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Hemocyanins in the Immunotherapy of Superficial Bladder Cancer

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1. Introduction

Chemo- and immunotherapeutic approaches have been used to prevent recurrence of transitional cell carcinoma (TCC), the most common type of superficial bladder cancer (SBC). The bacillus Calmette-Guérin (BCG) vaccine for tuberculosis, which consists of an attenuated form of *Mycobacterium bovis*, is the most commonly used immunotherapeutic agent (Morales et al., 1976). Despite the successful results achieved with BCG, its serious side effects have led researchers to investigate other immunostimulatory substances. In the early 1970s, Olsson and collaborators reported that subcutaneous stimulation with keyhole limpet hemocyanin (KLH) from the Californian marine gastropod *Megathura crenulata* significantly reduced SBC recurrence frequency in TCC patients without any toxic side effects, making it ideal for long-term repetitive treatments (Olsson et al., 1974). These results provided promising support for the use of mollusk hemocyanins as alternative agents in SBC immunotherapy.

Hemocyanins, blue respiratory glycoproteins that were discovered in 1878 by Léon Fredericq (Ghiretti-Magaldi & Ghiretti, 1992), are found freely dissolved in the blood of some mollusks and arthropods. These proteins are giant structures with molecular weights between 4 and 8 MDa, and they exhibit some of the most complex and sophisticated quaternary structures known. Hemocyanins are part of the type-3 group of copper proteins that includes phenoloxidases and tyrosinases (Decker & Tuczec, 2000). These proteins contain active copper-containing sites in which the Cu(I,I) state is oxidized to the Cu(II,II) state, thus accounting for their distinctive deep blue color. Because of these properties, the biochemistry of hemocyanins has been intensively studied (van Holde & Miller, 1995). The pioneering work of Weigle in the 1960s on the immunochemical properties of KLH demonstrated its remarkable immunostimulatory properties in an experimental animal model (Weigle, 1964). These results were quickly incorporated into clinical studies to evaluate its immunological effects.

Because the primary amino acid sequences of mollusk hemocyanins are highly divergent from mammalian sequences, they are strongly recognized by the immune system, resulting in potent immunogenicity; these proteins can be used therapeutically as non-specific immunostimulants with beneficial clinical outcomes. Moreover, hemocyanins have been extensively used as carriers to generate antibodies against diverse hapten molecules and

peptides and to induce antigen-specific CD8⁺ and CD4⁺ T cell responses (Harris & Markl, 1999). Currently, hemocyanins are used as carrier-adjuvants for several tumor-associated antigens (TAAs), such as glycolipid and glycoprotein (mucin-like) antigens, in experimental therapeutic vaccines against certain cancers, including melanomas, sarcomas, breast, prostate, ovary and lung (Musselli et al., 2001; Schumacher, 2001; Zhu et al., 2009; Del Campo et al., 2011). Other therapeutic strategies that use hemocyanins include dendritic cell (DC) vaccines pulsed with tumor lysates to enhance interferon gamma (IFN- γ) production by tumor-reactive T cells (Timmerman & Levy, 2000; Shimizu et al., 2001; Millard et al., 2003; Lopez et al., 2009; Jacobs et al., 2010; Lesterhuis et al., 2011) and anti-idiotypic vaccines for some types of B cell malignancies (Leitch & Connors, 2005; Kafi et al., 2009). KLH has been the gold standard for these applications for over 40 years simply because it was used in earlier studies instead of other hemocyanins (Harris & Markl, 1999). The first studies used a research-grade KLH (non-GMP) containing different levels of endotoxin (Vandenbark et al., 1981); since then, several companies have produced clinical-grade KLH.

The versatile properties of KLH in biomedical and biotechnological applications have led to increasing commercial demand and growing interest in finding new, alternative hemocyanins with similar or more potent immunomodulatory properties. Although the KLH gene has been cloned, and its amino acid sequence is known, it has not been possible to express a heterologous protein, mainly because of its complex structure (Lieb et al., 2001; Markl et al., 2001; Altenhein et al., 2002). Therefore, this protein can be obtained only from its natural source. Several hemocyanins from other species of mollusks have been studied biochemically and immunologically, including *Haliotis tuberculata* (HtH, Abalon) (Markl et al., 2001); *Helix vulgarix* (HpH, Vineyard snail), *Rapana venosa* (RvH, Asian rapa whelk), and *Rapana thomasiana* (RtH, Black sea murex) (Dolashka-Angelova et al., 2003; 2008; 2010); and *Concholepas concholepas* (CCH, Loco), which is found on the Pacific Chilean coast (De Ioannes et al., 2004). Only CCH has been pre-clinically evaluated in a murine experimental model of SBC and may be considered a safe alternative therapy (Molledo et al., 2006; Atala, 2006). Although KLH and CCH have different origins and structure they have similar immunostimulatory capacities, suggesting that a conserved pattern common to both hemocyanins induces an ancient immunological mechanism (Molledo et al., 2006). Interestingly, we have described a new hemocyanin from *Fissurella latimarginata* (FLH) that exhibits higher immunogenicity than either CCH or KLH, opening a new avenue for research on the use of hemocyanins (Espinoza et al., 2006; Arancibia et al., 2010).

Notwithstanding the biomedical interest in mollusk hemocyanins, the molecular and cellular bases of their adjuvant/immunostimulatory capacity in SBC remain poorly understood. Currently, we know that hemocyanins are able to drive the differentiation of T helper (Th) cells toward a Th1 phenotype, characterized by increased secretion of IFN- γ and the production of IgG2a isotype antibodies (Molledo et al., 2006).

In this chapter, we will review what is currently known about the experimental and clinical uses of mollusk hemocyanins as non-specific immunostimulants to prevent SBC recurrence, including the details of their intricate structure and the immunologic mechanisms that have been proposed to explain their antitumor activity.

2. Structure of the mollusk hemocyanins

Because of their enormous size, mollusk hemocyanins are easily observed by transmission electron microscopy (TEM) using negative staining. These molecules have a cylindrical form

with an external diameter of approximately 350 nm and length of approximately 400 nm. Fig. 1 shows the characteristic appearance of gastropod hemocyanins under TEM.

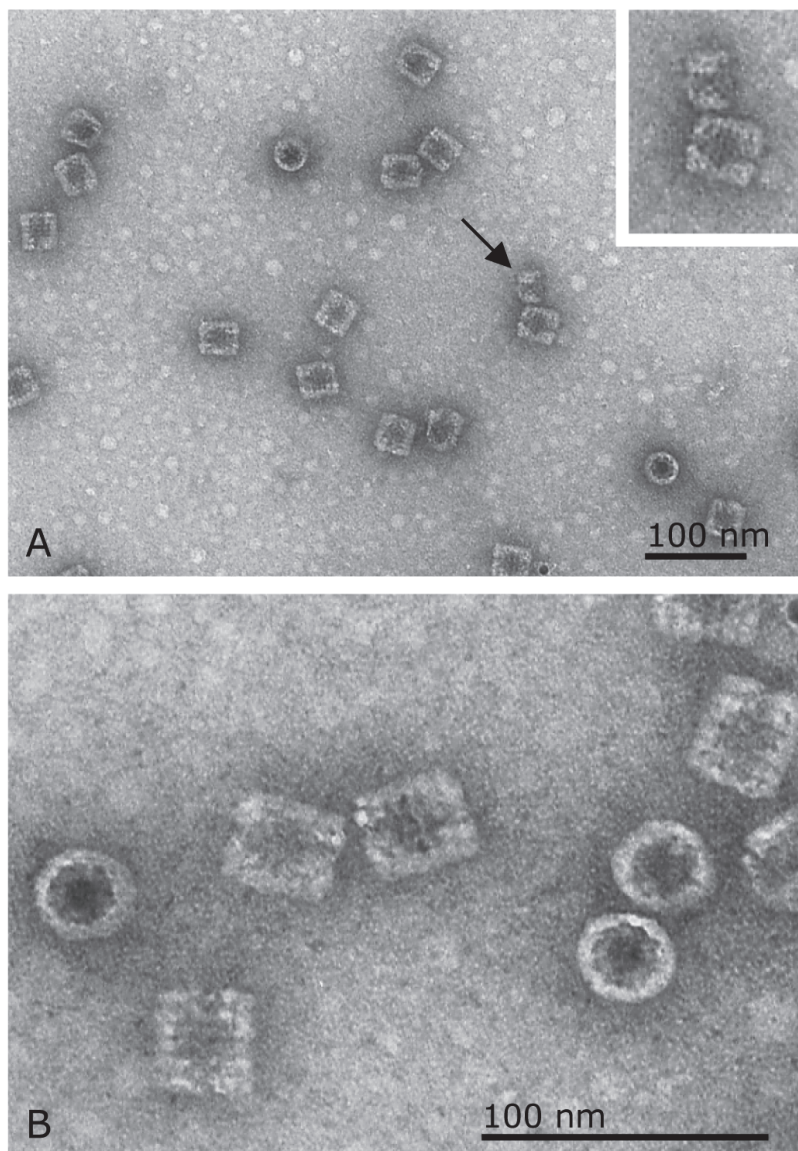


Fig. 1. Electron microscopy of negatively stained *C. concholepas* hemocyanin molecules. **A.** Low magnification micrographs of a preparation of the protein showing their characteristic hollow cylinder form. The images show the top (circles) and lateral (rectangles) views of the molecule. The arrow shows a decamer. **B.** High magnification images of hemocyanin molecules showing their intricate structure. The side views show the proteins' characteristic didecameric form with subunits arranged in layers.

Many experimental studies on hemocyanins, using different dissociation and association conditions and physicochemical and biochemical methods, have helped to elucidate their hierarchically organized structure (van Holde & Miller, 1995; Harris & Markl, 1999). As shown in Fig. 2, the basic structure of hemocyanins is composed of ten subunits that are self-assembled into a hollow cylinder, a structure known as a decamer, with a lumen that is narrowed by a complex collar (Harris et al., 1993; Cuff et al., 1998; Decker et al., 2007). In

gastropods, the decamers can self-associate face-to-face to form stable dimers or didecamers, which display an intricate internal arrangement and result in the formation of extremely large structures with approximate D5 symmetry (Orlova et al., 1997). Hemocyanin subunits have a molecular weight ranging from 350 to 450 kDa and are composed of a string of seven or eight globular domains called functional units (FUs), each with a molecular weight between 35 and 50 kDa. These FUs are connected by a short flexible linker peptide strand of 10 to 15 amino acid residues. Each FU has two well-separated copper atoms that reversibly capture O₂ molecules; one is called the A site, which is located towards the N-terminus, and the other is called the B site and is located downstream of the polypeptide (van Holde et al., 2001).

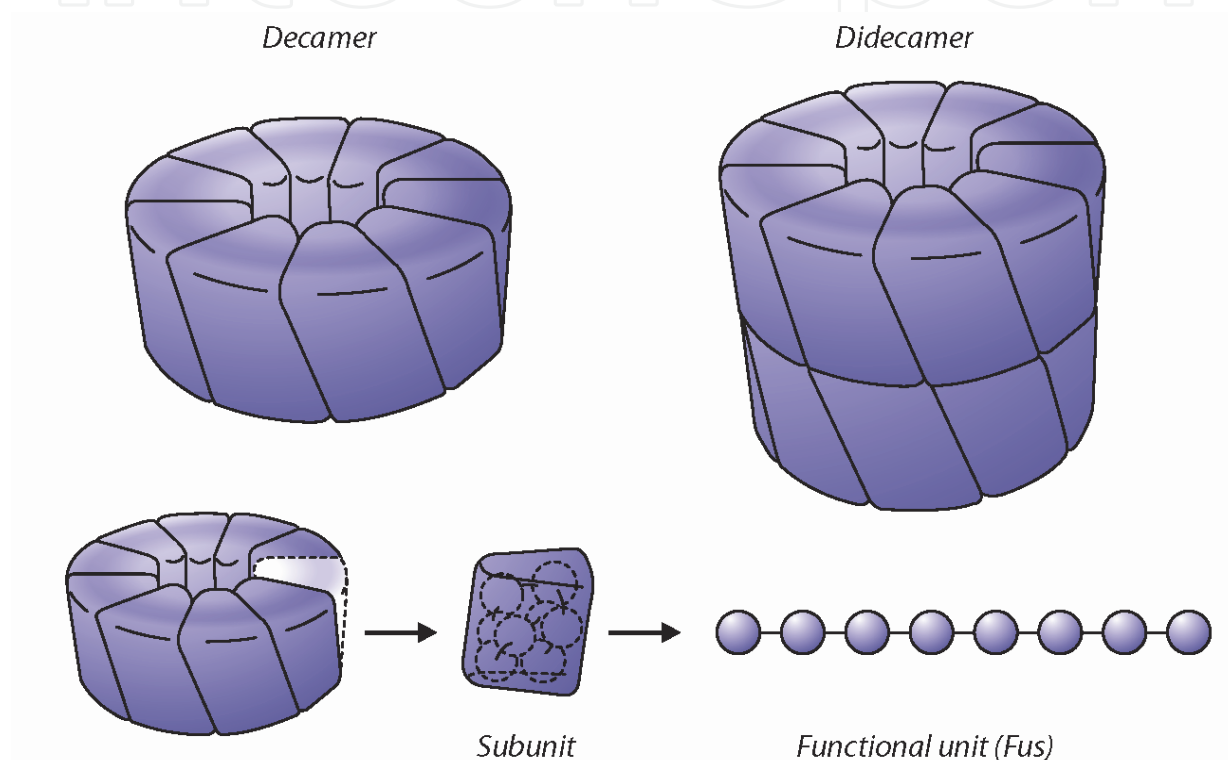


Fig. 2. Model of the structure of mollusk hemocyanin. The basic structure of a mollusk hemocyanin is a decamer, which is formed by the association of 10 polypeptides or subunits. In hemocyanins from some species of mollusk, such as gastropods, including KLH and CCH, the decamers are associated in pairs to form very large molecules called didecamers. The subunit consists of seven or eight globular domains linked by a peptide spacer consisting of 10 to 15 amino acid residues, similar to a pearl necklace. Each of these globular domains has a pair of copper atoms that reversibly bind one oxygen molecule, which is why they are called functional units.

Knowledge of the carbohydrate moieties present in mollusk hemocyanins has been essential for understanding their organization, antigenicity and biomedical properties (Paccagnella et al., 2004; Siddiqui et al., 2007). In fact, several authors have reported that hemocyanin carbohydrates may play a role in their immunostimulatory effects. The high carbohydrate content of hemocyanins, up to 9% (w/w), has been measured by different methods, including the use of lectins and high-pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The presence of numerous N-glycosylation sites and a reduced number of O-

glycosylation sites has been established (Dolashka-Angelova et al., 2003; Gielens et al., 2004; Idakieva et al., 2004; Gatsogiannis & Markl, 2009; Dolashka et al., 2010). Mollusk hemocyanins contain diverse sugar moieties, including mannose, D-galactose, fucose, N-acetyl-D-galactosamine and N-acetyl-glucosamine residues, with mannose being the most abundant (Harris & Markl, 1999). Hemocyanins also contain monosaccharides that are not usually found in animal proteins, such as xylose (Lommerse et al., 1997).

2.1 KLH and CCH

Although KLH and CCH each have two subunits that constitute the basic structure known as a decamer, closer analysis revealed unique differences. Native gel electrophoresis has shown that KLH is made up of two different non-covalently linked subunits called KLH1 (350 kDa) and KLH2 (350 kDa) that do not display shared epitopes (Swerdlow et al., 1996). Using the same approach, it was demonstrated that CCH is also made up of two different subunits, CCHA (405 kDa) and CCHB (350 kDa), that contain common and specific epitopes (Oliva et al., 2002; De Ioannes et al., 2004). In KLH, the subunits form homodidecamers (i.e., the molecules are formed from either KLH1 or KLH2 subunits). However, in CCH the subunits form heterodidecamers (i.e., the molecules are formed by pairing the two different subunits). In addition, purified KLH requires divalent cations in storage buffers to maintain the stability of its quaternary structure, whereas CCH does not (De Ioannes et al., 2004); this is probably a consequence of the higher hydrophobicity of CCH (Leyton et al., 2005). Despite these differences, the immunogenic properties of CCH and KLH are similar. CCH has been successfully used as a carrier protein to generate antibodies against hapten molecules and peptides (Becker et al., 1998; Torres et al., 1999; Mura et al., 2002; Duvillie et al., 2003; Manosalva et al., 2004; Cancino et al., 2007; Gravotta et al., 2007; Matus et al., 2007; Grenegard et al., 2008); as a carrier in vaccines (Miller et al., 2006; Mauldin & Miller, 2007; Pilon et al., 2007) and as an experimental antigen (Becker et al., 2007; Moltedo et al., 2009). Several studies have demonstrated that KLH contains approximately 3.2% (w/w) carbohydrate residues, displaying specific structural motifs on N-glycans, such as Fuc(α1-3)GalNAc, Gal-(β1-6)Man-, Gal(β1-4)Fuc-, and Gal(β1-4)Gal(β1-4)Fuc-, which are thought to contribute to its non-specific immunostimulatory capacities in SBC (Wuhrer et al., 2004). Our knowledge of the corresponding oligosaccharide composition of CCH is very limited. However, we have demonstrated using selective glycosidase treatments and electrophoretic analysis that sugar moieties account for 3.1% (w/w) of the mass of CCH. A comparative analysis using lectin staining indicated that mannose is the only exposed carbohydrate common to CCH and KLH (Becker et al., 2009). It is important to note that, despite the differences in carbohydrate composition between KLH and CCH, both proteins have similar immunogenicity and immunotherapeutic capacity in SBC, suggesting that other factors are responsible for this effect. We assume that the primary structure of these proteins contains the determining factor because they share regions of high sequence homology (van Holde et al., 2001; Manubens et al., 2010). These regions were confirmed in antibody cross-reactivity experiments that revealed the presence of common or mimetic epitopes in CCH and KLH (Oliva et al., 2002).

3. Use of hemocyanins in experimental SBC

Rats and various strains of mice have been used as *in vivo* SBC models to evaluate therapeutic agents because bladder tumors in these rodents have similarities with human

tumors. In addition, tumor cells can be established subcutaneously (heterotopically) or in the bladder (orthotopically) by either transplantation or chemical induction, allowing the investigation of clinical aspects such as pharmacokinetics and toxicity (Gunther et al., 1999; Linn et al., 2000; Arentsen et al., 2009).

The first controlled study of a hemocyanin as immunotherapy in the treatment of superficial bladder cancer was published in the 1980s by the Lamm group (Lamm et al., 1981). They developed the mouse bladder tumor-2 cell (MBT-2) transplantable murine model of SBC and demonstrated that pre-immunization with 200 µg of KLH three weeks prior to subcutaneous injection with MBT-2, followed by intralesional immunotherapy with 50 µg one and seven days after inoculation, resulted in a significant reduction in tumor growth and a prolongation of animal survival. Later, other studies by the same researchers evaluated non-specific immunotherapeutic regimens (Lamm et al., 1982). Animals received an intradermal MBT-2 inoculation, and the immunotherapy was administered intralesionally one day after tumor transplantation. Tumors were excised at a volume of 400 mm³, and the animals were re-challenged with tumor cells, treated again, and followed for tumor incidence, growth rate and survival. This study demonstrated that KLH had a weak antitumor effect compared with the response to BCG. In 1986, Lau and collaborators studied the same response, this time comparing intraperitoneal and intralesional administration of the agents. C3H/He mice were injected subcutaneously with 5×10^4 tumor cells. After that, the mice received either intraperitoneal or intralesional treatments (50 µg KLH); these experiments demonstrated that the intralesional route was more effective than intraperitoneal administration for tumor growth inhibition (Lau et al., 1986).

Lamm's group also evaluated the possible additive and/or synergistic effects of KLH immunotherapy in the MBT-2 model in conjunction with other treatments, such as IFN-α. Tumor cells were transplanted subcutaneously without prior immunization. Treatment was given intraperitoneally twice weekly for three weeks, except for BCG, which was administered once a week. Significant reductions in tumor incidence relative to the controls were observed in groups receiving KLH (42%), IFN-α (42%) and KLH + IFN-α (17%) (Riggs et al., 1992). The following year, the same group compared two alternative immunotherapies in the MBT-2 model: crude KLH and Immucothel, a clinical-grade KLH from Biosyn Arzneimittel GmbH. Mice were sensitized with 50 or 100 µg KLH, and 21 days later, 10^3 tumor cells were injected. Intralesional treatment with 50 or 100 µg KLH was performed on days 1, 7 and 13 or 14. Crude KLH required either immunization before tumor transplant or frequent therapy after transplantation to be effective. In addition, Immucothel required pre-immunization to be effective, even with an increased frequency and dosage of the post-transplant immunizations. In a subsequent study, the endotoxin contamination of KLH was demonstrated to be partly responsible for the antitumor activity because treatment with endotoxin alone resulted in a significant reduction of tumor growth and mortality (50% survival) (Lamm et al., 1993). Moreover, KLH + 100 Endotoxin Units (EU) resulted in complete inhibition of tumor growth and 100% survival. KLH + 1000 EU appeared to reduce the antitumor response (50% survival), suggesting that endotoxin may interfere with the response to purified KLH. Finally, endotoxin-free KLH induced antitumor responses (50% survival). However, pre-immunization was required for KLH to exert a significant (75% survival) antitumor effect (Lamm et al., 1993).

Walsh and collaborators studied KLH immunotherapy in two different models with no promising results. First, they transplanted 2.5×10^6 MBT-2 tumor cells subcutaneously after pre-immunization 20 days prior. Treatment was given on days 1, 8 and 18 in the form of

subcutaneous or intravesical injection of 50 µg KLH. The results showed no difference between the control and treated groups in terms of either tumor growth or animal survival. Alternatively, they transplanted 2.1×10^6 MBT-2 tumor cells into the bladder of C3H/He mice. The bladder was irrigated with 1.5 mg N-methyl-N-nitrosourea 48 hours prior. The treatment group was injected with 50 µg KLH on day 1, and the bladders were instilled with 200 µg KLH on days 14 and 21. There was no significant difference from the control group (Walsh et al., 1983). Using a similar model, Marsh and collaborators demonstrated that intravesical immunotherapy with *Corynebacterium parvum* and *Allium sativum* was more effective than KLH and slightly more effective than BCG. MBT-2 cells were delivered into the bladder transurethrally using a small catheter, and the immunotherapy was administered directly into the bladder via this catheter on day 1 or day 6, or both. The authors associated the lack of a significant effect with inappropriate dosage or insufficient stimulation of the immune system (Marsh et al., 1987). Later, the antitumor activity and potential toxicity of a clinical grade KLH preparation named KLH-Immune Activator (KLH-IA) was examined. Mice were immunized subcutaneously with KLH-IA two weeks prior to intravesical implantation with 2×10^4 MB-49 tumor cells. Treatment consisted of intravesical KLH-IA (10 or 100 µg) 1, 4, 7, 14 and 21 days after implantation. By four weeks after implantation, tumor outgrowth in the treated groups was significantly decreased. Prior subcutaneous immunization was required to elicit the antitumor activity of KLH-IA. Animals treated with a dissociated form of KLH showed decreased tumor outgrowth, but this was not significant. A separate toxicity study in which KLH-IA was given subcutaneously (4 mg/kg), intraperitoneally (40 mg/kg) or intravesically (40 mg/kg) reported no significant gross or histopathological abnormalities, except for mild to moderate papillary hyperplasia in all catheterized animals (Swerdlow et al., 1994).

A third model developed by Recker and collaborators also showed the effectiveness of KLH. Bladder carcinoma was induced in Wistar rats using N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). Stimulation of the rats with 12.5 mg of KLH administered intravesically and 0.5 mg administered subcutaneously twice weekly after sensitization with 1 mg subcutaneous KLH resulted in a reduction in BBN-induced bladder tumors. These results confirmed that effective induction of an immune response is important for the control of tumor development because immune-suppressed rats treated with cyclosporine A (CsA) showed enhanced bladder tumor expansion compared with rats treated with 0.05% BBN alone (Recker & Rubben, 1989). A subsequent study distinguished between intravesical and subcutaneous application to determine the most effective treatment regime. Five weeks after the completion of tumor induction with 0.05% BBN solution, exophytic bladder tumors appeared in all control animals. In group 2, which was given KLH via intravesical instillation, tumors developed in 73.5% of cases. In group 1, with subcutaneous administration, tumors developed in only 50% of cases. The tumor growth was significantly slower in group 1 than group 2 (Linn et al., 2000).

The results described above demonstrated promising potential for the use of KLH in SBC therapy. More recently, preclinical studies have proven hemocyanin from *Concholepas concholepas* (CCH) to be a reliable alternative to KLH (Molledo et al., 2006). C3H/He mice were primed with CCH before subcutaneous implantation of MBT-2 cells. Treatment consisted of a subcutaneous dose of CCH (1 mg or 100 µg) at different intervals after implantation. The results demonstrated a significant antitumor effect, as indicated by decreased tumor growth and incidence, prolonged survival and a lack of toxic effects. These results were similar to those achieved with KLH.

Model	Priming ¹	Via Administration	Therapeutic Dosage and Schedule ²	Results	Reference
Mouse, MBT-2	Yes 200 µg	Intralesional	50 µg Days: 1 and 7.	Significant reduction of tumor growth and survival with KLH.	Lamm et al.1981
	No	Intralesional	Day: 1	KLH presented a minor antitumor effect compared with BCG.	Lamm et al. 1982
	Yes	Subcutaneous or intralesional	50 or 200 µg Days: 1, 8 and 18 or 1, 14 and 21.	KLH do not show difference with controls in tumor growth or animal survival.	Walsh et al. 1983
	No	Intraperitoneal v/s intralesional	50 µg	Intralesional route of inoculation of KLH was more effective.	Lau et al. 1986
	No	Intravesical	50 µg Days: 1 or 6, or both.	Immunotherapy with <i>C. parvum</i> and <i>A. sativum</i> was more effective than KLH.	Marsh et al.1987
	No	Intraperitoneal	50 µg Twice weekly for 3 weeks.	Better response in the animals treated with KLH more INF-α.	Riggs et al. 1992
	Yes 50 or 100 µg	Intralesional	50 or 100 µg Days: 1, 7 and 13 or 14.	Required pre-immunization of KLH and Immucothel to be effective.	Lamm et al. 1993a
	Yes 50 or 100 µg	Intralesional	50 or 100 µg Days: 1, 7 and 13 or 14.	Endotoxin contamination of KLH was responsible in part for the antitumor activity.	Lamm et al. 1993b
	Yes 200 to 400 µg	Subcutaneous	1 mg Days: 1 to 6 or 100 µg Days: 1, 3, 5, 7 and 9.	Significant reduction of tumor growth and survival with CCH.	Molledo et al. 2006
	Yes 200 µg	Subcutaneous	100 µg Days: 1, 3, 5, 7 and 9.	Better antitumor effect with CCHA subunit than CCHB subunit.	Becker et al. 2009
Mouse, MB-49	Yes 100 µg	Intravesical	10 or 100 µg Days: 1, 4, 7, 14 and 21.	Prior immunization of KLH-IA was required to elicit antitumor activity.	Swerdlow et al. 1994
Rats, tumor induction with BBN ³	Yes 1 mg	Intravesical and subcutaneous	12.5 mg and 500 µg Twice weekly.	Reduction of bladder tumors with KLH.	Recker et al. 1989
	Yes 1 mg	Intravesical v/s subcutaneous	500 µg Twice weekly for 8 weeks.	Subcutaneous route of KLH was more effective than intravesical route.	Linn et al. 2000

¹ Priming: Usually, around two weeks prior to tumor challenge. ² Immunotherapy after tumor transplantation. ³ BBN: N-butyl-N-(4-hydroxybutyl) nitrosamine

Table 1. Preclinical studies in different animal models of SBC with KLH or CCH as an immunotherapeutic agent.

Later, the individual contributions of the CCHA and CCHB subunits of CCH as immunotherapeutic agents in the same bladder cancer model were studied. C3He/He mice were subcutaneously primed with CCHA or CCHB; whole CCH and PBS were used as positive and negative controls, respectively. After day 15, mice were challenged with a subcutaneous injection of 2×10^5 MBT-2 cells, and the antitumor treatment was started; treatment consisted of a subcutaneous dose of either subunit or a control on alternate days for 9 days. Surprisingly, either subunit alone showed an antitumor effect in the MBT-2 model. However, the tumor incidence was lower in animals treated with CCHA (44% incidence) than with CCHB (60% incidence) or whole CCH (62.5% incidence). Moreover, the survival probability increased in mice under immunotherapy with CCHA (69.5%) compared with CCHB- (64%), CCH- (60%) and PBS-treated (46.5%) mice. In conclusion, this study indicated that the CCHA subunit accounts for the most important immunogenic effects of CCH (Becker et al., 2009). Together, these preclinical studies (summarized in Table 1) demonstrated that hemocyanins have beneficial effects in animal models of SBC that resemble human disease without the negative side effects of BCG (Schenkman & Lamm, 2004).

4. Use of hemocyanins in clinical studies of SBC

Surgical procedures such as transurethral resection (TUR) are commonly used as the first option to treat SBC. However, there are some tumors that must be treated by other strategies, due to the difficulties of fully removing them and the high risk of recurrence. Thus, intravesical administration of chemotherapeutic and biological agents has been demonstrated to be an effective method in the early stages of the disease, either to treat an existing tumor or to prevent recurrence and tumor progression after TUR (Perabo & Muller, 2004). BCG is one biological therapy that is used as a non-specific immunostimulant to treat several malignant tumors (Edwards & Whitwell, 1974; Milas & Withers, 1976), including SBC (Morales et al., 1976). BCG has become the first-line treatment and the most effective intravesical immunotherapy, lowering the risk of recurrence to an average of 27% of cases (Nseyo & Lamm, 1997). Despite these successful results, BCG therapy causes numerous side effects, such as dysuria, urinary frequency, cystitis (90% of cases), hematuria and, in rare cases, sepsis, indicating the need for new approaches that provide the same or a better response without toxic effects (Lamm, 2003).

In a 1974 delayed-type hypersensitivity (DTH) experiment to measure the immune competence of patients with TCC, Olson and collaborators reported the unexpected result that patients subcutaneously primed with 5 mg of KLH and then subcutaneous immunized with 200 μ g of KLH had a significantly diminished tumor recurrence rate over a study period of two years. Those patients that were DTH positive to KLH, and therefore immune competent, had almost no recurrences (Olsson et al., 1974). This outstanding effect was confirmed many years later in a controlled study of patients in stages Ta and T1 who had previously been subject to TUR. In this study, the ability of KLH to prevent tumor recurrence was compared to mitomycin C (MMC). The patients were subcutaneously immunized with 1 mg of KLH and then received monthly intravesical administrations of 10 mg of KLH. Only 14% of the patients treated with KLH had recurrences, in contrast to the MMC patients, 39% of whom reported recurrences, demonstrating that KLH was significantly more effective than MMC (Jurincic et al., 1988).

A prospective randomized trial compared the effects of ethoglucid and KLH in patients who were unresponsive to the chemotherapeutic treatments, doxorubicin or MMC. The recurrence rate and the tumor progression rate for the two therapies showed no statistical differences (Flamm et al., 1990). Wishahi et al., reported that the incidence of recurrence in patients with TCC associated with urinary schistosomiasis was 15% after KLH treatment compared with 77% before therapy (Wishahi et al., 1995). This result was similar to the results obtained by Olson et al., (1974) and Jurincic et al., (1998) confirming the outstanding immunotherapeutic properties of KLH (Olsson et al., 1974; Jurincic et al., 1988). The efficacy of this treatment in patients with carcinoma *in situ* (CIS) grade 3 was studied in a long-term follow-up. The patients received an intravesical instillation of KLH weekly for 6 weeks, monthly for 1 year and bimonthly for the following 2 years. Patients who were unresponsive to KLH were treated with BCG. CIS long-term remission was observed only in a limited number of cases, and most cases progressed over time, indicating the aggressiveness of this disease (Jurincic-Winkler et al., 1995a). In Table 2, we summarize the clinical studies previously described.

Currently, Immucothel, a clinical-grade KLH preparation, is being evaluated in a Phase III clinical trial in Germany for its efficacy in SBC treatment (Biosyn). The Food and Drug Administration (FDA) has also authorized another Phase III trial to evaluate the efficacy and safety of KLH BCI-Immune Activator (Intracell, USA) versus doxorubicin in BCG refractory or intolerant patients with carcinoma *in situ*, with or without resected SBC. However, this study has been suspended.

The mechanism associated with the immunotherapeutic effect of KLH in this disease is still poorly understood. However, there are immunohistological studies on biopsies of TCC patients treated with KLH that show strong cellular activation characterized by the infiltration of large numbers of mononuclear cells and CD4+ lymphocytes, and to a lesser extent, CD8+ T cells and granulocytes, nine months after the beginning of therapy (Jurincic-Winkler et al., 1995b). This result suggests that the effect of KLH might be strongly related to a non-specific immunostimulation of the immune system leading to the development of an antitumor response.

5. Immunologic mechanisms involved in the immunotherapy of SBC with hemocyanins

Although hemocyanins are widely used as thymus-dependent model antigens, the relationship between the structure of hemocyanins and the molecular and cellular basis of their immunostimulatory capacity is still largely unknown. Investigations into the antitumor effect of hemocyanins in human and murine models of SBC have demonstrated a systemic activation of the immune response. In these experiments, priming with hemocyanins is crucial for the induction of antitumor activity (Lamm et al., 2000; Moltedo et al., 2006). This could partially explain why hemocyanins stimulate the immune system. In patients with TCC under intravesical KLH therapy, DTH reactions occur. As mentioned previously, studies on biopsies of TCC patients treated with KLH showed a higher increase in CD4+ cell infiltration than CD8+ T lymphocytes in the submucosa and urothelial cells (Jurincic-Winkler et al., 1995b). Currently, we know that such responses are characteristic of Th1 type responses, which mediate inflammatory functions critical for the development of cell-

mediated immune responses (Szabo et al., 2003). Other investigations demonstrated that during immunization with KLH, the T CD4+ lymphocyte response showed a mixed profile of IL-4 and IFN- γ with an increase in T CD8+ cells in the lymphatic nodules (Doyle et al., 1998).

Patients	Control Group	Priming	Therapeutic Dosage and Schedule	Recurrence Rate	Reference
19	10	5 mg subcutaneous	200 μ g Subcutaneous	11%	Olsson et al. 1974
44	23	1 mg subcutaneous	10 mg Intravesical, monthly for 21 months, approximately.	14%	Jurincic et al. 1988
84	46	1 mg subcutaneous	30 mg Intravesical, weekly for six weeks and then monthly for one year.	55%	Flamm et al. 1990
13	Own controls	1 mg subcutaneous for five days until DTH	10 mg Intravesical, for seven days.	15%	Wishashi et al. 1995
21	Own controls	No	20 mg Intravesical, weekly for six weeks and then monthly for one year or bimonthly for two years.	43% of patients presented long-term remission 57% had to be cystectomized because of CIS progression	Jurincic-Winkler et al. 1995a

Table 2. Clinical studies using KLH as an immunotherapeutic agent in SBC patients.

The fact that the non-specific immunotherapeutic effects of hemocyanins are not due to any super-antigen-like activity, but rather rely on adequate priming, strongly suggests that their therapeutic properties could be attributable to a bystander effect on the tumor due to either a loss of tolerance toward tumor antigens or an enhancement of the immune response to the tumor. This kind of response would favor a milieu that augments the antigen-specific activity of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cell responses. These hypotheses are supported by the observation that IFN- γ and IL-2 are secreted in the regional lymph nodes in response to hemocyanin treatment (Gilliet et al., 2003; Verdijk et al., 2009). NK cells are strongly stimulated by IL-2 secreted by T lymphocytes, leading to their differentiation into lymphokine-activated killer cells (LAK) and increasing the destructive elements acting on tumor cells. It has been reported that

MBT-2 cells do not grow when they are injected into the bladders of mice treated with a combination of IL-2 and the cytotoxic agent cyclophosphamide (Ikemoto et al., 1997). Moreover, KLH has been shown to enhance NK cell activity and stimulate IFN- γ secretion in SBC patients (Molto et al., 1991). Our later results confirm these observations; mice treated with KLH or CCH increase NK cell activity and serum levels of IFN- γ (Molledo et al., 2006). This is a very important result because, in primary tumors, IFN- γ is a tumor suppressor cytokine that coordinates T and NK cell activities (Kaplan et al., 1998). Indeed, it has been demonstrated that the depletion of NK cells abolishes the immunotherapeutic effect of BCG on bladder cancer in mice, confirming that these cells play a key role in the destruction of primary tumors (Brandau & Bohle, 2001).

In addition to the antitumor effect provided by the secretion of IFN- γ , NK cells can delay tumor growth by means of antibody-dependent cell-mediated cytotoxicity (ADCC), which induces effector cells to kill bladder tumor target cells. We have observed that, in the MBT-2 model, intralesional CCH or KLH induce an increase in the humoral immune response against cell surface tumor antigens in addition to the CCH or KLH antibody response. Biopsies taken from the surrounding bladder tissues in SBC patients treated with KLH showed an increase in the B lymphocyte population in the lymph follicles, suggesting that humoral mechanism are also involved in the immune response induced by hemocyanins (Jurincic-Winkler et al., 1995b).

Finally, the fact that the immunotherapeutic effects of KLH and CCH on bladder cancer do not require an adjuvant raises intriguing questions regarding the means by which hemocyanins initiate the non-specific anti-tumor immune response and which cells are involved. It is possible that hemocyanins interact with a putative receptor on the cell surface of antigen presenting cells, leading to their internalization and processing. A promising candidate was the mannose receptor because of the high levels of this sugar residue in KLH and CCH and the fact that this receptor is highly expressed in antigen presenting cells. However, experiments on endocytosis inhibition performed in human DCs cultured *in vitro* with an anti-mannose receptor antibody and KLH showed that while KLH incorporation by DCs was partially inhibited, KLH still promoted the activation and maturation of DCs 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). In contrast, Teitz-Tennenbaum and collaborators (2008) demonstrated that murine DCs pulsed with KLH for 18 hours *in vitro* did not undergo DC maturation, a result that is consistent with *in vivo* experiments (Teitz-Tennenbaum et al., 2008; Molledo et al., 2009) and our current results. We observed that DCs internalized (Fig. 3) but did not mature within 72 hours of culture *in vitro* with this protein.

It is not known whether hemocyanins might be processed and presented by bladder tumor cells themselves, leading to the stimulation of the cytotoxic killer cell antitumor activity. Murine bladder tumor cells have been shown to be able to present BCG antigens to specific CD4⁺ T lymphocytes in a classic MHC Class II (Ia)-dependent fashion (Lattime et al., 1992). Experiments performed in our laboratory demonstrated that primary cultures of mouse bladder epithelial cells and MBT-2 cells cultured *in vitro* incorporate hemocyanin; however, we did not observe any changes in the expression pattern of MHC I and MHC II antigens (Del Campo et al., 2007). In addition, *in vitro* anti-cancer effects of KLH against breast, esophageal, prostate and pancreas cancer has been reported (Riggs et al., 2002), also in melanoma (Somasundar et al., 2005), however if this effect have an *in vivo* implication is unknown.

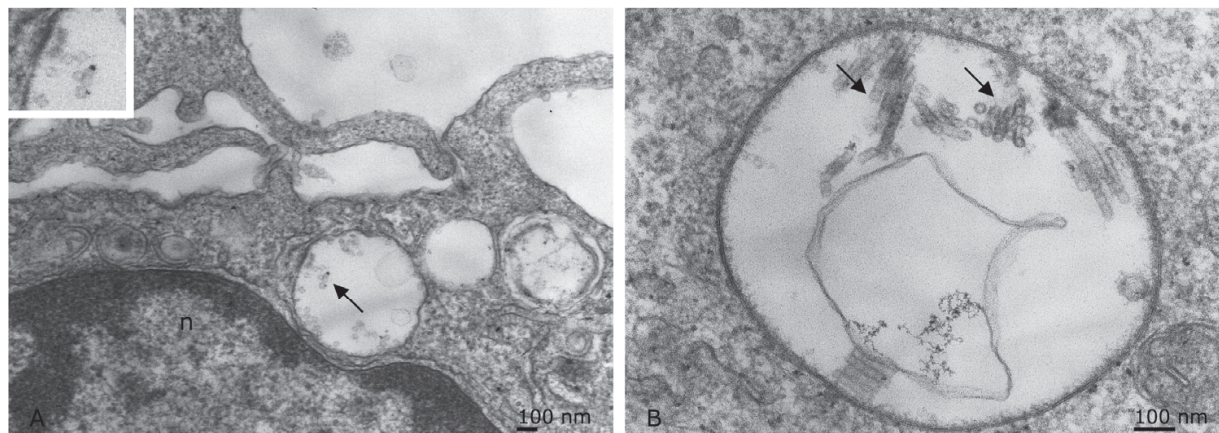


Fig. 3. Incorporation of *Concholepas* hemocyanin by mouse myeloid dendritic cells cultured *in vitro*, analyzed by transmission electron microscopy. Mouse myeloid DCs of 5th day of culture *in vitro* as described (Inaba et al., 1992), previously isolated by positive selection with immunomagnetic beads, and later culture with CCH during different times. **A.** Dendritic cell cultured during 30 minutes with CCH. The photograph shows its characteristic superficial membrane process, the nucleus (n), and hemocyanin molecules inside a clear vacuole (arrow) that resemble a primary lysosome. **B.** Because of the large size of CCH, and because of its peculiar structure as a hollow cylinder, we were able to identify the presence of whole hemocyanin molecules inside secondary lysosome like vesicles (arrows) containing membrane debris (Del Campo et al., 2007).

Macrophages are another potential cell type through which hemocyanins could initiate anti-tumor immune responses. Indeed, IL-1 α , a pro-inflammatory cytokine produced by activated macrophages, has been shown to be increased in the urine after intravesical instillation with KLH in patients with SBC (Jurincic-Winkler et al., 1995c). Similarly, this cytokine, in addition to other pro-inflammatory cytokines, has been detected in the urine after BCG instillation along with an influx of mononuclear cells into the bladder (Teppema et al., 1992; Brandau & Bohle, 2001; Brandau et al., 2001).

In summary, considering that BCG is a whole organism, whereas KLH or CCH are single molecules, it is amazing that it induces a similar response. In both cases, however, it is not clear which cytokines and cells contribute directly to the anti-tumor activity and which represent a secondary phenomenon.

6. Conclusions

Hemocyanins have proven to be safe and useful in the immunotherapy and prophylaxis of patients with superficial bladder cancer who have failed or are intolerant to the current BCG therapy. Moreover, KLH has been shown to produce a more predictable reaction than BCG, eliminating the risk of further infections. Despite the fact that biomedical interest in mollusk hemocyanins goes back more than 40 years, the precise molecular and cellular mechanisms underlying the non-specific immunostimulatory capacities of KLH and, more recently, CCH, are poorly understood. The current evidence shows that these huge proteins can induce an inflammatory milieu and activate innate immunity, driving a vigorous antitumor

adaptive immune response characterized by long-lasting HLA-DR+ cell infiltration into the bladder and the secretion of a Th1-type cytokine profile.

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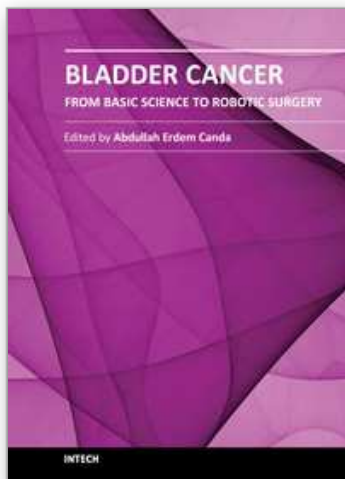
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This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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