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Advantages to the Use of Rodent Hepadnavirus Core Proteins as Vaccine Platforms

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Abstract

The hepatitis B core antigen (HBcAg) has been proposed as a useful particulate carrier platform for poorly immunogenic peptidic and carbohydrate B cell epitopes. However, biochemical and immunologic impediments have plagued this technology. Specifically, the “assembly” problem characterized by the low yield of unstable hybrid particles resulting from the insertion of foreign sequences and the “pre-existing immunity” problem due to the fact that the HBcAg is derived from a human pathogen have limited the development of this carrier technology. As a means of addressing the “pre-existing immunity” problem we have used the core proteins from the rodent hepadnaviruses. A number of advantages to the use of the rodent hepadnaviral core proteins as opposed to the HBcAg for vaccine design were defined including: equal or superior immunogenicity at the T and B cell levels; the use of the rodent core proteins does not compromise the anti-HBc diagnostic assay; the efficacy of the rodent core proteins as vaccine carriers will not be limited by pre-existing anti-HBc antibodies that are present in previously and currently HBV-infected persons; and the HBcAg-specific tolerance present in HBV chronic carriers can be circumvented by the use of the rodent core proteins.

Keywords

vaccine; carrier; hepadnavirus

1. Introduction

The ability to map neutralizing B cell epitopes on protein and carbohydrate antigens has created much interest in the potential use of these “hapten-like” antigens in vaccine development. There are a number of advantages to the use of peptides and small well-defined oligosaccharides (OS) for subunit vaccine design. For example, chemical purity and safety, ease of production, cost,

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stability, defined and targeted B/T cell epitopes and mutability. The promise of the “hapten-like” technology hasn't been fully realized because efficient and reproducible methods for the delivery of these small epitopes to the immune system are lacking. Peptidic and OS antigens often require conjugation to an immunogenic carrier for efficient immunogenicity in order to provide efficient T cell help for antibody producing B cells. Peptide antigens often do not contain Th cell epitopes and carbohydrate antigens are not recognized by T cells. We and others have suggested that the particulate hepatitis B core protein (HBcAg) represents an efficient carrier platform that possesses many of the characteristics uniquely required for the delivery of weak immunogens to the immune system [1,2].

The hepatitis B virus core protein is a 21 kDa polypeptide, and 240 polypeptides spontaneously assemble into a particulate structure (27nm) in the course of virion assembly and during heterologous expression in both prokaryotic and eukaryotic systems. Recent cryoelectron microscopy and crystallographic studies have elucidated the structure of HBcAg particles to a high resolution [3-5]. Dimers that align along a long vertical α -helical axis comprise the subunits of the particle. Dimer clustering of subunits produces spikes on the surface of the core shell, which consist of radial bundles of four long α -helices. We and others determined that the immunodominant B cell epitope on HBcAg is localized around amino acids 76-82 [6-8]. This structure is consistent with our finding that a heterologous sequence inserted into the internal 75-83 loop region of HBcAg was significantly more antigenic and immunogenic than the same sequence positioned at the N- or C-termini [7]. It appears that the spacing of the spikes on the core shell is optimal for B cell membrane immunoglobulin (mIg) receptor cross-linking [9]. The superior immunogenicity of insertions in the loop region of the HBcAg suggest that the inherent immunogenicity of the native HBcAg B cell epitopes can be successfully “substituted” by placement of heterologous epitopes in the same position as the native dominant epitopes (i.e., at the tip of the spike).

Although HBcAg is a highly immunogenic particulate antigen, the existing HBcAg-based platform technology is plagued with a number of biochemical and immunological problems that may limit its full potential as a vaccine carrier for the human population. The two main limitations of the current HBcAg platform technology can best be described as the “pre-existing immunity” problem and the “assembly” problem. Because HBcAg is derived from a human pathogen, preexisting anti-HBc antibodies present in individuals previously exposed to HBV infection may adversely affect immunogenicity. Further, the anti-HBc antibodies elicited by an HBcAg-based vaccine will compromise the usefulness of the anti-HBc assay currently employed as a diagnostic for current or recent HBV infection. Most importantly, T-cell immune tolerance towards HBcAg is present in individuals chronically infected with HBV (400 million globally). The “assembly” problem is illustrated by the observation that less than 50% of foreign epitopes can be accommodated by the HBcAg platform because of adverse effects on particle assembly [10]. This high failure rate is believed to be due to destabilization of particle assembly caused by inserting foreign sequences.

In previous studies we have addressed the “pre-existing” immunity” problem by suggesting the use of rodent hepadnavirus core proteins [i.e., the woodchuck hepatitis core antigen (WHcAg) or ground squirrel hepatitis core antigen (GSHcAg)] as vaccine platforms [11]. The use of the rodent hepadnavirus core proteins are likely to circumvent the pre-existing immunity problem because the rodent core proteins are largely non-crossreactive with the HBcAg at the antibody and T-cell levels [11]. The “assembly” problem has been addressed by developing a combinatorial approach to hepadnavirus core protein vaccine design, which consists of an expanded number of insertion sites, an expanded number of C-terminal modifications and insert-specific modifications that in combination allow for efficient hybrid core particle assembly [12]. In the current study we have examined in greater detail the ability of the rodent

hepadnavirus core proteins, in particular the WHcAg, to function as vaccine platforms and explored possible advantages to their use as opposed to the HBcAg.

2. Materials and Methods

2.1. Animals

The C57BL/10 (B10), B10.S and (B10 × B10.s) F1 mice were obtained from the breeding colony of the Vaccine Research Institute of San Diego (VRISD). The B10, H-2 congenic strains: B10.BR, B10.D1, B10.D2, B10.M, B10.PL, B10.RIII and Balb/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal care was performed according to National Institutes of Health standards as set forth in the *Guide for the Care and Use of Laboratory Animals* (1996).

2.2. Recombinant core proteins and synthetic peptides

Full length recombinant HBcAg of the *ayw3* subtype was produced as described below. The recombinant rodent core proteins, WHcAg, GSHcAg and hybrid-WHcAg particles were expressed or derived (GSHcAg) from the pUC-WHcAg vector expressing the full-length woodchuck core. Briefly, the full-length sequence of WHcAg (# accession NC_004107) served as a template to create synthetically the other rodent full-length core (GSHcAg # NP_040993). The core proteins were either full length or truncated at amino acid 149 plus a cysteine at residue 150. Addition of a cysteine at residue 150 has been previously shown to stabilize truncated HBcAg [13]. All insertions were accomplished using the EcoRI-XhoI sites specifically engineered to not disrupt the reading frame and to encode a linker Gly-Ile-Leu on the N-terminus, and Leu-Glu on the C-terminus of the inserted heterologous epitopes. The production of the HBcAg-based malaria vaccine candidate, V12.PF3.1 (HBc-M), has been described previously [13]. All the core constructs have been sequenced in both directions (Retrogen, Inc. San Diego). Production and purification of the plasmids (MoBio laboratories, Inc., San Diego) and ligation experiments (La Roche) were performed according to the manufacturer's procedures. Cloning and subcloning have been performed following protocols described in Sambrook et al. [14]. The Top10 E.coli strain was purchased from Invitrogen. Transformation of chemically-competent Top10 by heat shock was carried out according to the manufacturer's protocol (Invitrogen). For greater detail regarding constructs refer to a previous report [12].

2.3. Purification of core antigens

The core proteins were precipitated from the bacterial lysate by the addition of solid ammonium sulfate to 45% saturation (277 g/l). The precipitates were collected by centrifugation, redissolved in a minimum of buffer (10 mM sodium phosphate buffer, pH 6.8) and dialyzed extensively against the same buffer. The protein solutions were then applied to a BioRad BioGel HTP, hydroxyapatite column (5 × 5-10 cm, depending on amount of protein) and eluted with 50 mM sodium phosphate buffer, pH 6.8. The core antigens pass through unretained. The proteins were then applied to a Sepharose CL4B column (5 × 100 cm).

Endotoxin was removed from the core preparations by a modification of a phase separation with Triton X-114 [15,16]. A solution of the protein at a concentration of 5 mg/ml was made 1% Triton X-114 and incubated at 4°C for 30 min with constant stirring. The solution was then incubated at 37°C for 10 min and then centrifuged at 20,000 × g for 10 min. The protein solution was recovered from above the detergent. This procedure was repeated 4 times. Finally the protein was precipitated by lowering the pH to 5. Residual detergent remains in solution. The protein was recovered by centrifugation, and dissolved in endotoxin free buffer. Prior to Triton X-114 treatment the core preparations contained approximately 10-25 ng of endotoxin/μg and

after phase separation with Triton X-114 the endotoxin content was 0.1 ng/μg to undetectable as determined by the QCL-1000 Chromogenic LAL Endpoint Assay (Cambrex, NJ).

Synthetic peptides derived from the HBcAg, WHcAg or GSHcAg sequences were synthesized by the simultaneous peptide synthesis method as previously described [17].

2.4. Immunizations and serology

Groups of 3-5 mice were immunized intraperitoneally (i.p.) with the core proteins (usually 10–20 μg) emulsified in incomplete Freund's adjuvant (IFA) for both antibody production and T cell experiments. For antibody experiments, mice were bled retro-orbitally and sera pooled from each group. Periodically individual mouse sera were tested to confirm the fidelity of the pooled sera results. Anti-core or anti-insert immunoglobulin G (IgG) antibodies were measured in murine sera by an indirect solid-phase ELISA by using the homologous or heterologous core proteins (50 ng/well) or synthetic peptides (0.5 μg/well), representing the inserted sequence, as solid-phase ligands as described previously [7]. Serial dilutions of both test sera and preimmunization sera were made and the data are expressed as antibody titers representing the reciprocals of the highest dilutions of sera required to yield an optical density at 492nm (OD₄₉₂) three times an equal dilution of preimmunization sera. IgG isotype-specific ELISAs were performed by using IgG₁-, IgG_{2a}-, IgG_{2b} and IgG₃-specific peroxidase-labelled secondary antibodies (Southern Biotechnology, Birmingham, AL).

2.5. In vitro T cell proliferation and cytokine assays

Spleen cells from groups of 3 each of the B10 H-2 congenic mice were harvested and pooled 4-6 weeks after immunization with the various core proteins. Spleen cells (5×10^5) were cultured with varying concentrations of homologous or heterologous core proteins or synthetic peptides. For T cell proliferation assays spleen cells were cultured for 96 h and during the final 16 h with 1 μCi of [³H] thymidine (6-7 Ci/μmole; New England Nuclear). The cells were harvested onto filter strips for determination of [³H] thymidine incorporation. The data are expressed as cpm corrected for background proliferation in the absence of Ag (Δ cpm). For cytokine assays, culture supernatants were harvested at 48h for IL-2 determination and at 96 h for interferon-gamma (IFNγ) determination by ELISA. IFNγ production was measured by a two-site ELISA using mAb 170 and a polyclonal goat anti-mouse IFNγ (Genzyme Corp. Boston, MA).

3. Results and Discussion

3.1. Immunogenicity of hybrid WHcAg particles

In order to determine the feasibility of the WHcAg to function as a carrier platform we purified a number of hybrid WHcAg core particles containing five different B cell epitopes inserted at seven different positions with eight varying C-termini to evaluate immunogenicity. This evaluation included determining the *in vivo* humoral immune response to the inserted epitope as well as the WHcAg carrier. Figure 1 is a summary of antibody production 8 weeks after a primary (20 μg) and 6 weeks after a secondary (10 μg) immunization with various hybrid particles emulsified in IFA for both injections. Although a hierarchy of immunogenicity was observed, most hybrid WHcAg particles were quite immunogenic both in terms of anti-insert and anti-WHc antibody production. The primary anti-insert IgG serum titers ranged from 1:5000 to 1.3×10^6 and the secondary anti-insert IgG serum titers ranged from 1:25,000 to 1.75×10^6 . Note that the insertion of 2 distinct B cell epitopes, a CS repeat from *P. falciparum* (M) inserted on the NH₂ terminus and a CS repeat from *P. vivax* (MV) inserted at position 78 in the loop region of WHcAg, elicited anti-insert antibodies to both B cell epitopes (Fig. 1b). This demonstrates that multiple epitopes of differing specificities can be inserted into a single hybrid WHcAg particle.

To directly compare the WHcAg and the HBcAg two hybrid core particles (i.e., WHc-M and HBc-M) were produced, which carried the same *P. falciparum* malaria circumsporozoite (CS) repeat epitope [M, NANPNVDP(NANP)₃], and a single 20 µg dose of WHc-M in IFA elicited higher levels of anti-NANP antibodies with a better persistence profile than the same dose of HBc-M (Fig. 2). These data indicate that the WHcAg is a feasible alternative to the HBcAg as a vaccine carrier platform.

3.2. Absence of genetic nonresponder strains to a WHcAg-Malaria hybrid particle

Efforts to produce *P. falciparum* vaccine candidates based on the CS repeat sequences have been plagued by low immunogenicity and severe genetic restriction characterized by low responders in human clinical trials and low or nonresponder murine MHC genotypes in mouse immunization studies. Therefore, for malaria B cell epitopes as well as for other pathogen-specific B cell epitopes it is imperative that the carrier platform provides sufficient T cell helper function in the context of a wide variety of MHC haplotypes to guarantee the absence of antibody nonresponders.

To directly examine the issue of MHC-linked restriction of the antibody response to a WHcAg-based immunogen, B10 H-2 congenic murine strains expressing 8 different H-2 haplotypes were immunized with an intermediate (7.0 µg) dose of WHcAg-malaria hybrid particle (HyW-M78) in IFA and primary (1°, 6 weeks) and secondary (2°, 10 days post boost with 3.5 µg in IFA) anti-WHc and anti-NANP serum IgG antibody titers were determined (Fig. 3). First and importantly, all H-2 haplotypes responded and produced both anti-WHc and anti-NANP antibodies after a primary immunization with HyW-M78 and nonresponder H-2 haplotypes were not identified. Secondly, all strains at all time points produced an equal or greater antibody response to the insert (anti-NANP) as compared to anti-WHc, with exception of the secondary antibody response of the B10.S strain. The lack of genetic nonresponders to this experimental WHcAg-based immunogen is consistent with the absence of nonresponders to the WHcAg platform itself at the antibody and T cell levels [11].

3.3. Use of the WHcAg as a vaccine platform will NOT compromise the anti-HBc diagnostic assay

One reason we have decided to develop the WHcAg as a platform is because use of the HBcAg as a vaccine platform will elicit anti-HBc antibodies which would compromise the use of the commercial anti-HBc assay, which is used as a diagnostic marker of previous or current HBV infection. Because the WHcAg and HBcAg are not significantly crossreactive at the antibody level [11], anti-WHc antibodies produced to hybrid core particle vaccines should not interfere with the diagnostic assay. To directly examine this issue murine anti-HBc, anti-WHc and antisera to a HBcAg-based malaria vaccine candidate V12.PF3.1 [13] and several WHcAg-malaria hybrid core particles were tested in a commercial anti-HBc assay (DiaSorin, Italy). This assay is an inhibition assay and positivity is measured by % inhibition. As shown in figure 4, murine anti-HBc demonstrated 100% and antisera to the V12-PF3.1 malaria vaccine candidate demonstrated 50% inhibition of the assay, respectively. The anti-V12-PF3.1 antisera only inhibited 50% because the malaria insert in the loop disrupts one of only two dominant endogenous B cell epitopes on HBcAg [7]. Murine antisera to native WHcAg showed only low level inhibition (16%) as did antisera to one of the WHcAg-malaria hybrid core particles (150-3RC-M74). Antisera to a second WHcAg-malaria hybrid particle (HyW-M78) exhibited no inhibition (Fig. 4). Therefore the use of the WHcAg as a vaccine platform largely circumvents the problem of interference with the commercial anti-HBc assay.

3.4. Use of the WHcAg as a vaccine platform should be more effective than the HBcAg in HBV chronic carriers

Another extremely important consideration that convinced us to develop the WHcAg as an alternative to the HBcAg as a vaccine platform is the issue of immune tolerance that exists to the HBcAg in chronic carriers of the HBV. The HBV is endemic in many parts of the world with an estimated 300-400 million HBV chronic carriers worldwide. The HBcAg-specific CD4⁺ and CD8⁺ T cell responses in HBV chronic carriers are severely depressed and usually undetectable [18]. The WHV, of course, is not a human pathogen and CD4⁺ T cell recognition of WHcAg and HBcAg is mostly non-crossreactive ([11]; and see below). Therefore, an extensive WHcAg-specific Th cell repertoire should be available in HBV chronic carriers, whereas, the HBcAg-specific T cell repertoire is severely compromised by immune tolerance mechanisms. We have utilized an HBV-Tg mouse model of HBV chronic infection to investigate this phenomenon. HBeAg-Tg mice produce the secreted form of the HBcAg and HBeAg-Tg mice on a (B10.S × Balb/c)_{F1} background are tolerant to the HBcAg at the Th cell level, which mimics the immune status of HBV chronic carriers. The control (+/+) mice and HBeAg-Tg mice were immunized with either HBcAg, the HBcAg-based malaria vaccine candidate (Hbc-M, V12.PF3.1) or a WHcAg- malaria hybrid immunogen (WHc-M, HyW-M78). Both hybrid particles possess the same malaria CS-derived B cell epitope [i.e., NANPNVDP (NANP)₃]. The Hbc-M vaccine candidate also possesses a heterologous malaria-specific Th cell epitope referred to as the malaria “universal T cell site” (UTC) [19]. As shown in figure 5 (top), HBeAg-Tg mice are immune tolerant to the HBcAg and produce no anti-core antibody whereas, the control (+/+) mice produce anti-Hbc at weeks 2, 4 and 8 after immunization. Likewise, immunization with the Hbc-based V12.PF3.1 vaccine candidate also elicits significantly less anti-Hbc antibody production in HBeAg-Tg mice as compared to the control (+/+) mice (Fig. 5, left middle panel). Furthermore, the HBeAg-Tg mice produce significantly less (i.e., at least 10 fold) anti-NANP antibody as compared to control (+/+) mice (Fig. 5, right middle panel). Therefore, immune tolerance to the HBcAg adversely affects the ability of the HBcAg to perform as a vaccine platform for the malaria-specific B cell epitope. The low level of anti-core and anti-NANP antibodies that are produced may be due to the function of the UTC heterologous Th cell epitope [13] or a novel Th cell epitope(s) created at the junction between the HBcAg and the inserted sequence. The NANPNVDP(NANP)₃ sequence is not a T cell epitope in (B10.S × Balb/c)_{F1} mice. Importantly, the HBeAg-Tg mice demonstrated no diminished ability to produce anti-WHc or anti-NANP antibodies when the WHcAg-based malaria immunogen (HyW-M78) was used (Fig. 5, bottom). Therefore, the negative effects of immune tolerance to the HBcAg were circumvented by using the WHcAg as a vaccine platform for the malaria B cell epitope.

To better understand the mechanism enabling the WHcAg to circumvent immune tolerance to the HBcAg, we also examined the T cell responses of the wild-type (+/+) and HBeAg-Tg mice described in figure 5. The HBcAg-primed T cells of (B10.S × Balb/c)_{F1} mice predominantly recognize the 120-140 sequence, and since this sequence is highly conserved between HBcAg and WHcAg, the WHcAg, HBcAg and 120-140 all recall *in vitro* IFN γ production from HBcAg-primed T cells in +/+ mice (Fig. 6, top). However, 120-140-specific T cells are tolerized in HBeAg-Tg mice immunized with the HBcAg (i.e., no recall response with 120-140), which accounts for the poor *in vitro* recall responses elicited by both the HBcAg and the WHcAg compared to +/+ mice. Similarly, the *in vitro* T cell responses to the HBcAg and the WHcAg are significantly reduced in HBeAg-Tg mice as compared to +/+ mice immunized with the HBcAg-based V12.PF3.1 vaccine candidate because 120-140 specific T cells are tolerized in HBeAg-Tg mice (Fig. 6, middle). In contrast, an advantage to using the WHcAg as a carrier platform is the presence of T cell epitopes within the WHcAg that are unique to the WHcAg and not present on the HBcAg, for example, residues 60-80 (W) and 80-95 (W) (Fig. 6, bottom and see Figs. 8,9). Therefore, while the function of 120-140-specific T cells is

lost in HBeAg-Tg mice immunized with the WHcAg-based immunogen (HyW-M78) due to the tolerizing effects of the presence of HBeAg, T cell recognition of the WHcAg-specific T cell epitopes (60-80W and 80-95W) is unaffected and identical in control mice (+/+) and HBeAg-Tg mice. The ability of the WHcAg to recall IFN γ production in HBeAg-Tg mice immunized with WHc-M, is only minimally decreased as compared to the severe decrease in IFN γ production recalled by the HBcAg. The difference between the WHcAg and the HBcAg as recall antigens is due to the function of 60-80 (W) and 80-95 (W)-specific T cells, which are also sufficient to promote high levels of anti-WHc and anti-NANP antibody production in HBeAg-Tg mice as shown in figure 5. These data indicate that a vaccine based on a WHcAg platform will be significantly more effective in an HBV chronic carrier population than a vaccine based on a HBcAg platform.

3.5. Efficient CD4⁺ T cell response to the WHcAg and T cell fine specificity

It is important for a carrier platform to be an efficient Th cell immunogen and possess sufficient Th cell (CD4⁺) recognition sites to ensure that every MHC haplotype will be able to associate with at least one T cell site in order to avoid genetic (MHC-linked) nonresponsiveness. Figure 7 illustrates the CD4⁺ T cell proliferative response to WHcAg after immunization with WHcAg in Balb/c mice. The WHcAg was able to recall a proliferative response at a relatively low *in vitro* concentration of 0.12 ng/ml. Also note the low level of crossreactivity between the WHcAg and the HBcAg, which required an *in vitro* concentration of 80 ng/ml to recall a proliferative response from WHcAg-primed T cells (i.e., a 666-fold difference). This lack of quantitative T cell crossreactivity suggests that WHcAg-primed T cells in Balb/c mice (H-2^d) recognize a site(s) on WHcAg that is not conserved on the HBcAg, which is indeed the case (see Figs. 8 and 9).

We have examined the fine specificity of T cell recognition of the WHcAg and the HBcAg in 8 different H-2 congenic strains representing 8 separate MHC genotypes using panels of WHcAg- and HBcAg- derived synthetic peptides as *in vitro* recall antigens (Fig. 8). First, all 8 MHC genotypes responded to the WHcAg at the T cell level (i.e., no genetic nonresponders). Secondly, in general, most strains recognize totally distinct sets of T cell sites on WHcAg and HBcAg and even when similar regions are recognized WHcAg-primed T cells rarely crossreact with HBcAg- derived peptides and vice versa. The two exceptions are the H-2^s and the H-2^b haplotypes, which predominantly recognize the 120-131 (H-2^s) and 129-140 (H-2^b) sites, which are both highly conserved between the WHcAg and the HBcAg (Fig. 8b). However, even in H-2^s and H-2^b-bearing strains a number of additional non-crossreactive T cell sites are recognized by WHcAg or HBcAg-primed T cells. For example, the T cells of B10 (H-2^b) mice primed with WHcAg recognize non-crossreactive T cell sites at residues 60-80, 80-90 and 105-125 in addition to the 129-140 T cell site (Fig. 8b).

In comparing T cell (CD4⁺) recognition of WHcAg versus HBcAg several interesting observations are apparent that suggest the WHcAg is a more efficient T cell immunogen than the HBcAg. First, in most murine strains the WHcAg appears to possess more and stronger CD4⁺ T cell epitopes (Fig. 8). Second, comparing the amount of IFN γ produced by WHcAg-primed T cells recalled with WHcAg with the amount of IFN γ produced by HBcAg-primed T cells recalled by HBcAg reveals that WHcAg elicits greater IFN γ production in all but one strain (i.e., B10.D1, Fig. 8a). Third, when the WHcAg is used as the immunogen the ratio of IFN γ produced after *in vitro* recall with WHcAg as opposed to *in vitro* recall with HBcAg is always greater than 1 (ranges between 3.0 [B10 and B10.S strains] and 32 [B10.M strain]). In contrast, when the HBcAg is used as the immunogen the ratio of IFN γ production after *in vitro* recall with HBcAg as compared to recall with WHcAg is 1 or less in 5 of the 8 strains tested (Fig. 8). A T cell response, which is recalled more efficiently by a heterologous antigen than the priming antigen is referred to as a “heteroclitic” T cell response. The ability of the WHcAg

to elicit a heteroclitic T cell response from HBcAg-primed T cells in 3 strains (i.e., B10.D2, B10.PL and B10.RIII) suggest that the WHcAg may be processed and/or presented by antigen presenting cells (APCs) more efficiently than the HBcAg.

The analysis of T cell recognition was extended to a second rodent hepadnavirus core protein, the ground squirrel hepatitis core antigen (GSHcAg). The GSHcAg is 91% identical at the amino acid level to the WHcAg and 67% identical to the HBcAg. Previous studies demonstrated that the GSHcAg and WHcAg are significantly crossreactive at the antibody level, whereas, the GSHcAg and HBcAg are not [11]. Similarly, at the T cell level it appears that the GSHcAg and WHcAg are crossreactive, whereas, GSHcAg-primed T cells are only partially crossreactive for the HBcAg dependent on H-2 haplotype (Table 1). For example, the GSHcAg and the WHcAg demonstrate T cell crossreactivity in all 8 H-2 congenic strains immunized with the GSHcAg and the GSHcAg and the HBcAg demonstrate T cell crossreactivity in only 2 of 8 H-2 congenic strains (Table 1). The basis for this T cell crossreactivity is demonstrated by CD4⁺ T cell epitope mapping studies (Fig. 9). The GSHcAg and the WHcAg share at least 1 overlapping T cell epitope in all 8 H-2 congenic strains studied, whereas, the GSHcAg and the HBcAg share overlapping T cell epitopes in only 2 of 8 strains, aa 120-131 in the B10.S strain and aa 129-140 in the B10 strain (Fig. 9). In summary, CD4⁺ T cell crossreactivity between the GSHcAg, the WHcAg and the HBcAg is variable and is dependent on the conservation of the specific T cell epitope recognized and the MHC class II genotype (H-2 haplotype).

Although we have mapped CD4⁺ T cell recognition sites on the HBcAg and the rodent core proteins in a neutral species (mouse), predominant CD4⁺ T cell sites on the HBcAg recognized by humans have been mapped to aa 1-20 and aa 50-69 [20]. Because aa 1-20 and aa 50-69 are not well conserved between the rodent core proteins and the HBcAg, a low level of T cell crossreactivity between the rodent core proteins and the HBcAg by human CD4⁺ T cells is predictable. Furthermore, a number of Th cell sites have been mapped on WHcAg in outbred WHV-infected woodchucks [21]. A large variety of Th cell recognition sites were defined on WHcAg and the frequency and fine specificity of the sites recognized correlated with acute versus chronic WHV infection [21]. This study in woodchucks suggests that the number of Th cell sites on WHcAg will not be a limiting factor in the use of the WHcAg as a carrier in a human outbred population.

3.6. Avoiding the problem of pre-existing anti-HBc antibodies by using the WHcAg or GSHcAg platforms

Pre-existing anti-HBc antibodies are present in all chronic or acute HBV patients as well as persons previously exposed to HBV even though they have recovered. It is noteworthy that globally 2 billion people have been infected with the hepatitis B virus including the 350-400 million persistently infected chronic HBV carriers. Pre-existing antibodies to a carrier protein may have negative effects on the primary response to a carrier-hapten complex because the carrier-hapten complex may be prematurely cleared from the circulation due to the presence of circulating anti-carrier antibodies. Limited inhibition of T cell priming [22] and antibody production [23] by HBcAg (carrier) -specific antibodies have been reported in murine model systems. This is less of a concern after secondary boosting with the carrier-hapten complex because, although anti-carrier antibodies may have been elicited by the primary immunization, anti-hapten memory B cells are also present and require less antigen for the booster effect to occur. One way to avoid this problem of pre-existing anti-HBc antibodies is to use a carrier platform which will not be recognized by anti-HBc antibodies. As shown in Table 2, HBcAg particles are recognized by anti-HBc antibodies present in the serum of chronic HBV patients and acute HBV patients. Similarly, hybrid-HBcAg particles are also recognized by chronic and acute HBV patient sera (data not shown). We have previously reported that anti-HBc antibodies

in the sera of chronic but not acute HBV-infected patients can recognize the WHcAg and, in fact, suggested that this WHcAg crossreactivity could be exploited as a diagnostic test for chronic as opposed to acute HBV infection [24]. Indeed, as shown in Table 2, WHcAg is recognized by anti-HBc antibodies in chronic patient sera but not acute patient sera. This crossreactivity could compromise the efficacy of the WHcAg platform, at least in chronic HBV patients, just as the use of the HBcAg platform may be compromised in all HBV-exposed individuals possessing anti-HBc antibodies. However, as shown in Table 2, hybrid-WHcAg particles, which are contemplated as candidate vaccines, are not recognized by chronic HBV patient sera containing anti-HBc antibodies. Regardless if the heterologous B cell epitope is inserted in the loop region, 76-82, outside the loop region (i.e., 74, 75, 92) or N- or C-terminally on WHcAg, the anti-HBc crossreactive determinant on WHcAg is not present on hybrid-WHcAg particles. Therefore, pre-existing anti-HBc antibodies in the sera of HBV-exposed individuals will not compromise the efficacy of the WHcAg platform.

In summary, the advantages of utilizing the rodent hepadnavirus core proteins as opposed to the HBcAg as a carrier moiety for vaccine purposes include: i) the rodent core proteins are equally or more immunogenic than the HBcAg at the T and B cell levels; ii) the rodent core proteins will not substantially compromise the use of the anti-HBc diagnostic assay because they are not significantly crossreactive with HBcAg at the antibody level as shown herein and previously [11] iii) pre-existing anti-HBc antibodies in HBV chronically infected patients or in previously infected and recovered persons may limit the efficacy of the HBcAg platform, whereas, hybrid- WHcAg and hybrid- GSHcAg (data not shown) platforms do not bind pre-existing anti-HBc antibodies; (iv) the HBcAg-specific immune tolerance present in HBV chronic carriers can be circumvented by the use of the WHcAg and GSHcAg platforms because the HBcAg is only partially crossreactive at the T cell level with WHcAg and GSHcAg; and v) the WHcAg and GSHcAg combinatorial technologies are more versatile than the HBcAg in terms of accommodating the insertion of a greater variety of foreign epitopes [12].

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References

1. Milich DR, Peterson DL, Zheng J, Hughes JL, Wirtz R, Schodel F. The hepatitis nucleocapsid as a vaccine carrier moiety. *Ann N Y Acad Sci* 1995;754:187–201. [PubMed: 7542855]
2. Pumpens P, Borisova GP, Crowther RA, Grens E. Hepatitis B virus core particles as epitope carriers. *Intervirology* 1995;38(12):63–74. [PubMed: 8666525]
3. Bottcher B, Wynne SA, Crowther RA. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* 1997;386(6620):88–91. [PubMed: 9052786]
4. Conway JF, Cheng N, Zlotnick A, Wingfield PT, Stahl SJ, Steven AC. Visualization of a 4-helix bundle in the hepatitis B virus capsid by cryo-electron microscopy. *Nature* 1997;386(6620):91–4. [PubMed: 9052787]
5. Wynne SA, Crowther RA, Leslie AG. The crystal structure of the human hepatitis B virus capsid. *Mol Cell* 1999;3(6):771–80. [PubMed: 10394365]
6. Salfeld J, Pfaff E, Noah M, Schaller H. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. *J Virol* 1989;63(2):798–808. [PubMed: 2463383]
7. Schodel F, Moriarty AM, Peterson DL, Zheng JA, Hughes JL, Will H, et al. The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J Virol* 1992;66(1):106–14. [PubMed: 1370083]
8. Belnap DM, Watts NR, Conway JF, Cheng N, Stahl SJ, Wingfield PT, et al. Diversity of core antigen epitopes of hepatitis B virus. *Proc Natl Acad Sci USA* 2003;100(19):10884–9. [PubMed: 12954985]

9. Milich DR, Chen M, Schodel F, Peterson DL, Jones JE, Hughes JL. Role of B cells in antigen presentation of the hepatitis B core. *Proc Natl Acad Sci USA* 1997;94(26):14648–53. [PubMed: 9405667]
10. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, Kundig T, et al. A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine* 2002;20(2526):3104. [PubMed: 12163261]
11. Billaud JN, Peterson D, Schodel F, Chen A, Sallberg M, Garduno F, et al. Comparative antigenicity and immunogenicity of hepadnavirus core proteins. *J Virol* 2005;79(21):13641–55. [PubMed: 16227284]
12. Billaud JN, Peterson D, Barr M, Chen A, Sallberg M, Garduno F, et al. Combinatorial approach to hepadnavirus-like particle vaccine design. *J Virol* 2005;79(21):13656–66. [PubMed: 16227285]
13. Milich DR, Hughes J, Jones J, Sallberg M, Phillips TR. Conversion of poorly immunogenic malaria repeat sequences into a highly immunogenic vaccine candidate. *Vaccine* 2001;20(56):771–88. [PubMed: 11738741]
14. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning, a laboratory manual*. second ed.. Cold Spring Harbor Laboratory Press; Plainview, New-York: 1989.
15. Aida Y, Pabst MJ. Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J Immunol Methods* 1990;132(2):191–5. [PubMed: 2170533]
16. Liu S, Tobias R, McClure S, Styba G, Shi Q, Jackowski G. Removal of endotoxin from recombinant protein preparations. *Clin Biochem* 1997;30(6):455–63. [PubMed: 9316739]
17. Sallberg M, Ruden U, Magnus LO, Norrby E, Wahren B. Rapid “tea-bag” peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids applied for antigenic mapping of viral proteins. *Immunol Lett* 1991;30(1):59–68. [PubMed: 1720419]
18. Ferrari C, Penna A, Bertoletti A, Valli A, Antoni AD, Giuberti T, et al. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J Immunol* 1990;145(10):3442–9. [PubMed: 2230128]
19. Calvo-Calle JM, Hammer J, Sinigaglia F, Clavijo P, Moya-Castro ZR, Nardin EH. Binding of malaria T cell epitopes to DR and DQ molecules in vitro correlates with immunogenicity in vivo: identification of a universal T cell epitope in the *Plasmodium falciparum* circumsporozoite protein. *J Immunol* 1997;159(3):1362–73. [PubMed: 9233633]
20. Ferrari C, Bertoletti A, Penna A, Cavalli A, Valli A, Missale G, et al. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. *J Clin Invest* 1991;88(1):214–22. [PubMed: 1711541]
21. Menne S, Roneker CA, Roggendorf M, Gerin JL, Cote PJ, Tennant BC. Deficiencies in the acute-phase cell-mediated immune response to viral antigens are associated with development of chronic woodchuck hepatitis virus infection following neonatal inoculation. *J Virol* 2002;76(4):1769–80. [PubMed: 11799172]
22. Ruedl C, Schwarz K, Jegerlehner A, Storni T, Manolova V, Bachmann MF. Virus-like particles as carriers for T-cell epitopes: limited inhibition of T-cell priming by carrier-specific antibodies. *J Virol* 2005;79(2):717–24. [PubMed: 15613299]
23. Schodel F, Wirtz R, Peterson D, Hughes J, Warren R, Sadoff J, et al. Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. *J Exp Med* 1994;180(3):1037–46. [PubMed: 7520465]
24. Maruyama T, Schodel F, Iino S, Koike K, Yasuda K, Peterson D, et al. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. *Gastroenterology* 1994;106(4):1006–15. [PubMed: 7511551]

IMMUNOGENICITY

Figure 1.

The immunogenicity of hybrid-WHcAg particles. The indicated hybrid-WHcAg particles were injected (i.p.) (20µg, 1°) and boosted (10µg, 2°) in IFA in groups of 3-5 mice. After the primary and 6 weeks after the secondary immunization, sera was collected, pooled and IgG anti-insert and anti-WHc antibody end-point titers were determined by ELISA. The hybrid-WHcAg particles are designated by the C-terminus, the inserted epitope (e.g., M, malaria *P. falciparum* CS repeat; MV, malaria *P. vivax* CS repeat; HV4, HIV gp120 THGIRPVVSTQLLNGSLAEEEE; IM2(-), FLU-M2, SLLTEVETPIRNEWGARANDSSD; CE, cholesteryl ester transfer protein, FGFPEHLLVDFLQSL) and the position of the insert (e.g., 74). Full descriptions of the C-termini and the inserted epitopes have been previously described (12). ND, not determined.

Figure 2.

The comparative immunogenicity of HBcAg-based (HBc-M) and WHcAg-based (WHc-M) (NANP)_n containing immunogens. Groups of three mice each were immunized (i.p.) with a single dose of 20μg of the indicated particles in IFA and sera were collected and pooled at the indicated time points for determination of IgG, anti-NANP end-point titer by ELISA.

Figure 3.

Absence of genetic (MHC-linked) nonresponders to a hybrid-WHcAg particle. Groups of three mice each of the eight indicated B10, H-2 congenic murine strains were immunized (i.p.) with 7.0µg and boosted with 3.5µg of a WHcAg-malaria hybrid particle (HyW-M78) in IFA. Six weeks after primary (1°) immunization and 10 days after the boost (2°) sera were collected, pooled and anti-WHc and anti-NANP IgG, end-point titers were determined by ELISA.

Figure 4.

Anti-WHc antibodies do not interfere with the commercial anti-HBc diagnostic assay. The commercial anti-HBc assay was performed as recommended by the supplier with the exception that 1:10 dilutions of murine sera were used.

Figure 5.

The WHcAg can circumvent immune tolerance to the HBcAg. Wild type (+/+) and HBeAg-expressing transgenic (HBe-Tg) (B10.S×Balb/c)_{F1} mice were immunized (i.p.) with a single 20µg dose of either HBcAg, an HBc-based malaria vaccine candidate (HBc-M, V12.PF3.1) or a WHc-based malaria immunogen (WHc-M, HyW-M78) in IFA. Sera were collected at 2, 4, and 8 weeks after immunization and analyzed for anti-core antibody (left panels) and anti-NANP antibody (right panels) by ELISA.

Figure 6.

WHcAg circumvents immune tolerance to the HBcAg at the Th cell level. Groups of 3 (B10.S×Balb/c)_{F1} wild-type (+/+) or HBeAg-Tg mice were immunized (i.p.) with 10 μg of HBcAg, the HBc-based malaria vaccine candidate (HBc-M, V12.PF3.1) or a WHc-based malaria immunogen (WHc-M, HyW-M78). Two weeks later spleen cells were collected and cultured with varying concentrations (0.08-2.0 $\mu\text{g/ml}$) of HBcAg, WHcAg, peptide 120-140 which is highly conserved between HBcAg and WHcAg (W/H) or the WHcAg-derived peptides p60-80 (W) and p80-95 (W), which are not conserved on HBcAg. After 4 days of culture, supernatants were harvested and analyzed for IFN γ by ELISA. The results shown are from single mice and are representative of 3 +/+ mice and 3 HBeAg-Tg mice.

Figure 7.

There is a low level of cross-reactivity between the WHcAg and the HBcAg at the T cell level. Balb/c mice were immunized in the hind foot pad with WHcAg (5.0µg) in IFA. Ten days later draining lymph node (LN) cells were harvested and cultured with varying concentrations of WHcAg and HBcAg *in vitro* for 96 hours. T cell proliferation was measured by [³H]TdR uptake and corrected for background (ΔCPM).

Figure 8.

Comparative T cell immunogenicity of HBcAg and WHcAg and CD4+ T cell epitope mapping analysis in 8 H-2 congenic strains of mice. Groups of 3 mice were immunized (7.0µg) and boosted (3.5µg) (i.p.) with either WHcAg or HBcAg in IFA, and ten days later spleen cells were cultured with varying concentrations of WHcAg, HBcAg and a panel of WHcAg-derived or HBcAg-derived peptides *in vitro*. After 4 days, tissue culture supernatants (SN) were collected and IFN γ (ng/ml) was measured by ELISA. The relative strengths of the peptide T cell sites are indicated by the minimum peptide concentrations required to recall IFN γ production *in vitro*.

Figure 9.

A CD4⁺ T cell epitope mapping analysis of GSHcAg-primed mice in the eight indicated strains of H-2 congenic mice. Groups of 3 mice were immunized with GSHcAg (10µg in IFA) and 4 weeks later spleen cells were cultured with varying concentrations of GSHcAg-derived, WHcAg-derived or HBcAg-derived peptides *in vitro*. After 4 days, culture supernatants (SN) were collected and IFN γ production was measured by ELISA. The relative strengths of the peptide T cell sites depicted are indicated by the minimum peptide concentrations required to recall IFN γ production *in vitro*.

Table 1
Crossreactivity of GSHcAg-primed T cells with WHcAg and HBcAg.

Strain	H-2	IL-2 (U/ml)		
		GSHcAg	WHcAg	HBcAg
B10.BR	(k)	2,500	450	0
B10.D1	(q)	900	130	0
B10.D2	(d)	1,200	320	0
B10.M	(f)	600	290	0
B10.PL	(u)	2,000	400	0
B10.RIII	(r)	2,400	750	0
B10.S	(s)	2,500	1,800	750
B10	(b)	850	300	110

The indicated B10, H-2 congenic strains were immunized with GSHcAg (10 µg, IFA) and 4 weeks later spleen cells were incubated with GSHcAg, WHcAg or HBcAg (0.5 µg/ml) for 48 hours and IL-2 in the culture supernatant was measured by ELISA.

Table 2

Crossreactivity of HBV acute and chronic patient sera on HBcAg, WHcAg and Hybrid-WHcAg particles.

<u>Core Particles</u>	<u>Human Antisera</u>		
	<u>Chronic HBV (n=6)</u>	<u>Acute HBV (n=6)</u>	<u>Normal human sera (n=4)</u>
HBcAg	2.8 ± 0.18	2.5 ± 0.18	0.3 ± 0.02
WHcAg	2.3 ± 0.41	0.3 ± 0.10	0.17 ± 0.03
HYBRID-WHcAg			
150-M74	0.21 ± 0.05	0.18 ± 0.05	0.17 ± 0.02
150C-M75	0.19 ± 0.06	0.16 ± 0.05	0.18 ± 0.01
150C-M77	0.37 ± 0.08	0.24 ± 0.04	0.24 ± 0.02
150C-M78	0.31 ± 0.06	0.28 ± 0.07	0.21 ± 0.02
C-long-M78	0.32 ± 0.09	0.15 ± 0.02	0.20 ± 0.03
HyW-M (COOH)	0.25 ± 0.08	0.24 ± 0.09	0.23 ± 0.03
HyW-M (NH ₂)	0.30 ± 0.13	0.23 ± 0.07	0.23 ± 0.05
HyW-M92	0.33 ± 0.10	0.26 ± 0.07	0.24 ± 0.04
150C-IM2(-)-82	0.26 ± 0.04	0.22 ± 0.08	0.20 ± 0.05
HyW-SEB-75	0.29 ± 0.10	0.16 ± 0.04	0.25 ± 0.04

Human sera from chronic HBV patients (6), acute HBV patients (6) or normal non-infected blood donors (4) were diluted 1:500 and tested in ELISA using the indicated core particles as the solid-phase ligands (20 ng/well). The data are presented as mean O.D.₄₉₂ values ± standard deviations.