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Identification of DHBcAg as a potent carrier protein comparable to KLH for augmenting MUC1 antigenicity

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Abstract

MUC1 is expressed at the cell surface of epithelial cancers. We have shown previously that MUC1 conjugated to keyhole limpet hemocyanin (KLH) plus the saponin immunological adjuvant QS-21 induces consistent high titer IgM and IgG antibodies in patients after treatment of their primary or metastatic cancers. KLH however is poorly soluble and heterogeneous making it difficult to work with, and we hypothesize that changing carrier proteins mid-way through a vaccination schedule would further increase antibody titers. Consequently, there is need for an alternative potent carrier protein. Duck Hepatitis B core antigen (DHBcAg) has a molecular weight of approximately 25 kDa and is easily purified as a single band, but it self aggregates into particles of approximately 6.4×10^6 Da. Consequently, it is highly immunogenic, easy to work with and amenable to chemical and genetic conjugation to antigens such as MUC1. We compare here in mice the immunogenicity of MUC1 chemically conjugated to KLH or DHBcAg and MUC1-DHBcAg recombinant protein after an initial series of three vaccinations and then after an additional series of three vaccinations with the same or opposite carrier, all mixed with the saponin immunological adjuvant GPI-0100. High titer IgG antibodies were observed in all groups after the initial three vaccinations: MUC1-DHBcAg median ELISA titer 1/51200, RecMUC1-DHBcAg 1/25600 and MUC1-KLH 1/12800. This increased to 1/6553600 after the second set of three immunizations when the carrier remained the same in all three groups, but titers were significantly lower when the carriers were changed for the final three immunizations. These data demonstrate that DHBcAg is an excellent carrier protein and that changing carrier proteins does not further augment immunogenicity.

Keywords: Cancer vaccine; Carrier protein; DHBcAg

1. Introduction

One of the changes associated with malignant transformation is altered expression of cell surface glycolipids, glycoproteins and mucins [1,2]. Mucins are a heterogenous group of glycoproteins of high molecular weight. Epithelial cells in a wide range of organs including the gastro-intestinal, genitourinary and respiratory tracts express a dense layer of these mucins primarily at secretory borders [3,4]. Among the many mucins identified, the most widely studied is MUCIN-1 (MUC1). MUC1 contains a large number of 20 amino acid tandem repeats [5,6]. In normal tissues MUC1 is heavily glycosylated and its distribution is limited to the apical surface of ductal or luminal cells [3,4]. In malignant tissues there is an increased expression of under-glycosylated MUC1 distributed over the entire cell surface [4,7]. Under-glycosylation of MUC1 in malignancies unmasks novel epitopes that may not be recognized on normal tissues. Several different epitopes in the tandem repeat portion of under-glycosylated MUC1 have been shown to be immunogenic in cancer patients [7,8].

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Since MUC1, like most tumor antigens, is an autoantigen also found in a variety of normal tissues (though differentially glycosylated), it is poorly immunogenic. We have screened a variety of approaches to augmenting the immunogenicity of defined peptide and carbohydrate cancer antigens. Approaches tested to date include incorporation of antigens into a variety of liposomes or *Neisseria meningitidis* outer membrane protein vesicles termed Proteosomes [9], adherence to the surface of Bacillus Calmette-Guerin (BCG) or Salmonella mutant R595 [10], conjugation to a variety of carrier proteins [11] and the use of a variety of immunological adjuvants [12,13]. Conjugation of these defined peptide or carbohydrate antigens to immunogenic carrier proteins plus the use of a potent immunological adjuvant has consistently been the optimal approach. In our experience, keyhole limpet hemocyanin (KLH) was the optimal carrier and saponins such as QS-21 or GPI-0100 the optimal adjuvants. With regard to MUC1 peptide vaccines in the mouse, mixture of MUC1 peptide with BCG or QS-21 or the use of MUC1-KLH conjugate in the absence of a potent immunological adjuvant results in a minimal serologic response (<1/900) while the use of the MUC1-KLH conjugate plus QS-21 or GPI-0100 resulted in optimal responses with titers averaging over $1/10^6$ [12,14]. This same approach to immunization with MUC1 peptide was found to induce consistent antibody responses against MUC1 peptide and cancer cells expressing partially glycosylated MUC1 when tested in breast cancer patients, but unlike the case in the mouse where MUC1 is a xenoantigen, the peak antibody titers averaged 1/10,000 or lower and generally decreased in titer to 1/1280 despite continued immunizations [15]. Comparable or more pronounced decreases in antibody titer despite continued vaccinations have also been consistently seen with carbohydrate antigens in glycolipid (GM2, GD2, GD3, Globo H and Lewis Y antigen) and glycoprotein (Tn, sTn and TF antigens)-KLH conjugate vaccines [16,17].

While KLH was the most effective carrier we had tested, there are significant drawbacks to its use. It is poorly soluble, large and heterogenous. There is a need for a more immunogenic carrier protein that is more soluble and less heterogenous. Furthermore, it was our expectation that higher or more prolonged high antibody titers would result from changing the carrier molecule mid-way through the immunization schedule. We test here the duck hepatitis B virus core protein (DHBcAg) as a candidate for this needed carrier protein. This is based on previous studies which have indicated that the structurally similar human hepatitis B core antigen (HBcAg) is a highly immunogenic and effective carrier protein that self aggregates into particles of approximately 6.4×10^6 Da [18–23]. Additionally, DHBcAg can be recombinantly or chemically modified to incorporate novel antigens on the particle surface, a prime location for a carrier to stimulate a protective antigen specific humoral response [24]. We demonstrate here that DHBcAg fulfills our carrier protein needs. It is made from well defined small monomers, is soluble in particle form, and is as potent an immunological carrier as KLH.

2. Materials and methods

2.1. Reagents

MUC 1 peptide containing 41 amino acids and a terminal cysteine (CAPPAHGVTSAPDTRPAPGSTAPPA-HGVTSAPDTRPAPGST–OH) was synthesized using the Applied Biosystems Model 431A automated peptide synthesizer [15]. KLH carrier protein was obtained from Sigma, (St. Louis, MO). Duck hepatitis core antigen (DHBcAg) and recombinant MUC1-DHBcAg (recMUC1-DHBcAg) were provided by BioCache Pharmaceuticals Inc. (Richmond, VA). Monoclonal antibody HMFG-1, a mouse IgG reactive with MUC1 was kindly provided by Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, UK). GPI-0100, a semisynthetic saponin adjuvant was obtained from Advantogen Limited (San Diego, CA) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) from Pierce Co. (Rockford, IL).

2.2. Conjugation of MUC1 to KLH and DHBcAg

MUC1 peptide was covalently conjugated to KLH using an MBS linker as previously described [15]. Briefly, 5 mg MBS in 70 μl dimethylformamide (Sigma Chemical Co., St Louis, MO) was added to 9 mg KLH in 1 ml 0.01 M phosphate buffer, pH 7.0. After an hour incubation at room temperature, the MBS/KLH solution was applied to a Sephadex G 15 column equilibrated with 0.1 M phosphate buffer pH 6.0. The first peak at an absorbance of OD 280 nm (MBS-KLH) was collected and mixed with 5 mg MUC1 peptide and stirred for 2 h at room temperature. The unconjugated peptide was separated using a Centriprep 30 concentrator (Amicon Inc., Beverly, MA). The conjugated ratio of MUC1:KLH was 2505:1, calculated based on the initial amount of peptide and KLH, the amount of unconjugated peptide in the filtrate, and a KLH molecular weight of 8.6×10^6 Da.

A similar approach was applied to conjugate MUC1 to DHBcAg carrier protein. The conjugate ratio of MUC1:DHBcAg particle was 505:1, assuming a DHBcAg molecular weight of 6.4×10^6 Da.

2.3. RecMUC1-DHBcAg

The Duck Hepatitis core gene (corresponds to bases 2647-411 of the duck hepatitis B virus genome, Genbank accession no. M60677) was cloned into the pET3d vector creating pET3d-DHBcAg. DNA encoding two consecutive mucin-1 VNTR sequences plus flanking sequences encoding *Kpn*I restriction sites were constructed from commercially synthesized oligonucleotides (Integrated DNA Technologies, Coralville, IA). Oligonucleotides were annealed to form double-stranded DNA and ligated into *Kpn*I linearized pET3d-DHBcAg cloning vector. Recombinant DHBcAg-MUC1 (recDHBcAg-MUC1) was produced by *E.*

coli HMS174 cells transformed with the pET3d-DHBcAg-MUC1 plasmid. Typically, 8 liter of culture was grown in medium containing 10 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl, 1 g/l glucose, and 50 mg/l ampicillin. The bacteria were grown for 24 h at 37 °C and then harvested. The recDHBcAg-MUC1 was produced as a soluble intracellular protein and was purified using the method previously detailed in Zheng et al. [25]. Typically, 159–300 mg of purified protein is obtained from 8 l culture.

2.4. Vaccinations

Six-week-old female CBF1 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Groups of five mice were immunized subcutaneously (s.c.), initially three times at 1 week intervals with MUC1-KLH, MUC1-DHBcAg or RecMUC1-DHBcAg containing 3 μg of MUC1 plus 200 μg GPI-0100. Animals were bled before, 10 days and 52 days after the third immunization. An interval of 6 weeks was allowed to let antibody titer drop before the second round of 3 weekly immunizations. Mice were again bled 10 days after the second round of immunizations.

2.5. Serological assays

ELISA assays were performed to determine IgM and IgG serum antibody titers against MUC1 as previously described [14]. In brief, MUC1 0.1 μg per well in carbonate buffer was coated on ELISA plates and incubated overnight at 4 °C. Nonspecific sites were blocked with 3% human serum albumin (HSA) for 2 h. Serially diluted antiserum was added to each well. After 1 h incubation, the plates were washed and alkaline phosphatase labeled goat anti-mouse IgM or IgG added at 1:200 dilution (Southern Biotechnology Associates Inc., Birmingham, AL). The antibody titer was defined as the highest dilution with absorbance of 0.1 or greater over that of normal control mouse sera.

Flow cytometry with cultured MCF7 breast cancer cells which naturally express MUC1 was performed as previously described [26]. In brief, single cell suspensions of 1×10^6 MCF7 cells per tube were washed in PBS with 3% fetal bovine serum (FBS). Twenty five microlitres of sera diluted at 1:800 was added and incubated for 30 min on ice. Murine monoclonal antibody HMFG-1 against MUC1 served as positive control. After wash in 3% FBS, 25 μl of 1:25 diluted goat anti-mouse IgM or IgG labeled with fluorescein-isothiocyanate (FITC, Southern Biotechnology, Birmingham, AL) was added, and the mixture incubated for another 30 min on ice. After a final wash, the positive population and mean fluorescence intensity of stained cells were differentiated using FACS Scan (Becton & Dickinson, San Jose, CA). Pre- and post-vaccination sera were analyzed together. Pre-vaccination sera were used to set the FACScan result at 10% as background for comparison to percent positive cells with post-vaccination sera.

2.6. Complement-mediated cytotoxicity (CDC) assay

CDC assays were done at 1:200 serum dilution with MCF7 cells (MUC1 positive) and guinea pig complement as previously described [27]. All assays were carried out in triplicate. Controls were culture medium, complement, antisera while spontaneous release was calculated based on the chromium released by target cells incubated with complement alone. A positive control used was PGNX mAb against GM2 ganglioside. Maximum release was determined by incubating target cells with complement and 1% Triton X-100. Percent cytolysis was calculated according to the formula:

$$\text{Specific release}(\%) = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous}} \times 100$$

3. Results

3.1. Antibody response to MUC1

ELISA antibody titers against MUC1 in sera from mice immunized with MUC1 conjugated by using MBS to KLH or DHBcAg, and recombinant MUC1-DHBcAg were determined. The results are summarized in Table 1. There was no detectable anti-MUC1 IgG or IgM antibodies in sera obtained prior to vaccination or in sera from the negative control groups after vaccination (Groups 1 and 2). Sera obtained 2 weeks after the first round of three immunizations had median anti-MUC1 IgG titers ranging from 12,800 to 51,200 and IgM titers ranging from 800 to 6400. There was no significant difference between the groups of 10–15 mice using MUC1-KLH (groups 3 + 4 + 9), MUC1-DHBcAg (groups 5 + 6) or recMUC1-DHBcAg (groups 7 + 8). Mice were bled 6 weeks later, before the second set of (booster) immunizations. IgG and IgM antibody titers had fallen 2–8 fold during the 6 weeks.

In the second set of three booster immunizations, there was a switch in the carrier in groups 1, 2, 4, 6, 8 and 9 while the same carrier was used in groups 3, 5 and 7. There was a considerable increase in anti-MUC1 IgG titer in all groups. Mice boosted with the same conjugate (MUC1-KLH, MUC1-DHBcAg and RecMUC1-DHBcAg) had a median IgG titer of 6,553,600 which was significantly higher than the IgG titer for the groups switching carriers (see Table 1). The IgM antibody response was also significantly higher in the 3 groups boosted with the same carriers (groups 3, 5 and 7) compared to the four groups boosted with different carriers (groups 4, 6, 8 and 9).

3.2. Cell surface reactivity

Cell surface reactivities of sera were tested by flow cytometry using MCF7, a MUC1 positive human breast cell line. The results are summarized in Table 2 and Fig. 1.

Table 1
ELISA titers (median of five mice) against MUC1[a] after vaccination with MUC1 conjugate vaccines all plus 200 mcg GPI-0100

Group	Post 3rd, IgG	Vaccine[b], IgM	Pre 4th, IgG	Vaccine[c], IgM	Post 6th[d], IgG	Vaccine, IgM
1. KLH, DHBcAg	0	0	0	0	0	0
2. DHBcAg, KLH	0	0	0	0	0	0
3. MUC1-KLH	12800	3200	6400	800	6553600	51200
4. MUC1-KLH, MUC1-DHBcAg	51200	800	6400	400	409600	1600
5. MUC1-DHBcAg	51200	1600	6400	400	6553600	51200
6. MUC1-DHBcAg, MUC1-KLH	51200	6400	12800	800	51200	1600
7. RecMUC1-DHBcAg	25600	6400	12800	800	6553600	12800
8. RecMUC1-DHBcAg, MUC1-KLH	25600	800	3200	800	102400	1600
9. MUC1-KLH, RecMUC1-DHBcAg	51200	800	12800	400	102400	1600

[a] Each number is the median reciprocal titer for the five mice vaccinated.
[b] Sera collected 10 days after three weekly vaccines.
[c] Sera collected after 6 weeks interval after the first three vaccines and before the second round of immunizations.
[d] Sera collected 10 days after the second round of three immunizations.

Table 2
FACS analysis of immune sera on MUC1 antigen positive MCF-7 cells[a]

Groups	Post 3rd IgG (%/MFI)	Post 3rd IgM (%/MFI)	Pre 4th IgG (%/MFI)	Post 6th IgG (%/MFI)	Post 6th IgM (%/MFI)
1. KLH, DHBcAg	11/12	18/89	10/9	18/12	8/121
2. DHBcAg, KLH	24/14	13/77	8/9	14/10	24/238
3. MUC1-KLH	89/29	23/94	70/13	86/30	32/249
4. MUC1-KLH, MUC1-DHBcAg	98/55	50/160	93/27	91/38	8/118
5. MUC1-DHBcAg	98/56	22/96	95/36	81/23	8/121
6. MUC1-DHBcAg, MUC1-KLH	97/46	16/87	93/30	65/21	13/133
7. RecMUC1-DHBcAg	78/17	22/74	68/13	31/13	8/120
8. RecMUC1-DHBcAg, MUC1-KLH	61/11	14/99	42/9	83/32	17/199
9. MUC1-KLH, RecMUC1-DHBcAg	92/38	28/111	76/15	72/23	18/144

[a] Each pair of numbers is the median percent of positive cells (%) and mean fluorescence, intensity (MFI) with post-vaccination sera from five mice in each group. Sera were obtained as explained in Table 1.

Sera from unimmunized mice showed minimal reactivity, median percent positive cells less than 10% and median mean immunofluorescence intensity (MFI) of 11. Negative control groups also showed minimal reactivity with median percent positive 13–18% for either IgG or IgM (median MFI was 13 for IgG and 72 for IgM). After the first three immunizations, sera from all vaccinated mice showed significant IgG reactivity. Median percent positive cells and MFI for mice vaccinated with MBS conjugates were 89% gated, 29 MFI and 98%, 56 MFI for MUC1-KLH and MUC1-DHBcAg, respectively, as compared to 78% and 17 MFI for mice vaccinated with recMUC1-DHBcAg. After the three booster immunizations all groups had higher IgG reactivity with no clear difference within different groups. IgM reactivity decreased in all groups, ranging between 8 and 32% regardless of the carrier or switch sequence.

3.3. Complement-mediated cytotoxicity

Sera that were found to have high reactivity for MUC1 antigen by ELISA and FACS did not induce significant CDC. The assays system was confirmed using PGNX anti-GM2 mAb as positive control (64% lysis). MUC1-KLH group 3 vaccine had a pre of 0.4% and a post of 3.9% similar to MUC1-DHBcAg group 5 with 0.4 and 1.6%, respectively.

4. Discussion

We compared the effectiveness of KLH and DHBcAg as antigen carriers using the immunological adjuvant GPI-0100. Vaccines containing unconjugated MUC1 peptide, KLH or DHBcAg, plus GPI-0100 did not elicit any antibody against MUC1. MUC1 covalently linked to either KLH or DHBcAg, and genetically constructed MUC1-DHBcAg fusion protein, plus GPI-0100 successfully induced high titer IgM and even higher IgG antibodies. These antisera reacted strongly with MCF7 cells, particularly the IgG. DHBcAg was as effective a carrier as KLH in facilitating MUC1 immune response. In this study, we also tested whether there was enhancement of the antibody response using the two carriers in sequence: immunization three times with MUC1-KLH followed by three times with MUC1-DHBcAg or vice versa. As expected there was some drop in titer after the 6 week interval between the third and forth vaccinations, particularly for IgM, and a strong further increase in titers after the booster vaccinations. However, comparing switching carriers to using the same carrier for all six vaccinations, the antibody titers were higher by ELISA or similar by FACS when the same carrier was used. Contrary to our expectations, there was no evidence that switching carriers resulted in higher antibody titers.

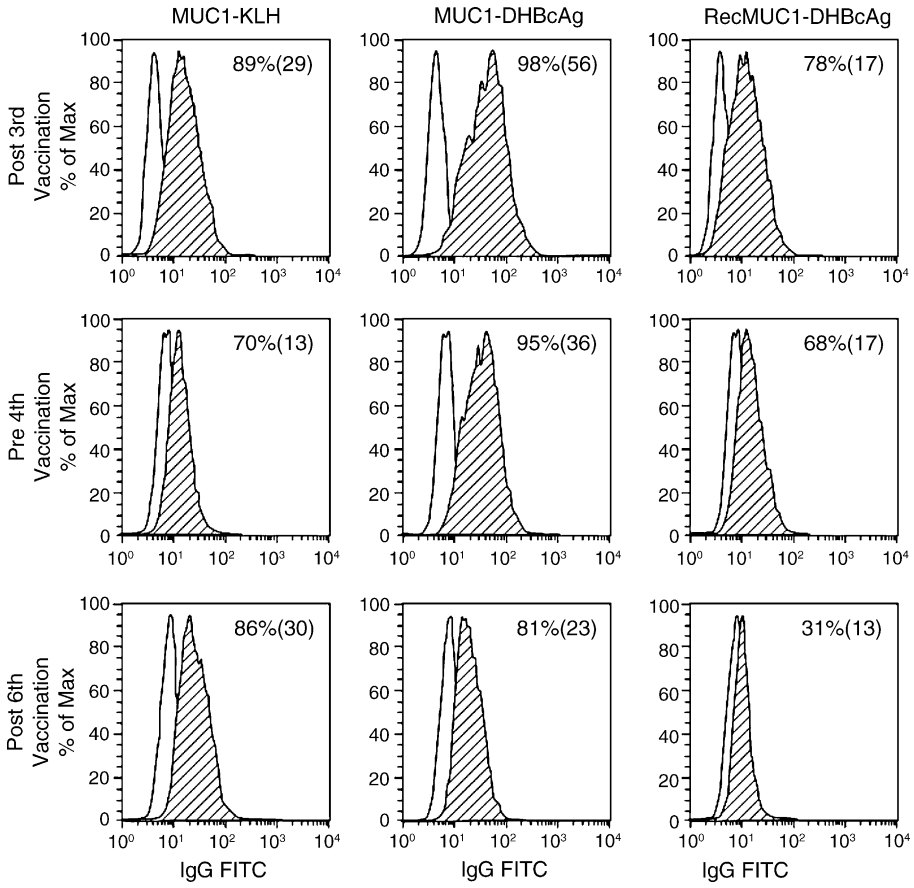

Fig. 1. Median IgG flow cytometry results pre (unshaded) and post (shaded) immunization with MUC1-KLH, MUC1-DHBcAg and RecMUC1-DHBcAg plus 200 μg GPI-0100. Sera were collected 10 days after the third vaccination, a day before the fourth vaccination and 10 days after the sixth vaccination. Data are presented as percent positive cells and mean fluorescence intensity (MFI). Gates were set so that pre treatment median percent positive cells were 10% with median MFI ranging between 4.5 and 7.

Our results indicate that the recombinant core antigen of the duck hepatitis B virus (DHBcAg) is a uniquely effective carrier protein for augmenting the immunogenicity of MUC1 peptide, at least as effective as KLH. This is surprising since it is more homogeneous than KLH, and not known to have mitogenic properties or to bind to Toll-like receptors. Human hepatitis B virus induces almost 100% seroconversion in patients, thus making it a viable candidate as an antigen carrier in vaccines [21]. However, the presence of hepatitis B core-specific antibody is a widely used method to screen hepatitis B virus (HBV)-positive donors from the blood donor population. Widespread use of HBcAg-conjugated vaccines would compromise HBV screening procedures. Because they share very little amino acid homology, HBcAg and DHBcAg do not cross react immunologically (Coleman, unpublished results). Therefore use of DHBcAg as a carrier should not produce anti-HBcAg antibodies and so should not interfere with the current HBV blood screening procedures (though this will eventually need to be confirmed by analysis of DHBc vaccinated patient sera).

DHBcAg shares many features with HBcAg such as core particle assembly and immunopotency [24]. The DHBcAg gene of the duck hepatitis B virus directs synthesis of a 262 amino acid protein, which spontaneously assembles into a particle containing approximately 240 monomers when expressed in *E. coli*. These particles have an apparent molecular weight of 7000 kD and are readily purified to homogeneity with high yields. The crystal structure of the human hepatitis B virus capsid has been determined [28]. While the DHBcAg crystal structure has not been solved, model building based upon 15 mammalian and 5 avian hepatitis B core protein sequences predicts that the DHBcAg capsid structure is very similar to that of HBcAg [29,30] with the major difference being two protrusions from the surface of DHBcAg due to the insertion of 45 residues following the immunodominant loop [31]. Since many of the properties attributed to HBcAg are related to its particle structure, it is likely that DHBcAg possesses similar attributes. In fact, DHBcAg has been shown previously to be an effective carrier protein for bacterial carbohydrate [24]. HBcAg particles are able to prime naive B-cells in a T-cell independent manner [32] and to interact with surface receptors on naive B-cells encoded by complementary-determining region 1 of the immunoglobulin VH domain [33]. These particles can cross-link several receptors, permitting the B-cell to become activated and to act as an efficient antigen-presenting cell.

Milich and McLachlan demonstrated that HBcAg particles are both T-cell-dependent and T-cell-independent antigens [34]. The HBcAg induced IgM and IgG antibodies in athymic (nude) mice. In euthymic mice it stimulated antigen specific T-cell proliferation and IL-2 production. The dual capacity as a T-cell-dependent and T-cell-independent antigen may be synergistic in vivo. If DHBcAg does possess this combination of properties, it may explain its strength as an immunologic carrier.

We demonstrate here in mice that with the human cancer antigen MUC1, DHBcAg is as potent an immunologic carrier protein as KLH, and that changing carrier proteins mid way through the immunization schedule does not further increase the antibody response. DHBcAg has several significant advantages over KLH: it is better defined, more soluble, and so easier to work with. However, while MUC1 is a poorly immunogenic autoantigen in humans, it is a highly immunogenic xenoantigen in mice. It remains to be demonstrated whether DHBcAg will prove as potent as KLH for augmenting the antibody response against autoantigens and carbohydrate antigens.

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