis et al., 1970); thus, its benefits for the treatment of recurrent superficial bladder cancer were discovered serendipitously (Olsson et al., 1974), as described later. In addition, at that time, through the coupling of small molecules (or haptens, defined as substances unable to induce antibodies by themselves) to KLH, it was possible to obtain specific antibodies against them. Thereby, KLH has become, and remains to this day, the most used carrier protein to produce polyclonal and monoclonal antibodies against small molecules, including peptides, drugs, hormones, toxins, antibiotics and countless chemicals. The optimal carrier properties of KLH add to its intrinsic immunostimulatory effects, leading to its current use in the development of several therapeutic vaccines for cancer through the generation of antibodies against TAAs; these antibodies act to eliminate cancer recurrence caused by circulating tumor cells and micrometastases. Moreover, KLH, when conjugated with idiotype antibodies, can induce strong anti-idiotypic antibody responses and cell-mediated responses to tumor antigen(s) *in vivo*, which has resulted in an objective outcome in patients with B-cell lymphoma (Nelson et al., 1996). In addition, the adjuvant/immunostimulatory properties of KLH have been amply supported through its use in vaccine studies as an immunological tracer protein due to its neo-antigen character. Thus, this protein serves as a strong "surrogate" antigen and an immunogenic "marker" for immunization studies using DC-based vaccines (Shimizu et al., 2001).

3.2. *Concholepas concholepas* hemocyanin (CCH)

The hemocyanin from the edible gastropod *Concholepas concholepas*, a specie distributed on the coasts of Chile and southern Perú, is structurally distinct from traditional KLH. The decamers are formed by two subunits, named CCHA (405 KDa) and CCHB (350 kDa), which are intermingled in the molecule and form heterodecamers. Consequently, their association in pairs results in heterodidecamers (De Ioannes et al., 2004). The carbohydrate content has been determined and has demonstrated that each subunit is differently glycosylated. Thus, CCHA (3.6% carbohydrate, w/w) has N-linked and O-linked glycans; CCHB (2.5% carbohydrate, w/w) has only sugar with N-linkages, while O-linked moieties are nearly absent (Becker et al., 2009). A feature that distinguishes CCH from the remaining hemocyanins is its great stability and solubility. In fact, in contrast with other hemocyanins, after its purification from the hemolymph, the stabilization of CCH does not require additional divalent cations, such as Ca^{2+} and/or Mg^{2+}, in the storage media to maintain its structure (De Ioannes et al., 2004). This quality facilitates chemical coupling reactions when CCH is used as a carrier protein. Thus, CCH has been successfully used as a carrier protein to generate antibodies against hapten molecules (Becker et al., 1998; Torres et al., 1999) and peptides (Duvillie et al., 2003; Grenegard

et al., 2008; Matus et al., 2007; Mura et al., 2002). Additionally, CCH has been used as carrier of a gonadotropin-releasing hormone (GnRH) in a contraceptive vaccine to control reproduction in deer, providing a longer-lasting contraceptive effect (Miller et al., 2008; Pilon et al., 2007). Furthermore, CCH has been pre-clinically evaluated in a murine experimental model of superficial bladder cancer and may be considered a safe alternative therapy to KLH (Atala, 2006; Moltedo et al., 2006), as described later.

Finally, the most important support for clinical attention to CCH in future biomedical developments includes the results of a recent study of its use as an adjuvant in a DC-based cancer vaccine for castration-resistant prostate cancer (CRPC) patients. This study demonstrated that CCH was able to induce an immune memory response, as measured by the delayed-type hypersensitivity (DTH) test, and did not produce toxic or allergic adverse reactions when administered subcutaneously in the patients. These results led to the conclusion that CCH may be considered as an alternative to KLH for providing safe and effective adjuvanticity in cancer vaccines (Reyes et al., 2013).

3.3. FLH

The hemocyanin from the black keyhole limpet *Fissurella latimarginata* (FLH) was discovered most recently (Arancibia et al., 2014). The experimental data demonstrate that FLH didecamers are composed of a single type of polypeptide with a molecular mass of approximately 350 kDa. Although the total carbohydrate content has not yet been estimated, carbohydrate staining with specific lectins has shown that FLH has exposed N-and O-linked oligosaccharides, similar to KLH (Arancibia et al., 2014). The evaluation of the humoral immune responses in different mouse strains immunized with CCH, FLH and KLH indicated that FLH is intrinsically more immunogenic than CCH and KLH and reaches titers an order of magnitude higher than those of CCH and KLH. Moreover, FLH had potent *in vivo* anti-tumor activity against melanoma in the B16F10 mouse model, delaying tumor growth and increasing the survival of mice challenged with these cells in a prophylactic setting (i.e., with the aim of preventing tumor growth). The most striking effect was observed in a therapeutic setting (specifically, therapy for established tumors in animals without previous FLH priming) (Arancibia et al., 2014). To elucidate the early immunologic mechanisms involved in this anti-tumor effect, we investigated the effect of FLH on murine DCs cultured *in vitro*. These studies demonstrated that FLH, but not CCH or KLH, is able to rapidly induce the secretion of certain pro-inflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-12p40 and IL-23α, a phenomenon that may explain its enhanced immunological activities (Arancibia et al., 2014).

3.4. HtH

The hemocyanin from the European abalone *Haliotis tuberculata* (**HtH**), a species under commercial aquaculture conditions, is composed of two types of didecamers that coexist in the animal's circulation, HtH1 and HtH2. Immunological analysis demonstrated that these two didecamers are closely related (Keller et al., 1999). These isoforms are formed by the HtH1 and HtH2 subunits, and each has a molecular mass of approximately 392 kDa (Altenhein et al., 2002; Lieb et al., 2000). The carbohydrate content of HtH is 4.5% (w/w); a highly heterogeneous group of structures with appreciable amounts of 3-o-methyl-d-mannose and 3-o-

Table 1. Hemocyanins available today

PROPERTIES	FEATURE	KLH	CCH	FLH	HtH	RtH
Taxonomy	Phylum	Mollusk	Mollusk	Mollusk	Mollusk	Mollusk
	Order	Gastropoda	Gastropoda	Gastropoda	Gastropoda	Gastropoda
	Family	Fissurellidae	Muricidae	Fissurellidae	Haliotidae	Muricidae
	Genus and specie	*Megathura crenulata*	*Concholepas concholepas*	*Fissurella latimarginata*	*Haliotis tuberculata*	*Rapana thomasiana*
Distribution	Habitat	Coast of California	Pacific coast of Perú and Chile	Pacific coast of Perú and Chile	Waters of the Channel Islands	Japan sea , and the East China Sea
Biochemical	Molecular weights	≈ 8 MDa	≈ 8 MDa	≈ 8 MDa	≈ 8 MDa	≈ 8 MDa
	Size of molecule	≈ 360 Å Ø 400Å height	≈ 325Å Ø 392Å height	≈350 Å Ø 370 Å height	≈ 350 Å Ø 380Å height	≈ 350 Å Ø 380Å height
	Subunits composition	2	2	1	2	2
	Subunit organization	Homodecamer	Heterodecamer	Homodecamer	Homodecamer	Homodecamer
	Stability structure in solution	Required Ca^{+2} and Mg^{+2}	Not required Ca^{+2} and Mg^{+2}	Required Ca^{+2} and Mg^{+2}	Required Ca^{+2} and Mg^{+2}	Required Ca^{+2} and Mg^{+2}
	Isoelectric point	5,7	6,0	ND	ND	ND
	% Oligosaccharides	3,2%	3,1%	ND	4,5%	2,6%
	Type of Oligosaccharides	N- and O-linked	N- and O-linked	N- and O-linked	N- and O-linked	N- and O-linked
Immunological	Immunogenicity	High	High	Very high	ND	ND
	Antitumor effects in animals models of SBC	Yes	Yes	ND	ND	ND
	Antitumor effects in animals models of melanoma	Yes	Yes	Yes	ND	ND
	Evaluated as hapten carrier	Yes	Yes	Yes	ND	ND
Biomedical uses	Used as adjuvant in DC vaccines	Yes	Yes	ND	ND	ND
	Used as immunostimulant in bladder cancer	Yes	No	No	No	No

ND: Not determinate

methyl-d-galactose has been identified in HtH (Idakieva et al., 2004; Velkova et al.). It is important to note that, although there are numerous studies on the biochemical characteristics of HtH, comprehensive studies about its immunologic and therapeutic properties are essentially absent. However, a stable preparation of abalone hemocyanin with high antiviral activity has recently been formulated and needs to be evaluated as therapeutic agent in future clinical applications (Zanjani et al., 2013).

3.5. RtH

The hemocyanin of the marine snail *Rapana thomasiana* (RtH), a species native of the China Sea and transferred to the West coast of the Black Sea, was described in the 1990s (Idakieva et al., 1993). RtH is a mixture of two hemocyanin isoforms, RtH1 and RtH2, that coexist in the animal's circulation (Gebauer et al., 1999b). Both have carbohydrate contents of 2.6% (w/w), with very similar monosaccharide compositions featuring type N-glycosylation (Idakieva et al., 2004). The biomedical applications of RtH as an adjuvant have been explored and have demonstrated that mice immunized with influenza vaccine developed a specific humoral and cellular immune response characterized by the induction of specific antibodies to viral proteins and a cytotoxic response lasting at least 5 months (Gesheva et al., 2011). Furthermore, antimicrobial activities against different strains of the genital herpes simplex virus have been reported for the RtH2 subunit (Genova-Kalou et al., 2008).

4. Hemocyanins as non-specific immunostimulants in recurrent superficial bladder cancer (SBC)

A significant decrease in the recurrence of superficial bladder cancer in 10 patients with transitional cell carcinoma (TCC) treated with KLH without toxic side effects was reported early on in the field of hemocyanin research (Olsson et al., 1974). This study was followed by tens of papers, which collectively provided encouraging support for the use of KLH and CCH as alternatives in SBC immunotherapy. The most frequently used immunotherapeutic agent to prevent recurrence of TCC is the Bacillus Calmette-Guerin (BCG) vaccine for tuberculosis (Morales et al., 1976). This attenuated form of *Mycobacterium bovis* has also been used as a therapy in other types of cancers (Edwards and Whitwell, 1974; Milas and Withers, 1976). However, despite the successful results (Nseyo and Lamm, 1997), the severe side effects of the BCG vaccine have led scientists to explore new immunostimulants (Lamm, 2003).

4.1. Experimental studies

Three different *in vivo* models have been primarily used to evaluate the therapeutic properties of hemocyanins in SBC, with the therapy being administered either subcutaneously, intraperitoneally or intravesically according to the site where the tumor was established (Lamm, 2003; Schenkman and Lamm, 2004). The mouse bladder tumor-2 cell (MBT-2) transplantable murine model of SBC was the first of these models to be developed in 1981 (Lamm et al., 1981). Mice were pre-immunized with 200 μg of KLH after a previous subcutaneous inocula-

tion with MBT-2 and were then intralesionally immunized with 50 µg at one and seven days after implantation, leading to a significant decrease in tumor growth and a prolongation of animal survival (Lamm et al., 1981). Further studies showed that priming was essential for achieving a therapeutic effect (Lamm et al., 1993b; Lamm et al., 1982; Marsh et al., 1987). However, other researchers have studied KLH immunotherapy in the same model without promising results. The priming and transplantation of MBT-2 tumor cells, either subcutaneously or into the bladder, with immunotherapy of 50 or 200 µg of KLH did not yield different results compared to controls (Walsh et al., 1983). In a later study, the administration routes of KLH were compared. Without priming, the intralesional route was more effective than intraperitoneal administration in terms of inhibiting tumor growth (Lau et al., 1986). After that, an additive effect was observed in animals that received KLH and interferon (IFN)-α intraperitoneally without prior immunization, compared with KLH and IFN-α alone (Riggs et al., 1992). However, in a subsequent study, the presence of endotoxin in KLH preparations partially accounted for its anti-tumor effect (Lamm et al., 1993a).

The syngeneic orthotopic murine bladder cancer model MB-49 was evaluated in 1994 (Gunther et al., 1999; Swerdlow et al., 1994). Subcutaneous immunization with KLH-Immune Activator, a clinical-grade KLH preparation, two weeks prior to intravesical implantation of tumor cells, followed by intravesical administration of 10 or 100 µg of KLH, resulted in significantly decreased tumor growth (Swerdlow et al., 1994). Prior immunization was required, and no significant histopathological abnormalities were observed. A third model was developed in 1989 that consisted of the induction of a bladder carcinoma in Wistar rats using N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) (Arentsen et al., 2009). Subcutaneous sensitization with 1 mg of KLH, followed by intravesical and subcutaneous administration twice weekly, led to a decrease of BBN-induced bladder tumors (Recker and Rubben, 1989). A subsequent study demonstrated that subcutaneous administration was more effective than intravesical instillation in terms of tumor growth and rats bearing tumors (Linn et al., 2000).

Finally, more recent studies have evaluated the effect of CCH in the MBT-2 murine model (Becker et al., 2009; Moltedo et al., 2006). Priming with CCH previous subcutaneous implantation of tumor cells, succeeded by subcutaneous immunization with 1 or 0.1 mg of CCH, led to decreases in the incidence and growth of tumors, prolonged survival and the absence of toxic effects (Moltedo et al., 2006). In a subsequent study of the contributions of CCH-A and CCH-B subunits, it was determined that each subunit alone has an anti-tumor effect. However, in terms of tumor incidence and animal survival, CCH-A had the maximum effect (Becker et al., 2009).

4.2. Clinical studies

As a result of complications of complete bladder removal and the possibility of cancer recurrence, some SBCs have been treated with intravesical administration of biological and chemotherapeutic agents in the initial stages of the disease to either treat an established tumor or avoid progression and recurrence after transurethral resection (Perabo and Muller, 2004). The first study in humans was performed in 1974, in which 10 immunocompetent patients subcutaneously primed with 5 mg of KLH and followed with immunization using 200 µg of

KLH had significantly reduced tumor recurrence rates over a period of two years (Olsson et al., 1974). More than 10 years later, a controlled study corroborated that KLH was more effective than mitomycin C (Jurincic et al., 1988). Then, it was determined that the incidence of recurrence in patients with TCC related to urinary schistosomiasis was diminished after KLH treatment (Wishahi et al., 1995). However, studies with patients who were unresponsive to chemotherapeutic agents (Flamm et al., 1990) or who had carcinoma *in situ* (CIS) (Jurincic-Winkler et al., 1995c; Jurincic-Winkler et al., 2000) have not shown any significant effects after KLH treatment.

A Phase III clinical trial was conducted by Intracel Resources (USA) to assess the safety and effectiveness of the KLH BCI-Immune Activator compared to doxorubicin in BCG-intolerant or refractory patients with CIS with or without resected SBC. Nevertheless, although the study was completed, no results have been posted. Recently, Biosyn (USA) evaluated the efficacy and adverse effects of IMMUCOTHEL®, a clinical-grade KLH preparation developed by Biosyn Arzneimittel GmbH (Germany). Their outcomes showed almost no KLH-induced adverse effects, and the efficacy of IMMUCOTHEL® was comparable to mitomycin C.

4.3. Other models

Due to some of the successful results using hemocyanins in SBC therapy, their therapeutic effects have been evaluated in other cancers, such as melanoma, breast, pancreatic, esophageal and prostatic.

4.3.1. In vitro studies

The effectiveness of KLH has been demonstrated in diverse human cancer cell lines, such as estrogen-independent breast (ZR75-1), estrogen-dependent breast (MCF-7), esophagus (SEG-1 and BIC-1), pancreas (PANC-1), melanoma (HTB68 and HTB72) and prostate (DU145), through the inhibition of cellular growth in an apoptotic-dependent or independent manner (McFadden et al., 2003; Riggs et al., 2002; Riggs et al., 2005; Somasundar et al., 2005). In addition, KLH was shown to exert an additive effect with IL-2 and IFN-α in a combined therapy against melanoma, which encourages the use of this type of bivalent therapy as an effective treatment against these types of aggressive disease (McFadden et al., 2007).

4.3.2. In vivo studies

Diverse hemocyanins have been evaluated as a therapy in murine models of melanoma. First, the combined effect of KLH with IL-2 and IFN-α was evaluated in an HTB68 mouse melanoma model. KLH augmented the effect of IFN-α, one of the most common immunotherapeutic agents against melanoma (Rizvi et al., 2007). Then, the effect of diverse glycoconjugates (GCs), which induce NK-cell mediated cytotoxicity *in vitro*, were evaluated with or without KLH in a B16F10 murine melanoma model. However, no synergism was observed, which was attributed to the common epitopes shared between the protein and the GCs (Hulikova et al.). Recently, it was demonstrated that an oxidated-modified CCH, which had enhanced structural stability and immunogenicity, has the same anti-tumor effect in the B16F10 melanoma model

as its native counterpart (Arancibia et al., 2012a). In addition, we have recently demonstrated the outstanding immunomodulatory properties of FLH in a B16F10 murine melanoma model. FLH promotes significantly higher antibody titers than CCH and KLH, in addition to exhibiting potent anti-tumor activity that delays the growth of B16F10 melanoma cells and prolongs murine survival (Arancibia et al., 2014).

5. Hemocyanins as carriers/adjuvants in vaccines

Mollusk hemocyanins have been widely used in therapeutic and prophylactic vaccines, enhancing the immune response by building on hemocyanin's carrier properties and adjuvant capacities (Del Campo et al., 2011). In order to generate immunity against antigens, pathogens, TAAs and certain chemical substances, such as commonly abused drugs, it is necessary to chemically couple the compound to the hemocyanin and then immunize the patient. The hemocyanins can also be used as genuine adjuvants to further enhance T cell reactivity to tumors in DC vaccine therapy; in this case, hemocyanins contribute to the reversal of the DC tolerogenic profile in cancer patients toward an immunostimulatory profile (Presicce et al., 2008). Indeed, the generation of an optimal cytotoxic T cell (CTL) immune response requires the presence of T helper lymphocytes (CD4+) and the expression of both helper-and CTL-defined antigen determinants on the same DC (Shimizu et al., 2001; Timmerman and Levy, 2000). Thus, hemocyanins produce a bystander effect, inducing a potent specific memory T cell response associated with the secretion of cytokines that indirectly promote the specific cellular response against the antigen of interest, whether a tumor cell, a pathogen or a deleterious compound.

The following overview offers a brief look at the experimental vaccines that have used hemocyanins in their formulations.

5.1. Vaccines against pathogens

Pathogenic immunodominant peptides or polysaccharides coupled to hemocyanin have been tested in the development of experimental vaccines that are intended to prevent diseases caused by bacteria, viruses and fungi. We next present some examples.

5.1.1. Vaccines against bacteria

For *Pseudomonas aeruginosa*, which causes pulmonary infection, the use of synthetic peptides representing surface-exposed, linear B-cell epitopes of outer membrane protein F (Hughes and Gilleland, 1995) or mucoid exopolysaccharide (MEP) coupled to KLH demonstrated that significantly enhances its immunogenicity and the capacity to elicit opsonic antibodies in mice and rabbits (Theilacker et al., 2003). For *Streptococcus pneumonia*, which causes pneumococcal infection, administration of a DNA plasmid encoding the FLt3 ligand gene as a mucosal adjuvant plus phosphorylcholine (PC) conjugated to KLH demonstrated elicitation of PC-specific immune responses at the mucosal and serum levels (Baatarjav et al., 2011). For *Coxiella burnetii,* which causes acute and chronic Q fever in humans, a lipopolysaccharide (LPS)

mimetic peptide was coupled to KLH; immunized mice were able to inhibit *C. burnetii* infection and to develop significant protection against *C. burnetii* challenge (Peng et al., 2012).

5.1.2. Vaccines against viruses

For papillomavirus, peptide fragments were used that mimicked B-cell epitopes of the capsid protein L1 of human papillomavirus (HPV) type 31 coupled to KLH, generating specific anti-peptide antibodies in mice (Andreev et al., 2012). For influenza, a conjugate vaccine consisting of the peptide of the highly conserved M2 membrane protein coupled to KLH was highly immunogenic and able to confer protection against lethal challenge with either H1N1 or H3N1 virus in mice (Fan et al., 2004; Kiraly et al., 2011). For HIV-1, aiming to develop neutralizing antibodies in a caprine model, KLH was coupled with a synthetic peptide representative of the p17 functional epitope (AT20) derived from HIV-1 or with MPR peptide from the gp41 membrane proximal region to prevent transmission of the virus through colostrum. This conjugated vaccine effectively induced specific sIgA and IgG in the colostrum of a lactating species.(Dorosko et al., 2008).

5.1.3. Vaccines against fungi

For *Cryptococcus neoformans,* which causes meningoencephalitis in AIDS patients, one component of the cryptococcal capsular polysaccharide was coupled to KLH, producing antibodies that were protective against the pathogen (May et al., 2003). However, a protective epitope of *Candida albicans* conjugated to KLH induced a Th1-type cytokine expression pattern in C57BL/6J mice (Su et al., 2007).

5.2. Antitumor vaccines

The antitumoral vaccines are intended to treat an existing cancer by strengthening the body's natural defenses against cancer and have emerged as alternatives to anti-proliferative treatments, such as chemo-or radio-therapy. The design of such vaccines is focused on the search for specific epitopes in tumor cells to raise antibodies against them. These epitopes are usually aberrant branches of polysaccharides located on the cell surface. Some of them are glycolipids, such as Globo H, Lewis Y, GM2, GD2, GD3 and fucosyl-GM1, and others are glycoproteins, such as MUC-1, Tn, sialyl-Tn and TF (Galonic and Gin, 2007). The aim of these vaccines is to transform these tumor antigens into immunogens powerful enough to achieve an immune response. Furthermore, hemocyanins are used as immunomodulatory agents in DC vaccines in which these autologous presenting cells are loaded with tumor antigen, whether as tumor lysate, recombinant antigen or transfecting cell tumor RNA, in the presence of hemocyanins (Steinman and Banchereau, 2007). In this case, the patient's responses are measured by DTH tests against hemocyanins or tumor extract (Escobar et al., 2005). In addition to the extensive use of KLH for this purpose, promising results have been obtained with CCH as an adjuvant in a DC vaccine administered to patients with CRPC (Reyes et al., 2013). Next, we review selected antitumor vaccine clinical trials with hemocyanins that have been approved by the US National Institutes of Health (NIH).

5.3. Breast, ovary, fallopian tube and peritoneal cancers

Sialyl-Tn (STn) is the tumor antigen most frequently expressed in breast, ovary, colon, rectal, stomach and pancreas adenocarcinomas. This antigen, conjugated to KLH, has been named Theratope and is capable of generating positive serological results, inducing significant titers of IgM and IgG antibodies against STn (Gilewski et al., 2007). The results of a Phase III multicenter clinical trial, which included 1,028 women with metastatic breast cancer, showed that the sialyl-Tn (STn)-KLH vaccine was well tolerated by the patients, but no overall benefit in time to progression or survival was observed (Miles et al., 2011). However, women who also received endocrine therapy and showed greater antibody response to the STn-KLH vaccine had significantly longer median overall survival than those who had lower antibody responses, demonstrating that this dual treatment may improve clinical outcomes in women with metastatic breast cancer (Miles et al., 2011). Other monovalent vaccines have been developed in which a single carbohydrate antigen, including Globo-H, fucosyl GM1 and Lewis^y (Le^y), were coupled to KLH with saponin fractions (QS21 or OPT-82142) added as adjuvants; these vaccines have exhibited varying degrees of promise in early clinical settings (Ragupathi et al., 2011; Zhu et al., 2009). Additionally, multivalent vaccines have been formulated with GM2, Globo-H, Lewis Y, Tn (c), STn (c), TF (c) and Tn-MUC1, each individually conjugated to KLH. QS21 was added as an adjuvant to this cocktail of conjugates and was evaluated in patients with either epithelial ovarian, fallopian tube or peritoneal cancer in second or greater complete clinical remission, demonstrating that this heptavalent vaccine was safe and induced antibody responses against five of seven antigens (Sabbatini et al., 2007). Currently, more elaborate constructs have been designed in a new vaccine against ovarian cancer, based on clusters of a globotriaosylceramide (Gb3) antigen and incorporating a MUC5AC peptide epitope that mimics the surfaces of targeted tumor cells (Zhu et al., 2009).

5.4. Melanomas

Vaccines were designed with the gangliosides GM2, GD2 and GD3 conjugated to KLH plus the adjuvant QS21. These vaccines produced high titers of IgM and IgG in patients with melanoma compared to vaccines with GM2 and BCG as adjuvants (Ragupathi et al., 2000). Although it was initially postulated that immunotherapy against GM2 was effective in reducing the risk of relapse in post-surgery patients with stage III melanoma, the vaccine was ineffective in a Phase III clinical trial in 1314 patients (Eggermont et al., 2013). Additionally, gangliosides GD2-L and GD3-L have been used as therapeutic targets, generating higher titers of IgM and IgG (Ragupathi et al., 2003).

Regarding cellular vaccines, the first clinical study included patients with advanced melanoma who were treated with DCs loaded with tumor lysate or a mixture of melanoma peptides in the presence of KLH. Patients tolerated the vaccine and did not develop autoimmunity (Nestle et al., 1998). Currently, other antigens, such as MART-1/MelanA, tyrosinase, MAGE-3 and gp100 (Fay et al., 2006; Tittarelli et al.; Tittarelli et al., 2012), in addition to the heat-shocked melanoma lysate named TRIMEL (Aguilera et al., 2011), with KLH as an adjuvant have been loaded on DCs, yielding promising results in terms of the prolongation of overall survival time. Interestingly, in the search for parameters of clinical effectiveness of KLH-containing

vaccines and therapies, a quantitative enzyme-linked immunosorbent assay (ELISA) has been developed to measure KLH-specific antibodies as biomarkers in humans, demonstrating that it is possible to monitor the dynamics of both KLH-specific antibody levels and antibody class-switching in individuals who are repeatedly exposed to KLH (Aarntzen et al., 2012).

5.5. Prostate cancer

Numerous tumor antigens coupled to hemocyanins have been tested in monovalent, divalent or polyvalent vaccines (Zhu et al., 2009). To date, there are 10 clinical trials in various stages of development using primarily the antigen MUC-2 with KLH. When comparing preclinical studies between mono-and polyvalent vaccines, the controversy arises over which strategy is best and why, based on the observations that the monovalent vaccines induced high titers of antibodies, whereas the polyvalent vaccines reached a greater range of antibodies (Slovin et al., 2007). Recently, CCH, as an adjuvant in a cancer vaccine based on DCs loaded with prostate cancer cell lysates and TRIMEL, was used for the first time in CRPC patients. This study demonstrated that CCH is safe and is capable of inducing memory T cell responses, as determined *in vivo* by a DTH test (Reyes et al., 2013), thus indicating that CCH can complement or substitute KLH in such therapies.

5.6. Lung cancer

In the eradication of micrometastases in chemotherapy-resistant small cell lung cancer (SCLC), the ganglioside fucosyl-GM1 is used as a therapeutic target. Therefore, in a clinical study, 11 patients with SCLC were vaccinated by injection with this synthetic antigen coupled to KLH. Higher IgM titers against fucosyl-GM1 were detected in eight of the patients, and only one patient developed IgG with a similar titer; these results led to a Phase II study (Krug et al., 2004). Recently, vaccination with N-propionyl polysialic acid-KLH conjugate plus QS-21 in patients with SCLC has demonstrated that this vaccine is safe and results in consistent high-titer antibody responses. These results open up the possibility of incorporating polysialic acid into a polyvalent vaccine against SCLC with four glycolipid antigens that are also widely expressed in SCLC, such as GD2, GD3, fucosylated GM1 and globo H (Krug et al., 2012). In consideration of the fact that tumor angiogenesis is critical for tumor growth, infiltration and metastasis, another promising approach includes the generation of a mimotope of Bevacizu-mab (Avastin), a monoclonal antibody that reacts directly against vascular endothelial growth factor (VEGF) conjugated to KLH, which induces humoral immunity and results in the induction of anti-angiogenesis responses in mice (Li et al., 2013).

5.7. Lymphomas and myelomas

For the non-Hodgkins lymphomas, the strategy of conjugating the variable regions of the immunoglobulin to the tumorigenic clone with KLH has been used. These variable regions act as tumor antigens. The principle of these vaccines is that the clonal immunoglobulin idiotype displayed on the surface of most malignant B cells is not only tumor-specific; it is also a patient-specific antigen that can be used for therapeutic vaccination (Bendandi, 2006; de Cerio et al., 2007). These idiotype vaccines have shown some degrees of both biological efficacy and

improved clinical outcomes in lymphoma (Bendandi, 2009). The effect of this type of vaccine has been enhanced by incorporating granulocyte macrophage colony-stimulating factor (GM-CSF); showing DCs recruitment and activation of CD4+and CD8+T lymphocytes (Bendandi et al., 1999). These encouraging results led to the initiation of Phase III clinical trials to establish the clinical benefits induced by anti-idiotypic vaccines (Neelapu and Kwak, 2007). In addition, DC-based anti-idiotype vaccination using KLH as an adjuvant induces a specific cellular immune response in patients with stage 1 myeloma (Rollig et al., 2011).

5.8. Vaccines against abused drugs

Experimental vaccines have been developed to curb recidivism in the consumption of addictive drugs, including cocaine and nicotine. The aim of these vaccines is to generate antibodies that reduce blood concentrations of the given drug and its effect on the central nervous system by preventing the drug from crossing the blood-brain barrier. The experimental strategies to induce protective and specific antibodies against cocaine included subcutaneous and intranasal immunization (Hrafnkelsdottir et al., 2007). Preclinical studies using KLH as a carrier of these drugs are promising (Moreno and Janda, 2009). However, the only clinical evidence with good results for the effectiveness of these vaccines used cholera toxin as a carrier of the cocaine-B subunit (Martell et al., 2009). Another interesting experimental approach is the generation of two complementary but mechanistically distinct active vaccines (i.e., non-catalytic and catalytic hapten designs) using a cocaine-like hapten, GNE, and a cocaine transition-state analogue, GNT, coupled to KLH. Although GNT-KLH induced higher titers of catalytic antibodies, these titers were not sufficient to provide protection in mice challenged with cocaine (Cai et al., 2013). Additionally, an anti-nicotine vaccine that used nicotine coupled to KLH, which was named Niccine®, was no better than the placebo (Gorelick, 2012).

6. The mechanisms of hemocyanin immunostimulation

6.1. At the innate immunity level

The effects induced by hemocyanins during the early phases of the immune response and the identities of the target cell type(s) have been scarcely studied. Several authors have suggested that the oligosaccharides may play a role in this process. As mentioned, the carbohydrate compositions of hemocyanins are quite diverse; however, the most abundant monosaccharide is mannose, which is often found in high-mannose or hybrid structures of oligosaccharides. The mammalian innate immune system has a variety of cell types that express several receptors on the cell surface. In this context, specialized professional antigen-presenting cells (APCs), such as macrophages and DCs, are key players in immune surveillance. These cells present a broad range of germ-line encoded pattern recognition receptors that recognize conserved pathogen-associated molecular patterns. Among these receptors, the C-type lectin family of receptors recognizes several types of oligosaccharides present on pathogens and foreign molecules (McGreal et al., 2006; van Vliet et al., 2008). The biological role of the high mannose

oligosaccharides on mollusk hemocyanins has not been clearly demonstrated. However, it has been reported that KLH promotes the *in vitro* maturation of human DCs through its engagement of the mannose receptor, as assessed by the up-regulation of the cell surface expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules (Presicce et al., 2008). It seems that this phenomenon has been described only for human DCs; in mice, neither KLH nor CCH induce DC maturation *in vitro* through canonical mechanisms (i.e., via up-regulation of MHCII and co-stimulatory molecules) during the early hours of *in vitro* culture (Arancibia et al., 2012a). Moreover, Teitz-Tennenbaum and collaborators (2008) demonstrated that murine DCs pulsed with KLH for 18 h *in vitro* did not undergo DC maturation, a result that is consistent with *in vivo* experiments performed by Moltedo et al. 2009 (Moltedo et al., 2009; Teitz-Tennenbaum et al., 2008). Recently, we observed that mouse DCs internalized this hemocyanin but did not mature within 72 h of culture *in vitro* with CCH. Remarkably, FLH, unlike KLH or CCH, promoted the high secretion of pro-inflammatory cytokines, such as IL-6, IL-12p40, TNF-α and IL-23, from murine DCs *in vitro* (Arancibia et al., 2014). Moreover, the secretion of cytokines was dependent on the presence of oligosaccharides on FLH, indicating a role for lectin-like receptors in the response. These results indicate the complexity of the immune response and demonstrate that different hemocyanins can activate diverse molecular and cellular pathways. In addition, the divergence regarding the role of DCs in hemocyanin recognition highlights the importance of studying those mechanisms in human cells.

Once hemocyanins are internalized by APCs, the proteolytic machinery of the cell degrades them slowly in comparison to classical antigens, thus increasing antigen persistence. This process permits the presence of hemocyanins for longer periods of time, resulting in augmented immunogenicity, as demonstrated for CCH (Arancibia et al., 2012a). In fact, using electron microscopy, we have determined that CCH molecules are internalized by DCs mainly through macropinocytosis and are then localized intact in lysosomes for up to 24 h. Moreover, we compared the antigen-processing kinetics of DCs for CCH and ovalbumin, a widely used antigen model, demonstrating the persistence of a band of approximately 49 kDa in DC cultures with CCH for 72 h; in contrast, in cultures of DCs pulsed with ovalbumin, the antigen was completely degraded by this time.

6.2. At the adaptive immunity level

Hemocyanins are thymus-dependent antigens; therefore, they require the interaction between T and B lymphocytes to initiate antibody production. In fact, it has been established that non-specific immunotherapeutic effects of hemocyanins in superficial bladder cancer rely on adequate priming, emphasizing the importance of the adaptive immune response in this property. Considering that the antitumor effect is induced by hemocyanins themselves without adjuvant, we assume that KLH and CCH have common structural features that promote inflammation and maintain innate immunity, leading to the onset of an antitumor adaptive immune response. These common characteristics of hemocyanins may rely on carbohydrates, which may act as natural adjuvants. A proposed alternative mechanism is that CD4+T lymphocytes reacting to preserved xenogenic peptidic sequences stimulate T lympho-

cytes to secrete Th1 cytokines that, in turn, break tumor tolerance via a bystander effect, thus braking tolerance to tumor antigens and enhancing the immune response against the tumor. This hypothesis is supported by the high secretion of cytokines, such as IFNγ and IL-2, observed in the regional lymph nodes (Arancibia et al., 2012b) after hemocyanin challenge. Accordingly, mice that were primed with CCH or KLH, challenged with MBT-2 cells and then subjected to immunotherapy with the respective hemocyanin increased natural killer cell activity and exhibited cytokine environment polarization toward a Th1 response (i.e., IFNγ production increased significantly in mouse sera), which correlated with antibodies belonging to the Th1 isotypes (Moltedo et al., 2006). Moreover, in patients with superficial bladder cancer under intravesical KLH therapy, a significant increase of CD4+T lymphocytes in the submucosa and among urothelial cells was observed, in contrast to a slight increase in CD8+T lymphocytes (Jurincic-Winkler et al., 1995a; Jurincic-Winkler et al., 1995b). In addition, KLH-conjugated vaccines against cancer using mucin-like or ganglioside epitopes have induced tumor-specific antibodies of the IgG1 and IgG3 isotypes (Musselli et al., 2001).

7. Future prospects

The information available shows that the mollusk hemocyanins presented herein – including KLH, the traditional and most utilized hemocyanin in biomedicine, and **CCH**, **RtH**, **HtH** and **FLH,** the most recently characterized hemocyanins – differ in their immunogenicity and non-specific immunostimulatory properties, opening basic questions on the structural characteristics responsible for these differences. Moreover, it is important to note that although several subunits of these hemocyanins have been sequenced via cDNA and that the complete amino acid sequences for these subunits are currently available, until now, it has not been possible to express heterologous or synthetic hemocyanins, mainly because of their size, complex structures and glycosylations. Therefore, all of the hemocyanins mentioned in this review can be obtained only as purified biological products from their natural sources, emphasizing the importance of pre-clinically evaluating diverse sources of hemocyanins for biomedical purposes. In this respect, only CCH hemocyanin from *Concholepas*, which has been pre-clinically evaluated in a murine experimental model of superficial bladder cancer (Moltedo et al., 2006) and has been recently used as an adjuvant in DC immunotherapy of patients with prostate cancer, is considered a safe complement or alternative to KLH (Reyes et al., 2013; Salazar-Onfray et a. 2013).

Finally, from a basic science perspective, a detailed molecular understanding of the receptors and signaling pathways involved in hemocyanin-induced immunostimulatory processes in mammals will be required to determine the basis of the qualitative and quantitative differences in the effects of the different hemocyanins on tumor immunology. Undoubtedly, this knowledge will support the development of new and better biomedical applications of these proteins, as hemocyanins exert their effects as immunopotentiators without the unwanted inflammatory side effects of classical adjuvants that drive cell-mediated immune responses.

Acknowledgements

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