Immunity to Malaria Elicited by Hybrid Hepatitis B Virus Core Particles Carrying Circumsporozoite Protein Epitopes

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Summary

The hepatitis B virus (HBV) nucleocapsid antigen (HBcAg) was investigated as a carrier moiety for the immunodominant circumsporozoite (CS) protein repeat epitopes of *Plasmodium falciparum* and the rodent malaria agent *P. berghei*. For this purpose hybrid genes coding for $[NANP]_4$ (C75CS2) or $[DP_4NPN]_2$ (C75CS1) as internal inserts in HBcAg (between amino acids 75 and 81) were constructed and expressed in recombinant *Salmonella typhimurium*. The resulting hybrid HBcAg-CS polypeptides purified from *S. typhimurium* were particulate and displayed CS and HBc antigenicity, however, the HBc antigenicity was reduced compared to native recombinant HBcAg. Immunization of several mouse strains with HBcAg-CS_1 and HBcAg-CS_2 particles resulted in high titer, *P. berghei*– or *P. falciparum*–specific anti-CS antibodies representing all murine immunoglobulin G isotypes. The possible influence of carrier-specific immunosuppression was examined, and preexisting immunity to HBcAg did not significantly affect the immunogenicity of the CS epitopes within HBcAg-CS_1 particles. Similarly, the choice of adjuvant did not significantly alter the immunogenicity of HBcAg-CS hybrid particles. Immunization in complete or incomplete Freund's adjuvant or alum resulted in equivalent anti-HBc and anti-CS humoral responses. Examination of T cell recognition of HBcAg-CS particles revealed that HBcAg-specific T cells were universally primed and CS-specific T cells were primed if the insert contained a CS-specific T cell recognition site. This indicates that the internal site in HBcAg is permissive for the inclusion of heterologous pathogen-specific T as well as B cell epitopes. Most importantly, 90 and 100% of BALB/c mice immunized with HBcAg-CS_1 particles were protected against a *P. berghei* challenge infection in two independent experiments. Therefore, hybrid HBcAg-CS particles may represent a useful approach for future malaria vaccine development.

The ability to define peptidic B cell epitopes on protein antigens and characterize carbohydrate B cell epitopes has created interest in the potential use of these "haptenlike" antigens in vaccine development. However, peptide antigens as well as carbohydrate antigens often require conjugation to an immunogenic T cell carrier moiety for efficient immunogenicity. A number of observations suggest that the hepatitis B virus (HBV)[1] nucleocapsid antigen (HBcAg) may represent an efficient carrier moiety (for a review see reference 1). The HBcAg is a particle composed of 180 subunits of a single 21.5-Kd polypeptide, and is highly immunogenic in humans as well as in experimental animal models (2). Studies in mice have provided insight into this enhanced immunogenicity. For example, HBcAg can directly activate B cells (i.e., T cell independence) (3), as a protein antigen HBcAg also elicits strong T cell responses (4), and HBcAg-specific Th cells can cooperate with envelope (HBsAg)-specific B cells to produce anti-HBs (5). Whereas there is a clear MHC-associated hierarchy of anti-HBc responsiveness, no nonresponder strain has been identified among MHC disparate murine strains (4). Furthermore, HBcAg can be expressed in *Escherichia coli* and other prokaryotes and self-assembles into core particles. For these reasons, HBcAg has been employed as a carrier moiety for chemically coupled or recombinant, translationally fused peptide epitopes (6–14). Recently,

[1] *Abbreviations used in this paper:* CS, circumsporozoite; HBcAg, hepatitis B virus nucleocapsid antigen; HBV, hepatitis B virus; TT, tetanus toxoid.

an internal position in HBcAg was identified which allowed insertion of heterologous B cell epitopes without interfering with particle assembly (11). The inserted peptidic epitopes became surface accessible and highly immunogenic. At the same time, incorporation of heterologous epitopes at the internal position of HBcAg had the useful effect of reducing HBcAg-specific antigenicity and immunogenicity. In this report we have used recombinant HBcAg to present the *Plasmodium berghei* and *P. falciparum* dominant circumsporozoite (CS) repeat epitopes inserted at this internal position.

Immunization with irradiated sporozoites can protect animals and humans against malaria (15–17). This protection is thought to be mediated by CTLs and CS-specific antibodies (18–26). The dominant B cell epitopes of the CS antigens have been identified in repeat regions and synthetic or recombinant peptides representing these amino acid sequences have been prepared as candidate vaccines (27–29). The level of protection against *P. falciparum* challenge achieved in volunteers after immunization with a recombinant CS protein or a synthetic peptide containing the CS repeat coupled to tetanus toxoid (TT) was disappointingly low (27, 28). The anti-CS antibody responses achieved in these studies were also correspondingly low, and the toxicity of the carrier made dose increases undesirable. Therefore, the ability of a high titer polyclonal anti-CS antibody response to protect against malaria infection remains unclear. The low immunogenicity of the TT-coupled synthetic peptides in human volunteers may have been linked to carrier-specific suppression of the antihapten response in the face of preexisting anti-TT antibodies (28–30). Conceptually, preexisting high titer CS-specific antibodies may protect against infection, whereas, T cell–mediated protection against disease requires at least a limited degree of infection and presentation of antigen. Therefore, it would be desirable to elicit a protective antibody response in naive vaccinees, possibly in combination with adequate T cell immunity.

One approach that succeeded in eliciting high titer CS repeat-specific antibodies, which were protective in an animal model, has been the synthesis of multiply branched CS-derived peptides or multiple antigen peptide systems (MAPs) (31). This prompted us to insert CS repeat epitopes into recombinant hybrid HBcAg particles. In this report, the B and T cell immunogenicity of such hybrid particles incorporating *P. berghei* and *P. falciparum* CS repeat sequences was analyzed. Specific questions addressed included: (*a*) would HBcAg-CS_1 immunization induce protective immunity against challenge with *P. berghei* in mice; (*b*) are the HBcAg-CS particles immunogenic in an adjuvant acceptable for human use; (*c*) what is the impact of preexisting high titer, anticarrier-specific antibodies on the immunogenicity of HBcAg-CS particles; and (*d*) what is the source of T cell helper function? If HBcAg is to be used as a carrier moiety for non-HBV vaccine development purposes, it may be desirable to include Th sites from the pathogen of interest (i.e., *P. falciparum*) for T cell memory recall. We therefore analyzed whether CS-specific Th cell recognition sites as well as HBcAg-specific Th cell sites would be functionally active in HBcAg-CS hybrid particles.

Materials and Methods

Mice. Inbred murine strains were obtained from the breeding colony at the Scripps Research Institute. Female mice 6–8 wk of age at the initiation of the experiments were used. For the malaria protection experiments, 6-wk-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were divided into 3 groups of 10 and immunized with 20 μg HBcAg-CS_1, 20 μg of HBcAg, or PBS emulsified in CFA, and boosted with the same doses of antigen in IFA 2 wk later. The mice were challenged with *P. berghei* as described below 3 mo after the primary immunization. The protection experiment was performed twice in an identical fashion.

Recombinant HBcAg and HBc-CS Hybrid Particles and Synthetic Peptides. Recombinant HBcAg subtype *ayw* was produced and purified as previously described (11). Two sets of synthetic oligonucleotides representing the coding sequences for the *P. berghei* and the *P. falciparum* CS repeat sequences, $[DP_4NPN]_2$ and $[NANP]_4$, ligated into plasmid pNS14PS2 (32) were digested with HpaI and XbaI and transformed into the *Salmonella typhimurium cya crp* vaccine strain x4064 (33). The oligonucleotide sequences used were: $[NANP]_4$ 1:5′-AAC GCT AAC CCG AAT GCT AAC CCG AAC GCT AAC CCG AAC GCT AAC CCG-3′; $[NANP]_4$ 2:5′-CTA GAC GGG TTA GCG TTC GGG TTA GCG TTC GGG TTA GCA TTC GGG TTA GCG TT3′; $[DP_4NPN]_2$ 1:5′-GAC CCG CCG CCG CCG AAC CCG AAC GAC CCG CCG CCG CCG AAC CCG AAC T-3′; and $[DP_4NPN]_2$ 2:5′-CTA GAG TTC GGG TTC GGC GGC GGC GGG TCG TTC GGG TTC GGC GGC GGC GGG TC-3′.

The resulting plasmids pC75CS1 and pC75CS2 (see Fig. 1) were analyzed by agarose electrophoresis, restriction analysis, and dideoxy DNA sequencing. Recombinant *S. typhimurium* were grown in Luria Bertani (LB) medium supplemented with 100 μg/ml ampicillin at 37°C under aeration and gentle shaking (200 rpm). Expression of the hybrid HBcAg-CS particles was confirmed by Western blot analysis (see Fig. 2). For immunoblotting, cells from overnight cultures were taken up in 2× sample buffer and boiled for 10 min. Proteins were separated by 14% SDS-PAGE. The proteins were subsequently transferred to nitrocellulose, incubated with a *P. falciparum* CS-specific mAb (pF2A10) (34), a rabbit anti–*P. berghei* sporozoite serum (kindly provided by Dr. Daniel Gordon, Walter Reed Army Institute of Research), or a mAb recognizing HBV pre-S2 (4408, courtesy of Dr. Makoto Mayumi [Jichi Medical School, Tochigi-Ken, Japan]) (35) and developed with peroxidase-coupled goat anti–mouse IgG (Medac, Hamburg, Germany) visualized on x-ray film (Kodak) after incubation with a chemiluminescent substrate (ECL; Amersham Corp., Arlington Heights, IL). Hybrid HBcAg-CS particles were purified from recombinant *S. typhimurium* by hydroxylapatite and Sepharose 4B chromatography as previously described for HBcAg from recombinant *E. coli* (11). Based on Edman degradation and SDS gel electrophoresis, the hybrid HBcAg-CS particle preparations were at least 95% pure. Peptides derived from the dominant repeat sequences of the CS proteins of *P. berghei* $[DP_4NPN]_2$ and *P. falciparum* $[NANP]_4$ were synthesized on an automated peptide synthesizer or by the simultaneous multiple peptide synthesis method (36), and were subjected to HPLC on a C18 reverse phase column. The peptides used eluted as a single major peak (>90%). Synthetic peptides were produced at the Torrey Pines Molecular Biology Institute (La Jolla, CA) and were generously provided by Richard Houghten.

mAbs and Antibody Assays. HBcAg-specific mAb 3105 and 3120 (37), and HBeAg-specific mAb 904 and 905 (38) were supplied by M. Mayumi. Polyclonal rabbit anti-HBc/HBe was obtained commercially (Dako Corp., Carpinteria, CA). The recombinant HBcAg

and HBcAg-CS hybrid particles were analyzed by liquid-phase sandwich ELISA. mAbs coated on microtiter wells were used as the capture reagents followed by addition of varying concentrations of recombinant antigen. Polyclonal rabbit anti-HBc/HBe was used as the detecting antibody and peroxidase-labeled goat anti-rabbit IgG as the probe. Anti-HBc and anti-peptide antibodies were measured in pooled, murine sera by indirect solid-phase ELISA using HBcAg (50 ng/well) and CS-derived peptides (1.0 μg/well) as solid-phase ligands and goat anti-mouse IgG (or IgG isotype-specific antibodies) as second antibody, and were developed with a peroxidase-labeled swine anti-goat IgG. The data are expressed as antibody titer representing the highest dilution of serum required to yield three times the OD_{492} of preimmunization sera. Mice were immunized for determination of in vivo antibody production by intraperitoneal injection of HBcAg or HBcAg-CS hybrid particles (10 μg) either emulsified in CFA or absorbed to alum for primary immunization and in IFA for subsequent immunizations.

Malaria Sporozoite Production and Antibody Assay. Salivary gland sporozoites used to measure anti-CS-specific antibody in immunofluorescent antibody and ELISA assays were produced by feeding laboratory-reared *Anopheles stephensi* mosquitoes on *P. berghei* ANKA-infected mice or *P. falciparum* NF54-infected cell cultures. Midgut oocyst and salivary gland sporozoite infection rates were monitored and sporozoites from infected glands were harvested in Medium 199 (Sigma Chemical Co., St. Louis, MO), washed in a protein-free buffer (for ELISA use), counted using a hemacytometer, and frozen at −70°C for use in ELISAs, or immediately transferred to multiwell (2,000–4,000 sporozoites/spot) printed slides, air dried at room temperature, and stored at −20°C until used in immunofluorescent antibody assays.

For ELISA, sporozoites were diluted in carbonate buffer, pH 9.0, and 1,000 sporozoites were added per microtiter plate well, and anti-CS antibody was measured in pooled mouse sera by indirect solid-phase ELISA as described above. The immunofluorescent antibody assays were initiated by spreading 30 μl of mouse serum diluted in Dulbecco's PBS, pH 7.4, into the slide well containing the dried sporozoites. The slide was incubated in a moist chamber at room temperature for 30 min, the well was aspirated dry and washed twice with 30 μl PBS followed by a 30-μl aliquot of fluorescein-labeled anti-mouse IgG (H + L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) (1:100 in PBS). After 30 min, the well was washed as above before mounting in glycerol/PBS (10:1). Slides were examined under ultraviolet light at a magnification of 500 for fluorescence and graded from negative for no fluorescence to 4+ for strong uniform fluorescence. The data are expressed as antibody titer representing the highest dilution of serum to yield at least a 1+ fluorescence score.

T Cell Proliferation Assay. Groups of mice were primed with either 10 μg of HBcAg or 50 μg of synthetic peptide in CFA by hind footpad injection. These amounts represented optimal immunization doses. 10 d after immunization, draining lymph node cells were harvested, pooled, and 5×10^5 cells in 0.1 ml of Click's medium were cultured with 0.1 ml of medium containing either HBcAg, various synthetic peptides, or medium alone. Cells were cultured for 96 h at 37°C in a humidified 5% CO_2 atmosphere, and during the final 16 h, 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq: New England Nuclear, Boston, MA) was added. The cells were then harvested onto filter strips for determination of [^{3}H]thymidine incorporation. The data are expressed as counts per minute corrected for background proliferation in the absence of Ag (Δ cpm). The T cell nature of the proliferation was confirmed by analyzing nylon-wool column-enriched T cells in selected experiments. All T cell activation experiments were performed at least twice, and representative experiments are depicted.

Malaria Challenge Experiments. *A. stephensi* mosquitoes used to challenge immunized mice were infected with *P. berghei* ANKA by feeding on infected mice. Mosquitoes used for this challenge had salivary gland sporozoite infection rates of 50–100% 18–22 d after the infectious blood meal.

Mice were anesthetized by injection of Rompun/Ketamin/sterile water (3:15:48) (0.1 ml/mouse) (Rompun, 100 mg/ml; Mobay Corp., Shawnee, KS; Ketamine HCl, 100 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA). Anesthetized mice were placed on a holding platform and the tails laid on top of a screened mosquito container. Mosquitoes were permitted to feed until blood was observed in the gut of five mosquitoes. Beginning 3 d after challenge, mice were checked daily for *P. berghei* infection by microscopic examination of Giemsa-stained thin-smear tail bleeds. A minimum of 25 fields (×400) were read before a mouse was determined negative for infection. After two consecutive positive blood smears, mice were euthanized and serum collected. Mice with negative blood smears were observed for a minimum of 11 and up to 25 d after challenge.

Results and Discussion

Antigenicity of Hybrid HBcAg-CS Particles. The DNA sequences coding for the immunodominant amino acid repeats in the CS proteins of *P. berghei* or *P. falciparum* were cloned into the vector pNS14PS2 at an internal site of the HBcAg gene (for the structure of the hybrid gene translation products see Fig. 1). *S. typhimurium cya crp* vaccine strain x4064 was transformed with the resulting DNA sequence-verified plasmids pC75CS1 and pC75CS2. The recombinant *S. typhimurium* which contained the plasmids synthesized HBcAg fusion proteins displaying CS antigenicity by Western blot analysis (Fig. 2). The purified hybrid HBcAg-CS particles were analyzed by electron microscopy (Fig. 3). Both hybrid HBcAg-CS_1 and HBcAg-CS_2 proteins were able to form corelike particles. To investigate the antigenicity of the recombinant particles they were compared to recombinant HBcAg and HBeAg (a nonparticulate form of the HBV nucleocapsid) in a liquid-phase sandwich assay using mAbs specific for HBcAg and HBeAg, respectively, as solid-phase capture reagents (Table 1). Insertion of either of the two CS repeat sequences studied

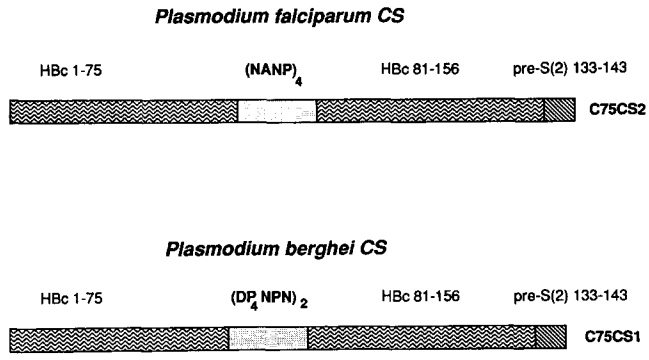

Figure 1. Structure of hybrid HBcAg-CS proteins. Numbers indicate amino acid positions of native HBcAg and pre-S(2) (subtype *ayw*). (*Right*) Plasmid designations. The amino acid sequence of the CS repeat epitope inserts is indicated in the one-letter code.

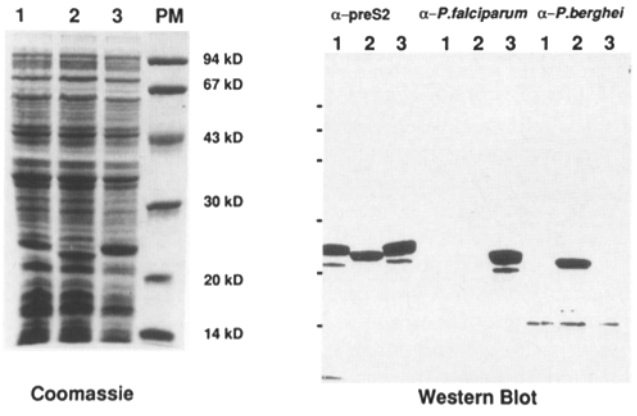

Figure 2. Expression of hybrid HBcAg-CS genes in *S. typhimurium* χ4064 (*pC75CS1*) and χ4064(*pC75CS2*). χ4064(*pNS27-53PS2*) (32) expressing a hybrid HBcAg/pre-S gene is added for specificity control and comparison of apparent molecular size. Proteins in whole bacterial cell lysates from overnight cultures of χ4064(*pNS27-53PS2*) (lane *1*), χ4064 (pC75CS1) (lane *2*), and χ4064(*pC75CS2*) (lane *3*) were separated by 14% SDS-PAGE and stained with Coomassie Brilliant blue (*left*) or transferred to nitrocellulose and visualized by immunostaining with a monoclonal anti-pre-S(2) antibody (4408, kindly provided by M. Mayumi), with a monoclonal anti-*P. falciparum* CS repeat antibody (pF2A10), or a polyclonal rabbit anti-*P. berghei* CS serum (*right*).

Table 1. *Monoclonal Analysis of HBcAg-CS Hybrid Particles*

		Monoclonal (OD_{492})			
		Anti-HBc		Anti-HBe	
Antigen	Concentration	3105	3120	904	905
	μg/ml				
HBcAg	1.0	0.56	1.5	0.29	0.14
	0.2	0.30	1.1	0.14	0.04
HBeAg	1.0	0.36	0.17	2.0	2.0
	0.2	0.19	0.03	2.0	2.0
HBcAg-CS_1	1.0	0.03	1.3	1.7	1.0
	0.2	0.02	0.45	1.4	0.86
HBcAg-CS_2	1.0	0.01	1.4	0.4	0.82
	0.2	0.01	0.4	0.16	0.65

The indicated recombinant antigens were analyzed in a liquid-phase sandwich ELISA. mAbs specific for HBcAg (mAbs 3105 and 3120) and HBeAg (mAbs 904 and 905) were used as solid-phase capture reagents. Concentrations (1.0 and 0.2 μg/ml) of recombinant antigens were added, and the second antibody was a rabbit polyclonal antibody, which recognizes both HBcAg and HBeAg epitopes, and the assay was developed with a peroxidase-labeled goat anti-rabbit Ig. Data are expressed as OD_{492} values corrected for background.

ablated recognition of hybrid HBcAg-CS particles by mAb 3105. Lack of recognition by mAb 3105 confirms our previous finding that HBcAg residues 75 to 81 are critically involved in this immunodominant HBcAg epitope (11). Another confirmation-dependent HBcAg-specific epitope defined by mAb 3120 is partially preserved by insertions within amino acids 75 and 81. Hybrid HBcAg-CS particles are recognized by HBeAg-specific mAbs 904 and 905. Truncation of HBcAg beyond amino acid position 149 exposes HBeAg epitopes without disrupting particle formation (39). Whether exposure of these HBeAg epitopes results from a conformational change within the particles or is predominantly due to liberation of HBeAg-like material from less stable truncated particles (40) is unclear.

Immunogenicity of HBcAg-CS Particles. To determine the immunogenicity of the hybrid HBcAg-CS particles, five MHC discordant mouse strains were immunized with HBcAg-CS_1 particles and three mouse strains were immunized with HBcAg-CS_2 particles. The HBcAg-CS_1 particles elicited high titer primary and secondary IgG serum antibodies reactive with the inserted *P. berghei* CS repeat sequence in all strains tested. In fact, the anti-CS responses measured on synthetic $[DP_4NPN]_2$ were significantly higher than the anti-HBc titers (Table 2). The anti-$[DP_4NPN]_2$ response correlated well with the anti-CS response (Table 2) and was not cross-reactive with the *P. falciparum* CS repeat sequence $[NANP]_4$. Similarly, immunization with HBcAg-CS_2 particles elicited high titer anti-$NANP_4$ IgG serum antibodies that correlated with the anti-CS response and were not crossreactive with

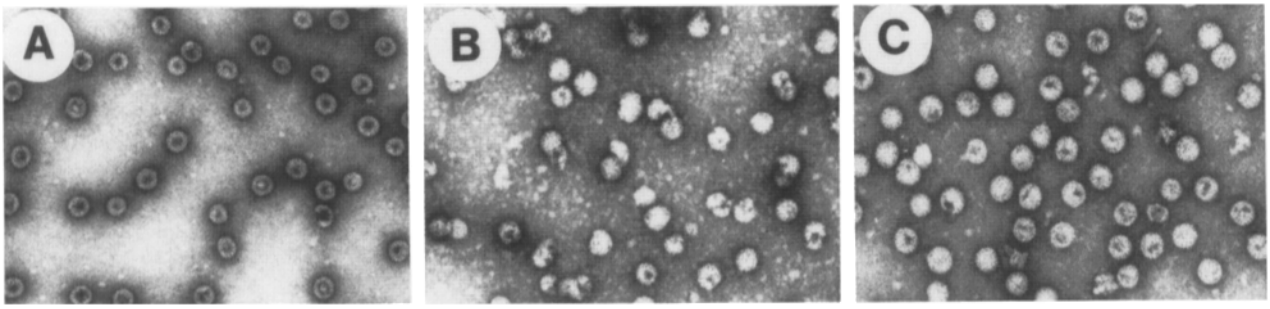

Figure 3. Electron micrographs of HBcAg and HBcAg-CS hybrid particles. (*A*) HBcAg; (*B*) HBcAg-CS_1; and (*C*) HBcAg-CS_2. Particles (100 μg/ml) were applied to grids and fixed with 1.5% glutaraldehyde plus 10% paraformaldehyde in cacodylate buffer, pH 7.4, for 10 min. The samples were then negatively stained with aqueous uranyl formate.

Table 2. *Immunogenicity of HBcAg-CS$_1$ Particles in Mice*

Strain	H-2	Time	Antibody titer (1/dilution)			
			HBcAg	CS	$[DP_4NPN]_2$	$[NANP]_4$
C57BL/10	*b*	10 d	2,560	0	10,240	0
(B10)		24 d	10,240	640	163,840	0
		2°	655,360	163,840	>655,360	0
B10.S	*s*	10 d	2,560	0	10,240	0
		24 d	2,560	640	163,840	0
		2°	163,840	163,840	>655,360	0
B10.M	*m*	10 d	2,560	0	10,240	0
		24 d	2,560	640	40,960	0
		2°	40,960	10,240	163,840	0
B10.BR	*k*	10 d	0	0	10,240	0
		24 d	2,560	640	163,840	0
		2°	40,960	10,240	655,360	0
BALB/c	*d*	10 d	160	0	10,240	0
		24 d	2,560	640	40,960	0
		2°	163,840	40,960	>655,360	160

Groups of three mice each of the indicated strains were immunized with 10 μg of hybrid HBcAg-CS$_1$ particles in CFA and boosted with 10 μg in IFA. Sera were collected before immunization, 10 and 24 d after primary and 2 wk after secondary (2°) immunization. Sera were pooled and analyzed by solid-phase ELISA: HBcAg (50 ng/well); CS represents *P. berghei* sporozoites (1,000/well); the *P. berghei* repeat $[DP_4NPN]_2$ (1.0 μg/well) and the *P. falciparum* repeat $[NANP]_4$ (1.0 μg/well) were the solid-phase ligands.

Table 3. *Immunogenicity of HBcAg-CS$_2$ Particles in Mice*

Strain	Time	Antibody titer (1/dilution)			
		HBcAg	CS	$[NANP]_4$	$[DP_4NPN]_2$
C57BL/10	10 d	10,240	200	2,560	0
(B10)	24 d	40,960	1,600	10,240	0
	2°	655,360	6,400	655,360	40
B10.S	10 d	10,240	200	640	0
	24 d	40,960	400	1,280	0
	2°	>655,360	400	20,480	0
BALB/c	10 d	10,240	0	0	0
	24 d	40,960	200	640	0
	2°	>655,360	1,600	40,960	0

Groups of three mice each of the indicated strains were immunized with 10 μg of hybrid HBcAg-CS$_2$ particles in CFA and boosted with 10 μg in IFA. Sera were collected before immunization, 10 and 24 d after primary and 2 wks after secondary (2°) immunization. Sera were pooled and analyzed by solid-phase ELISA: HBcAg (50 ng/well); the *P. falciparum* repeat $[NANP]_4$ (1.0 μg/well); and the *P. berghei* repeat $[DP_4NPN]_2$ (1.0 μg/well) were the solid-phase ligands. The anti-CS antibody titer was determined by immunofluorescent antibody assay.

Table 4. *Effect of Adjuvant on Immunogenicity of HBcAg-CS$_1$ Particles*

Immunogen	Adjuvant	Time	Antibody titer (1/dilution)	
			HBcAg	$[DP_4NPN]_2$
		wk		
HBcAg	CFA	2	163,840	0
		4	655,360	0
	IFA	2	163,840	0
		4	655,360	0
	Alum	2	163,840	0
		4	655,360	0
HBcAg-CS$_1$	CFA	2	2,560	10,240
		4	10,240	163,840
	IFA	2	10,240	2,560
		4	10,240	163,840
	Alum	2	10,240	10,240
		4	40,960	655,360

Groups of three BALB/c mice were immunized with either HBcAg (10 μg) or hybrid HBcAg-CS$_1$ particles (10 μg) prepared in three different adjuvants: CFA, IFA, or alum. Sera were collected before immunization and 2 and 4 wk after primary immunization. Sera were pooled and analyzed by solid-phase ELISA using HBcAg (50 ng/well) and $[DP_4NPN]_2$ (1.0 μg/well) as solid-phase ligands.

the *P. berghei* repeat sequence (Table 3). Immunization with HBcAg-CS$_2$ particles elicited more anti-HBc relative to antiinsert antibodies as compared with immunization with HBcAg-CS$_1$ particles. The reason for this disparity is unknown, but it suggests the potential for insert sequence–specific effects on particle assembly or stability. Therefore, alternative hybrid HBcAg-NANP constructs are currently being examined in order to optimize the immunogenicity of the NANP sequence. The greatest anti-$[NANP]_4$ response occurred in B10 (I-A^b) mice for which the inserted NANP sequence contains a Th as well as a B cell epitope (41, 42). In a separate experiment we found that $[NANP]_4$ within HBcAg-CS$_2$ particles represents a functional T cell site for B10 mice (see below). Therefore, it appears that $[NANP]_4$ may act as an additional source of Th cell function in this particular strain. The IgG subclasses elicited by immunization with hybrid HBcAg-CS$_1$ particles were also examined. Similar to the distribution of isotypes after immunization with native HBcAg (3), immunization of BALB/c mice with HBcAg-CS$_1$ particles elicited a primary anti-$[DP_4NPN]_2$ response comprised of all IgG isotypes. The IgG1, IgG2a, IgG2b, and IgG3 anti-$[DP4NPN]_2$ titers were at least 1:40,960 24 d after immunization with a single HBcAg-CS$_1$ particle dose (data not shown). This demonstrates that HBcAg is capable of eliciting a broad IgG isotype response specific for the inserted epitope, and will be a useful carrier moiety regardless of which isotype is desired.

Effect of Adjuvant on Immunogenicity. For vaccine purposes, a vaccine carrier moiety must be immunogenic in an adjuvant suitable for human use. The only currently licensed adjuvant for use in humans is alum. We therefore investigated whether the enhanced immunogenicity of the carried CS epitope in hybrid HBcAg-CS$_1$ particles would be preserved when employing alum as an adjuvant. To address this question we immunized BALB/c mice with either HBcAg or HBcAg-CS$_1$ particles formulated with CFA, IFA, or alum, and analyzed the primary serum anti-HBc and anti-$[DP_4NPN]_2$ IgG responses (Table 4). The primary anti-HBc and anti-$[DP_4NPN]_2$ IgG responses were nearly identical regardless of the adjuvants employed. The data demonstrate that HBcAg can be used as a vaccine carrier moiety with alum, an adjuvant acceptable for human use. A direct comparison of anti-HBc responses after immunization in all three adjuvants with native recombinant HBcAg or HBcAg-CS$_1$ particles also illustrates the reduced anti-HBc response (i.e., ~16-fold) after immunization with hybrid particles with an internal deletion at HBcAg position 75 to 81 (Table 4). Further characterization and deletion of the residual native HBcAg epitope(s) may make it possible to create hybrid HBcAg particles without any native HBcAg antigenicity at the B cell level. This would eliminate anticarrier-specific antibody production, and would not diminish the diagnostic value of anti-HBc antibody as a marker of past or present HBV infection.

Effects of Preexisting Carrier-specific Immunity. Preexisting immunity to a carrier protein may negatively affect the protein's ability to efficiently present new haptens (30). This presents a potential problem when identical carrier moieties are used for several different haptens as exemplified by the increasing use of conjugated oligosaccharide vaccines. Simi-

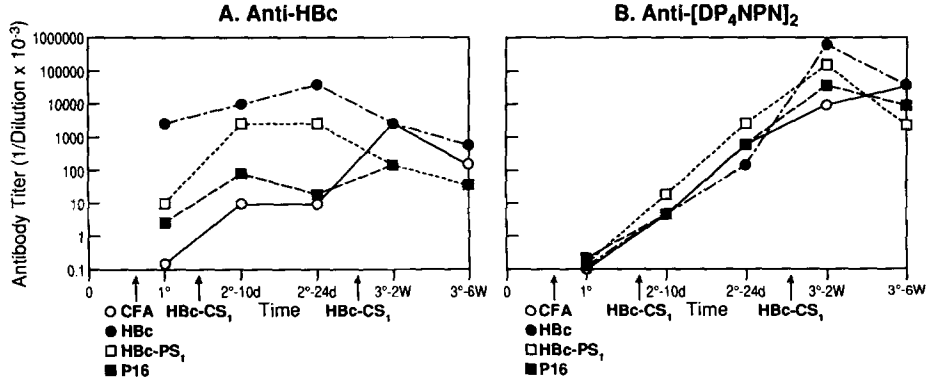

Figure 4. Effects of carrier-specific suppression on the anti-$[DP_4NPN]_2$ response after HBcAg-CS_1 immunization. Groups of three BALB/c mice were primed with either CFA only (O–O), or 10 μg of the following antigens in CFA: HBcAg (●–●); a heterologous HBcAg-PS_1 hybrid particle (□–□); or a truncated HBcAg monomer, P16 (■–■) (*first arrow*). 1 mo after priming, all groups were immunized with the HBcAg-CS_1 hybrid (10 μg; IFA) (*second arrow*). A second HBcAg-CS_1 injection (10 μg; IFA) was given 1 mo later (*third arrow*). Sera were collected before immunization, 1 mo after the primary injection (*1°*), 10 d (*2°-10d*) and 24 d (*2°-24d*) after the second injection, and 2 wk (*3°-2W*) and 6 wk (*3°-6W*) after the third injection. Sera were pooled and analyzed by solid-phase ELISA: HBcAg (50 ng/well) and the *P. berghei* repeat $[DP_4NPN]_2$ (1.0 μg/well) were used as solid-phase ligands. Antibody titers are expressed as the reciprocal of the highest dilution of sera to yield three times the OD_{492} reading of preimmunization sera.

larly, the use of TT or diphtheria toxin as carrier moieties may be affected by the high level of preexisting immunity to these childhood vaccine components. Anti-HBc from a preceding HBV infection or vaccination could theoretically limit the usefulness of HBcAg as a vaccine carrier moiety. We therefore analyzed the impact of prior immunization with native recombinant HBcAg on the antihapten responses obtained after immunization with HBcAg-CS_1 particles. For this purpose, BALB/c mice were primed with HBcAg or another HBcAg hybrid particle with an HBV envelope internal insert (HBcAg-PS_1) in CFA followed by two injections with HBcAg-CS_1 particles 1 and 2 mo later (Fig. 4). As a negative control, one group of mice was primed with CFA only. As a control for the effects of Th cell priming, another group was immunized with a structural polypeptide of truncated HBcAg (P16) which primes HBcAg-specific Th but not B cells due to the absence of the conformation-dependent B cell epitopes. Mice primed with HBcAg demonstrated the highest anti-HBc responses at all time points, as expected (Fig. 4 *A*). Note that priming with the heterologous HBcAg hybrid particle (HBcAg-PS_1) elicited significantly lower anti-HBc levels at the primary bleed (1°) and thereafter, as compared to wild-type HBcAg, due to the deletion of HBcAg residues 75 to 81. Priming with the truncated structural polypeptide (P16) of HBcAg elicited little anti-HBc before the first injection with HBcAg-CS_1 (Fig. 4 *A*). These varying levels of anti-HBc antibodies appeared to have little effect on anti-$[DP_4NPN]_2$ antibody production 10 d after the first injection with HBcAg-CS_1 particles (*2°-10d*) (Fig. 4 *B*). However, 24 d after the first injection (*2°-24d*) the HBcAg-primed group possessed the lowest anti-$[DP_4NPN]_2$ titer and the highest anti-HBc titer suggesting some degree of carrier-specific suppression. However, 2 wk after the second HBcAg-CS_1 injection (*3°-2W*), no evidence of carrier-specific suppression existed, and extremely high titer anti-$[DP_4NPN]_2$ antibody (6.7×10^8) was produced by the HBcAg-primed group (Fig. 4 *B*). The other groups also demonstrated very high titer anti-$[DP_4NPN]_2$ responses (1.04×10^7–1.7×10^8) at this time point. To our knowledge, serum antipeptide levels of this magnitude have not been previously reported. It is noteworthy that the $[DP_4NPN]_2$ peptide emulsified in CFA is nonimmunogenic in the BALB/c strain used in this experiment (data not shown; 43). Therefore, the enhanced antibody production to the peptidic $[DP_4NPN]_2$ epitope is due to HBcAg-specific carrier effects. 6 wk after the second injection with HBcAg-CS_1 particles (*3°-6W*), the anti-HBc and anti-$[DP_4NPN]_2$ titers began to decline relative to the peak responses 2 wk after the last injection with the exception of the anti-$[DP_4NPN]_2$ response of the unprimed group which was still rising at the (*3°-6W*) time point (Fig. 4). In general, these data indicate that hybrid HBcAg particles are extremely effective as vaccine carrier moieties even in the presence of preexisting immunity to HBcAg. This suggests that HBcAg may be used repeatedly as a vaccine carrier for multiple haptens.

T Cell Immunogenicity. It is clear from the preceding results that immunization with HBcAg-CS hybrid particles can elicit high titer serum antibodies specific for the CS repeat sequences. The source of T cell help for anti-CS antibody production is an important additional issue, especially in terms of non-HBV vaccine development. Will it be possible to insert functional pathogen-specific Th cell recognition sites as well as B cell epitopes into HBcAg? If Th cells exclusively recognize the HBcAg carrier moiety, a potential T cell site derived from the pathogen and included in an inserted sequence would not be functional. Previously it has been reported that a heterologous NH_2-terminally fused Th cell site was not functional (11), and a COOH-terminally fused Th cell site was functional (14) in the context of HBcAg hybrid particles. It was therefore of interest to examine the specificity of T cell recognition of HBcAg-CS hybrid particles. Three H-2 congenic murine strains were immunized with HBcAg-CS_1 and the corresponding synthetic CS repeat sequence $[DP_4NPN]_2$, and T cell proliferative responses were analyzed after in vitro restimulation with HBcAg and the synthetic peptide (Table 5). Immunization in all three H-2 congenic strains with HBcAg-CS_1 particles primed HBcAg- but not $[DP_4NPN]_2$-specific T cells. Immunization with the CS_1

Table 5. *T Cell Recognition of HBcAg-CS Hybrid Particles*

Strain	H-2	Immunogen	Antigens ([^{3}H]TdR Uptake, Δ cpm) HBcAg (1.0 lg/ml)	$[DP_4NPN]_2$ (100 μg/ml)	$[NANP]_4$ (1.0 μg/ml)	HBcAg-CS_1 or $_2$ (1.0 μg/ml)
B10	*b*	HBcAg-CS_1	30,917	1,050	–	78,250
		$[DP_4NPN]_2$	1,003	1,188	–	0
B10.S	*s*	HBcAg-CS_1	45,213	0	–	51,667
		$[DP_4NPN]_2$	2,509	407	–	0
B10.P	*p*	HBcAg-CS_1	24,348	0	–	55,352
		$[DP_4NPN]_2$	1,394	156	–	0
B10	*b*	HBcAg-CS_2	50,646	11,759	63,398	43,995
		$[NANP]_4$	6,968	0	74,637	33,825
BALB/c	*d*	HBcAg-CS_2	100,552	0	0	120,187
		$[NANP]_4$	8,845	0	0	0

Groups of three mice of the indicated strains were immunized with 10 μg of the HBcAg hybrid particles (HBcAg-CS_1 or HBcAg-CS_2) or 50 μg of the *P. berghei* repeat peptide $[DP_4NPN]_2$ or 50 μg of the *P. falciparum* repeat peptide $[NANP]_4$. Draining lymph node cells were collected, pooled, and cultured with the indicated antigens, and T cell proliferation was measured as [^{3}H]TdR uptake corrected for background proliferation in the absence of antigen (Δ *cpm*).

Table 6. *Protection Against P. berghei Challenge Infection*

Immunogen	Infected/noninfected (Percent protection) Exp. 1	Exp. 2
PBS	8/9 (11%)	9/10 (10%)
HBcAg	9/10 (10%)	10/10 (0%)
HBcAg-CS_1	0/10 (100%)	1/10 (90%)

Groups of 10 female BALB/c mice were immunized with PBS, 20 μg HBcAg, or 20 μg (*HBcAg-CS_1*) particles emulsified in CFA and boosted with the same doses of antigen in IFA 2 wk later. 3 mo after immunization, the mice were challenged with *P. berghei* by feeding *P. berghei* ANKA-infected *A. stephensi* mosquitoes on their tails. Parasitemia was monitored by microscopic examination of Giemsa-stained blood films daily for up to 25 d after challenge. The experiment was repeated a second time under identical conditions.

peptide revealed that the $[DP_4NPN]_2$ sequence did not contain a T cell recognition site relevant for these three strains, which explains the inability of HBcAg-CS_1 hybrid particles to prime CS-specific T cells. In contrast, the [NANP]n sequence of *P. falciparum* does represent a T cell recognition site in H-2^b haplotype-bearing strains (41, 42). Immunization of B10(H-2^b) mice with peptide $[NANP]_4$ confirmed T cell recognition of this sequence by B10 mice, and note that $[NANP]_4$-primed T cells were also recalled by HBcAg-CS_2 particles in vitro. In the reciprocal experiment, priming with HBcAg-CS_2 hybrid particles elicited HBcAg and $[NANP]_4$-specific T cell proliferative responses (Table 5). This result demonstrates that it is possible to insert a functional pathogen-derived T cell site between residues 75 and 81 of HBcAg. This expands the usefulness of HBcAg as a carrier vehicle to include both T and B cell recognition sites, and will allow the inclusion of additional malaria-specific Th sites which should alleviate the problem of MHC-restricted, CS-specific T cell recognition (44). To better understand positional effects on the priming of insert-specific T cells, future experiments will include the insertion of $[NANP]_4$ at different locations within HBcAg.

Protection Against Malaria. To study the protective efficacy of hybrid HBcAg-CS particles, we immunized BALB/c mice with HBcAg, CFA, or HBcAg-CS_1 particles and challenged them by feeding *P. berghei*–infected mosquitoes on their tails. This route of challenge was chosen as it represents a model that closely mimics the natural route of malaria transmission. 90– 100% of the control BALB/c mice immunized with HBcAg or CFA developed parasitemia within 4–6 d after challenge (Table 6). In contrast, in two independent experiments 10/10 mice and 9/10 mice were protected against *P. berghei* challenge after immunization with HBcAg-CS_1 particles. This demonstrates that the polyclonal, high titer anti-CS antibodies elicited by immunization with hybrid HBcAg-CS_1 particles are also protective. Protection is clearly mediated by CS-specific antibodies because immunization with HBcAg-CS_1 particles did not elicit CS-specific T cells (Table 5). More recently we have constructed hybrid HBcAg-CS particles carrying a *P. yoelii* CS repeat sequence (24) and confirmed that BALB/c mice were protected against *P. yoelii* challenge after immunization with these particles (F. Schödel, D.

Peterson, Y. Charoenvit, R. Wirtz, unpublished data). The availability of a carrier system which is known to be highly immunogenic in humans should now allow us to more conclusively test whether high titer, polyclonal and IgG isotype diverse anti-CS antibodies are protective against *P. falciparum* infection. If this proves to be the case, recombinant HBcAg-CS particles, possibly incorporating additional plasmodial T and B cell sites, may become a component of future malaria vaccines.

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