The effect of Hemocyanin Immuno-stimulatory in Innate and Adaptive Immunity and a potent tool in the development of Abs and Vaccines

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Abstract

Hemocyanins induce a potent Th1-dominant immune response with beneficial clinical outcomes when used as a carrier/adjuvant in vaccines and nonspecific immunostimulant in cancer.

Collectively, datas demonstrate that hemocyanins are able to trigger the release of proinflammatory factors with different patterns of cytokine expression, suggesting differential signaling pathways and transcriptional network mechanisms that lead to the activation of M1-polarized macrophages.

The immune response is triggered by a proinflammatory signal that arises from various components of the innate immune system that sense invading pathogens (52) or danger/alarm signals from injured cells (53), phenomena in which phagocytic cells and APCs are fundamental.

Macrophages can internalize almost any form of Ag (whether cell associated or soluble) either nonspecifically or via specific receptors, which allows them to stimulate T cells (55). hemocyanins were internalized by macrophages through pinocytic vesicles and by clathrin-dependent endocytosis, and these proteins were then slowly processed, an idea supported by their persistence for several days in vitro and in vivo.

All of the hemocyanins- maintained the down regulation of keyM2 cytokine genes, such as Il4, Il5, Il13, and Tgfb2, with differences in the type of gene and/or intensity.

There is a consensus that M1 macrophages activate a tumor-killing mechanism and antagonize the suppressive activity of M2 macrophages, which promote tumor growth and metastasis (16).

In this article, I discuss the Hemocyanins and the role in biomedicine, Clinical studies, In vivo studies, vaccines and the mechanisms of hemocyanin immunostimulation and the role of Hemocyanins in Cancer and autoimmune diseases in animal model.

Key Word: Hemocyanins, Innate Immunity, adaptive Immunity, Immunostimulatory, Vaccines, Viral Infections

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1. Introduction

Hemocyanins, the massive oxygen-transporting glycoproteins found freely dissolved in the hemolymph of some mollusks, are potent natural immune-stimulants 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 107 Da (1). 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 (2). Furthermore, hemocyanins can be used as non-specific immune-stimulants 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 (3). Biomedical interest in hemocyanins arose in the 1960s when their remarkable immunogenicity and immune-stimulatory properties in experimental animals, and in human beings, were discovered. Hemocyanins alone are capable of potently activating the immune system of mammals. Therefore, hemocyanins have been used as a biotechnological tool in the development of Abs and vaccines, and also as immunomodulators such as nonspecific immunostimulants in superficial bladder cancer (3). The hemocyanin purified from the giant keyhole limpet gastropod Megathura crenulata, commonly known as keyhole limpet hemocyanin (KLH), has traditionally been used for those purposes(4),(5),(6). However, the biodiversity of hemocyanins has prompted interest in developing new candidates with better biochemical and immunological properties because the supply depends on natural resources. Therefore, the immunostimulatory properties of these hemocyanins show differences in immunogenicity and immunomodulatory effects, indicating that different hemocyanins can activate diverse molecular and cellular pathways to promote Th1 immune responses. hemocyanins are incorporated by APCs, they induce a proinflammatory milieu that produces a bystander effect, which is a key event in their nonspecific immunostimulatory activity (7). Indeed, macrophages play an essential role in both innate and adaptive immunity as regulatory and effectors cells, and as one of the primary danger sensors in the host (8),(9). Macrophages display remarkable plasticity and flexibility; in response to Ags, they have the ability to polarize innate immune responses to produce various M1 or M2 patterns of cytokines that can be reversed in vitro and in vivo and that are stages that mirror the Th1/Th2 polarization of T cells (10),(11),(12). The hemocyanins used in this study present clear structural differences at this level, and experimental data have demonstrated that the KLH preparation is made up of two independent isoforms that coexist in variable proportions in the hemolymph of the animals, each composed of one type of subunit (13).

Another significant feature of the hemocyanin structure is the presence of carbohydrates, as heterogeneous N- and O-glycosylation ramifications, with mannose being the common and most abundant oligosaccharide (14),(15),(15),(16),(17),(18).

2. Structure of the mollusk hemocyanins

Mollusk hemocyanins are enormous glycoproteins (4–8 MDa) formed by an intricate arrangement 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 x 106 Da (19),(1),(20). An additional feature of hemocyanin structures are their carbohydrate contents, which play fundamental roles in their organization and their immunological efficacy (21). Gastropod hemocyanins contain variable, heterogeneous N-and O-glycosylation sites, with as high as 9% (w/w) mannose as the most abundant oligosaccharide (14). 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 (16),(4), as discussed later.

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 (22).

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) (23),(13). 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) (4). 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 (24).

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 (25), 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 (26),(17),(27).

In the 1970s, KLH was introduced in the clinic as part of a test to evaluate immunocompetence in immunosuppressed patients (28),(29), thus, its benefits for the treatment of recurrent superficial bladder cancer were discovered serendipitously (30), 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 (31). 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 (32).

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 (33). 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 (34). 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 Ca2+and/or Mg2+, in the storage media to maintain its structure (33). 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 (35),(36), and peptides (37),(38),(38),(39). 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 (40),(41). 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 (6),(42), 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 (43).

3.3. FLH

The hemocyanin from the black keyhole limpet *Fissurella latimarginata* (FLH) was discovered most recently (22). 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 (22). 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) (22). 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 (22).

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 (44). These isoforms are formed by the HtH1 and HtH2 subunits, and each has a molecular mass of approximately 392 kDa (26),(45).
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-methyl-d-galactose has been identified in HtH (24),(46). 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 (47).

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(48). RtH is a mixture of two hemocyanin isoforms, RtH1 and RtH2, that coexist in the animal's circulation (49). Both have carbohydrate contents of 2.6% (w/w), with very similar monosaccharide compositions featuring type N-glycosylation (24). 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 (50). Furthermore, antimicrobial activities against different strains of the genital herpes simplex virus have been reported for the RtH2 subunit (51).

4. 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 (52),(53). The mouse bladder tumor-2 cell (MBT-2) transplantable murine model of SBC was the first of these models to be developed in 1981(54). Mice were pre-immunized with 200 µg of KLH after a previous subcutaneous inocula- Mollusk Hemocyanins as Natural Immunostimulants in Biomedical Applications 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 (54). Further studies showed that priming was essential for achieving a therapeutic effect (55),(56),(57). 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 (58). 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 (59). 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 (60). However, in a subsequent study, the presence of endotoxin in KLH preparations partially accounted for its anti-tumor effect (61). The syngeneic orthotopic murine bladder cancer model MB-49 was evaluated in 1994 (25),(62). 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(62). 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) (63). Subcutaneous sensitization with 1mg of KLH, followed by intravesical and subcutaneous administration twice weekly, led to a decrease of BBN-induced bladder tumors (64). A subsequent study demonstrated that subcutaneous administration was more effective than intravesical instillation in terms of tumor growth and rats bearing tumors (65). Finally, more recent studies have evaluated the effect of CCH in the MBT-2 murine model (34),(42). 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(42). (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(34).

4.1. 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(66). 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 ofKLH had significantly reduced tumor recurrence rates over a period of two years(30). More than 10 years later, a controlled study corroborated that KLH was more effective than mitomycin C(67). Then, it was determined that the incidence of recurrence in patients with TCC related to urinary schistosomiasis was diminished after KLH treatment(68). However, studies with patients who were unresponsive to chemotherapeutic agents(69), or who had carcinoma in situ (CIS) (70),(71), 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. 4.3.1. 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 (72),(60),(73),(74). 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 (75).

4.2. 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 (76). 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 (77). 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 modelas its native counterpart (7). 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 (22).

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 (2). 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 (78). 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 CTLdefined antigen determinants on the same DC (32),(79). 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.

5.1. 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 (80), or mucoid exopolysaccharide (MEP) coupled to KLH demonstrated that significantly enhances its immunogenicity and the capacity to elicit opsonic antibodies in mice and rabbits (81). 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 PCspecific immune responses at the mucosal and serum levels (82). 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 (83).

5.2. 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 antipeptide antibodies in mice (84). 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 (85),(86). 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. (87).

5.3. 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 (88). However, a protective epitope of Candida albicans conjugated to KLH induced a Th1-type cytokine expression pattern in C57BL/ 6J mice (89).

5.4. 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 (90). 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 (91). In this case, the patient's responses are measured by DTH tests against hemocyanins or tumor extract (92). 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 (43).

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 (93),(94). The biological role of the high mannoseoligosaccharides 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(78). 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 (7). 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 (95),(96). 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 (22). 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 (7). 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 nonspecific 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-Mollusk Hemocyanins as Natural Immuno-stimulants in Biomedical Applications 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 (3), 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 (42). 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 (4),(97). In addition, KLHconjugated vaccines against cancer using mucin-like or ganglioside epitopes have induced tumor-specific antibodies of the IgG1 and IgG3 isotypes (98).

7. Conclusion and Future prospects

DCs and macrophages phagocytose Ags in a process that involves phagosomes, these structures can be modified over time by a range of cellular machinery before fusing with lysosomes to produce antigenic peptides (60).

Interestingly, the three hemocyanins induced significant elevations in the mRNA levels of CD40L (Cd40lg) in macrophages, which might support the observed nonspecific antitumor activity of hemocyanins in several cancers (6, 71). CD40–CD40L interactions promote the activation of NK cells by murine macrophages (72).

These cells play a fundamental role in innate immune surveillance via their direct cytolytic functions (e.g., destruction of nascent tumors) or regulatory capabilities (e.g., prevention of tumor metastasis dissemination via the expression of IFN-γ) (73,74).

Recent study showed a significant up-regulation of the IL-1α and IL- 1β genes in response to FLH and KLH and indicated the presence of these cytokines in the culture medium of macrophages; the ELISA Array detected small amounts Thus, the conformational and structural stability of a protein Ag may play crucial roles in its proteolysis, APC processing, T cell stimulation, and immunogenicity (79).

Macrophages might bind hemocyanins through receptors with different carbohydrate recognition domains that occur in certain C-type lectin receptors depending on the oligosaccharide (number, type, and substitution patterns of outer sugar branches) on the protein.

These receptors directly or indirectly trigger distinct signaling pathways, leading to the differential production of proinflammatory cytokines and promotion of the M1 immune response; thus, a bystander signaling pathway might be activated, increasing tumor cell killing or other latent-specific responses.

The transmission electron microscope (TEM) has contributed significantly to the understanding of KLH structure, primarily from negatively stained images. We give a brief account of TEM studies on the native KLH oligomers, the experimental manipulation of the oligomeric states, together with immunolabelling data and studies on subunit re-association

The major clinical use of KLH is specifically for the treatment of bladder carcinoma, with efficacy probably due to a cross-reacting carbohydrate epitope. KLH also has considerable possibilities for the treatment of other carcinomas, in particular the epithelially derived adenocarciomas, when used as a carrier for carcinoma ganglioside and mucin-like epitopes.

The widespread use of KLH as a hapten carrier and generalised vaccine component represent other major on-going aspects of KLH research, together with its use for the diagnosis of Schistosomiasis, drug assay and the treatment of drug addiction

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.

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