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The Position of Heterologous Epitopes Inserted in Hepatitis B Virus Core Particles Determines Their Immunogenicity†

FLORIAN SCHÖDEL,[1,2]* ANN M. MORIARTY,[3] DARRELL L. PETERSON,[4] JIAN ZHENG,[4] JANICE L. HUGHES,[2] HANS WILL,[1] DIDIER J. LETURCQ,[3] JULI S. McGEE,[3] AND DAVID R. MILICH[2]

Max-Planck-Institut für Biochemie, 8033 Martinsried, Germany[1]; Molecular Biology GA-1, The Scripps Research Institute, 10666 North Torrey Pines Road,[2] and R. W. Johnson Pharmaceutical Research Institute, Inc.,[3] La Jolla, California 92037; and Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia 23298[4]*

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The nucleocapsid (HBcAg) of the hepatitis B virus (HBV) has been suggested as a carrier moiety for vaccine purposes. We investigated the influence of the position of the inserted epitope within hybrid HBcAg particles on antigenicity and immunogenicity. For this purpose, genes coding for neutralizing epitopes of the pre-S region of the HBV envelope proteins were inserted at the amino terminus, the amino terminus through a precore linker sequence, the truncated carboxy terminus, or an internal site of HBcAg by genetic engineering and were expressed in *Escherichia coli*. All purified hybrid HBc/pre-S polyproteins were particulate. Amino- and carboxy-terminal-modified hybrid HBc particles retained HBcAg antigenicity and immunogenicity. In contrast, insertion of a pre-S(1) sequence between HBcAg residues 75 and 83 abrogated recognition of HBcAg by 5 of 6 anti-HBc monoclonal antibodies and diminished recognition by human polyclonal anti-HBc. Predictably, HBcAg-specific immunogenicity was also reduced. With respect to the inserted epitopes, a pre-S(1) epitope linked to the amino terminus of HBcAg was not surface accessible and not immunogenic. A pre-S(1) epitope fused to the amino terminus through a precore linker sequence was surface accessible and highly immunogenic. A carboxy-terminal-fused pre-S(2) sequence was also surface accessible but weakly immunogenic. Insertion of a pre-S(1) epitope at the internal site resulted in the most efficient anti-pre-S(1) antibody response. Furthermore, immunization with hybrid HBc/pre-S particles exclusively primed T-helper cells specific for HBcAg and not the inserted epitope. These results indicate that the position of the inserted B-cell epitope within HBcAg is critical to its immunogenicity.

Hepatitis B virus (HBV) encodes two polypeptides in its core gene. Initiation of translation at the first AUG within the gene results in a 25-kDa precore protein which is secreted as HBeAg after removal of the leader sequence at the amino terminus and 34 carboxy-terminal amino acids (4, 33, 48). Initiation at the second AUG (the first AUG on the core mRNA [C-mRNA]) leads to the synthesis of a 183-amino-acid, 21-kDa protein which assembles to form the virion nucleocapsid (HBcAg). HBeAg is colinear with HBcAg in its primary sequence; it has an additional 10 amino acids at the amino terminus and lacks the 34 carboxy-terminal amino acids of HBcAg (46). HBcAg can be expressed in *Escherichia coli* and self-assembles into core particles (9, 34, 44). The arginine-rich carboxy terminus of HBcAg, which mediates RNA binding in *E. coli*, is dispensable for particle formation (14). Although HBcAg is an internal component of the virion, it is highly immunogenic after HBV infection, regardless of the clinical outcome. Studies with mice have elucidated humoral and cellular mechanisms which explain the enhanced immunogenicity of HBcAg (22, 26, 27). For example, HBcAg is a particulate antigen that can function as a T-cell-independent antigen (i.e., it can directly activate B cells) (22), HBcAg can also efficiently immunize T cells (26), and HBcAg-specific T-helper (Th) cells can cooperate with envelope-specific B cells to produce anti-HBs (28). These characteristics predict that HBcAg may be exploited as an efficient T-cell carrier moiety for HBV and non-HBV vaccine design (28). Indeed, a number of investigators have utilized HBcAg for this purpose (3, 5–7, 25, 29, 38, 39, 42, 45). Foreign epitopes of HBV and non-HBV origins have been fused to the amino terminus of HBcAg through a precore linker sequence (5–7, 29) and to the carboxy terminus of truncated HBcAg (3, 42, 45). More recently, foreign epitopes were also inserted internally into HBcAg particles (6, 39, 42) at the site of a predicted outer loop. An outer loop was predicted at this site by amino acid sequence comparison of HBcAg with mengovirus VP3 and the existence of a 39-amino-acid insertion at this position in a related hepadnavirus, duck hepatitis B virus (1). In this study, we examined the effects of the position within HBcAg of the inserted epitopes on the antigenicity and immunogenicity of the hybrid HBcAg particles. For this purpose, neutralizing epitopes derived from the pre-S(1) and pre-S(2) regions of the HBV envelope proteins (17, 23, 24, 47) were inserted within HBcAg at the amino terminus, at the amino terminus through a precore linker sequence, at a truncated carboxy terminus, or at an internal position.

MATERIALS AND METHODS

Expression vectors and purification of hybrid HBc/pre-S particles. A vector (pFS14) expressing HBcAg of the authentic amino acid sequence (subtype *ayw*) (13) in *E. coli* under *tac* promoter control was described previously (42, 43). A

* Corresponding author.

† Publication 6899-MB of The Scripps Research Institute, La Jolla, Calif.

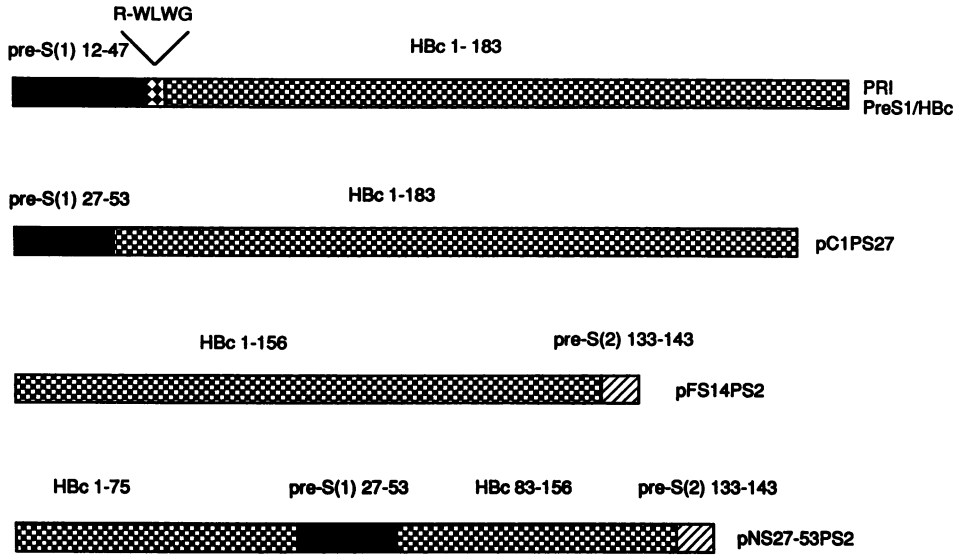

FIG. 1. Structure of hybrid HBc/pre-S proteins. Numbers indicate amino acid positions on the native proteins: HBcAg and pre-S(2) subtype *ayw* and pre-S(1) subtype *adw*. Plasmid designations are indicated at the right. The polylinker translation sequence (R) and the four carboxy-terminal precore amino acids (one-letter code) in plasmid PRI-PreS1/HBc are indicated (R-WLWG).

schematic presentation of the hybrid HBc/pre-S proteins is shown in Fig. 1. Vector pFS14PS2 directs the synthesis of pre-S2 amino acids 133 to 143 (sequence 133–143; subtype *ayw*) fused to amino acid position 156 of HBcAg [the resultant polypeptide will be designated HBc-(133–143)] (42). Vector pNS27-53PS2 codes for an insertion of pre-S1 amino acids 27 to 53 (sequence 27–53) between HBcAg amino acids 75 and 83 and a carboxy-terminal fusion of pre-S2 amino acids 133 to 143 after HBcAg amino acid 156, designated HBc-(27–53)-HBc-(133–143) (39). Vector pC1PS27 expresses a pre-S1 27–53 sequence inserted between the core methionine and the second core amino acid designated (27–53)-HBc. Vector pC1PS27 was derived from vector pFS14 by oligonucleotide-directed mutagenesis, by using a previously described polymerase chain reaction protocol (39, 40). The synthetic oligonucleotides used were C1PS27-3 (5′-AAA GAA TTC CAA CAA CCC GGA CTG GGA CTT CAA CCC GGT TAA AGA CGA CTG GCC GGA CAT CGA CCC TTA TAA AGA ATT TGG AGC-3′) and C1PS27-2 (5′-AAA GAA TTC GCA CCG AAA GCC GGG TCC AGC TGG TGG TCC ATA ATT CTG TTT CCT GTG TG-3′). Vector PRI-PreS1/HBc directs the synthesis of pre-S1 amino acids 12 to 47 (sequence 12–47) fused to the precore amino acid position 26. The resulting polypeptide is designated (12–47)-PC-HBc. Vector PRI-PreS1/HBc was constructed by subcloning a 575-bp *Sty*I-S1 fragment carrying the HBc gene plus 13 precore nucleotide residues (subtype *adw-2*) from a plasmid containing the entire HBV genome (AM6) (29). This precore-core fragment was subcloned into the *Sma*I site of pBluescript (Stratagene), and a pair of synthetic oligonucleotides specifying pre-S(1) amino acids 12 to 47 were inserted in the polycloning site of pBS upstream of the HBc gene and subcloned into the *tac* promoter vector pKK223-2 (Pharmacia), so that in the translation product the pre-S(1) sequence is fused to precore amino acid 26 through an additional arginine stemming from the polylinker. The coding region of the vectors was verified by restriction analysis and dideoxy sequencing, using standard procedures (36). Hybrid HBc/pre-S proteins and HBcAg were expressed in *E. coli* JM105 and purified to approximately 95% purity by chromatography on hydroxylapatite (19) and Sepharose 4B (Pharmacia) columns as previously described for woodchuck hepatitis virus core antigen (40). In contrast to the other particles, HBc-(27–53)-HBc-(133–143) was separated on a fast protein liquid chromatography Mono Q (Pharmacia) column with 10 mM Tris-HCl, pH 8.0, and an NaCl gradient from 0.01 to 1.0 M after hydroxylapatite chromatography. Fractions were monitored by spectrometry at 280 nm and assayed by using the Abbott HBeAg assay. The antigenic material eluted as a well-separated peak at about 0.6 M NaCl (results not shown). All preparations of hybrid HBcAg particles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting) (results not shown) as well as electron microscopy (Fig. 2).

Mice. BALB/c, C57BL/10 (B10), B10.S, and SJL/J murine strains were obtained from the breeding colony at the Scripps Research Institute. Female mice 6 to 8 weeks of age at the initiation of the experiments were used in all studies.

Synthetic peptide analogs. Peptides were synthesized by the Merrifield solid-phase method and were subjected to high-pressure liquid chromatography on a C-18 reverse-phase column. All peptides used eluted as a single major peak (>90%).

Monoclonal antibodies. Monoclonal antibodies specific for HBcAg were kindly provided by M. Mayumi (Institute for Immunology, Tokyo University, Tokyo, Japan) (3105 and 3120) (16, 46) and V. Bichko (Institute for Organic Synthesis, Riga, Latvia) (C1-5 and C3-1) or obtained from a commercial supplier (Green Cross Corporation, Osaka, Japan) (440 and 442). Monoclonal antibodies specific for pre-S(1) (18/7) (11) and pre-S(2) (4408) (24, 32) were generously provided by W. Gerlich (University of Giessen, Giessen, Germany) and M. Mayumi, respectively.

Immunization. For determination of serum antibody responses, groups of four mice were immunized intraperitoneally with 20 μg of hybrid particles or 100 μg of synthetic peptides in 0.2 ml of complete Freund's adjuvant. Mice were

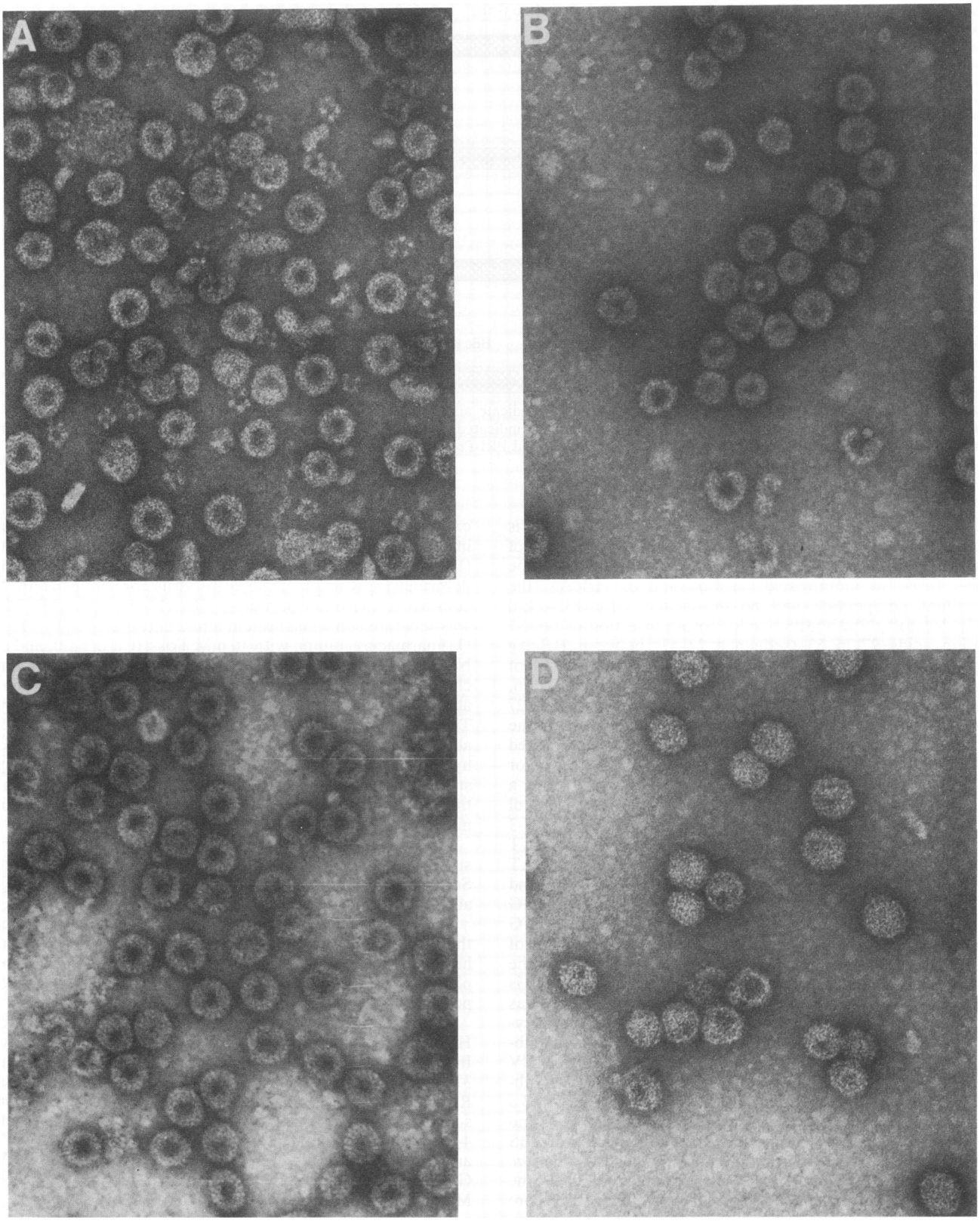

FIG. 2. Electron micrographs of hybrid HBc/pre-S particles. A, (12–47)-PC-HBc; B, (27–53)-HBc; C, HBc-(133–143); D, HBc-(27–53)-HBc-(133–143). Magnification, ×312,852. Samples were negatively stained by aqueous uranyl formate.

bled by retro-orbital puncture at 10 and 24 days after primary immunization and at 2 weeks after a secondary immunization in incomplete adjuvant. In vivo priming for T-cell proliferation was accomplished by footpad injection of 10 μg of hybrid particles or 100 μg of synthetic peptide in complete Freund's adjuvant.

Measurement of anti-HBc and anti-pre-S antibodies. Pooled murine sera were evaluated for anti-HBc antibody in a solid-phase enzyme-linked immunosorbent assay (ELISA). Purified HBcAg (100 ng per well) was coated to microtiter plates, incubated with test sera and then with goat anti-mouse immunoglobulin (Ig), and developed with peroxidase-labeled swine anti-goat Ig as previously described (23). The data are expressed as antibody titers representing the highest serum dilution to yield 4× the optical density at 492 nm (OD_{492}) of preimmune sera. For detection of anti-pre-S antibodies, synthetic peptides (1 μg per well) or plasma-derived, purified HBcAg/P39 (100 ng per well), which contains the pre-S(1) and pre-S(2) regions, was used as the solid-phase ligand.

T-cell proliferation assay. Groups of four mice were primed with 10 μg of hybrid particles or 100 μg of synthetic peptide in hind footpads, and draining popliteal lymph node cells were harvested and pooled 10 days after immunization. The popliteal lymph node cells (5×10^5) in 0.1 ml of Click's medium (8) were cultured with 0.1 ml of medium containing synthetic peptides or HBcAg for 96 h at 37°C in a humidified CO_2 atmosphere. During the final 16 h, 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added. The cells were then harvested onto filter strips for determination of [^{3}H]TdR incorporation. The data are presented as counts per minute corrected for background proliferation in the absence of antigen.

RESULTS

Antigenicity of hybrid HBc/pre-S particles. The purified hybrid HBc/pre-S polypeptides were analyzed by electron microscopy. All HBc/pre-S fusion proteins were able to form particles (Fig. 2). The HBc-(27–53)-HBc-(133–143) polypeptide formed particles which appear somewhat larger than normal core particles and display a slightly different electron density (Fig. 2D). To investigate HBc and pre-S antigenicities and the pre-S surface accessibility of the hybrid HBc/pre-S particles, we used monoclonal and polyclonal anti-HBc and anti-pre-S antibodies. The fused pre-S(1) or pre-S(2) epitopes were surface accessible under nondenaturing conditions when the particles were bound to ELISA plates, with the exception of the pre-S(1) sequence 27–53 fused to the authentic amino terminus of HBc, i.e., (27–53)-HBc (Table 1). A Western blot analysis of (27–53)-HBc confirmed that the pre-S(1) sequence was expressed (results not shown). However, a similar pre-S(1) sequence became surface accessible when it was fused to a precore linker sequence consisting of four amino acids (precore position 26) plus a linker arginine in (12–47)-PC-HBc particles (Table 1). The surface accessibility of pre-S sequences in hybrid HBcAg particles was further confirmed by liquid-phase capture assays with anti-pre-S monoclonal antibodies on the solid phase and polyclonal anti-HBc or monoclonal anti-pre-S antibodies as the probes (results not shown).

Fusion of heterologous sequences to the amino terminus or the truncated carboxy terminus of HBcAg preserved the major HBcAg antibody-binding sites recognized by a panel of anti-HBc monoclonal antibodies (Table 2). In contrast, all but one monoclonal anti-HBc antibody (3120) failed to recognize hybrid HBc particles with a pre-S(1) insertion between HBcAg amino acids 75 and 82, demonstrating the importance of this region in B-cell recognition of HBcAg. Furthermore, reactivity with HBc-(27–53)-HBc-(133–143) distinguished between the epitopes recognized by monoclonal antibodies 3105 and 3120 even though 3105 and 3120 reciprocally inhibit each other.

TABLE 1. Antigenicities of hybrid HBcAg particles[a]

Hybrid particle	OD_{492} of antiserum		
	Anti-HBc[b]	Anti-pre-S(1)[c]	Anti-pre-S(2)[d]
(27–53)-HBc	0.37	0	0
(12–47)-PC-HBc	0.53	0.7	0
HBc-(27–53)-HBc-(133–143)	0.14	2.0	1.7
HBc-(133–143)	0.7	0	1.7
HBcAg	0.6	0	0
HBsAg/P39[e]	0	1.4	0.6

[a] Microtiter plates were coated with particles (50 ng per well) and tested for HBc, pre-S(2), and pre-S(1) antigenicities with polyclonal and monoclonal reagents. OD_{492} readings corrected for background are shown.
[b] Human polyclonal anti-HBc.
[c] Monoclonal antibody 18/7; pre-S(1) specific; residues 38 to 47 (11).
[d] Monoclonal antibody 4408; pre-S(2) specific; residues 133 to 139 (24).
[e] Derived from serum; 0.5 μg per well.

Immunogenicity of hybrid HBc/pre-S particles. (i) Antibody responses. To determine and compare the immunogenicities of HBcAg and the inserted pre-S sequences of the various hybrid HBc/pre-S particles, BALB/c mice were immunized with equal doses (20 μg) of the four hybrid particles. The pre-S(2) 133–143 sequence fused to the truncated carboxy terminus of HBcAg induced high-titered anti-HBc and very low-titered anti-pre-S(2) antibodies in accordance with previously published data (Table 3). The pre-S(1) 27–53 sequence fused to the authentic HBcAg amino terminus was not surface accessible and predictably induced minimal anti-pre-S(1) antibodies. The anti-HBc response was intact and comparable to the anti-HBc response of the carboxy-terminal fusion. The pre-S(1) sequence 12–47, fused to a precore linker sequence, was surface accessible and induced high-titered anti-pre-S(1) as well as high-titered anti-HBc antibodies (Table 3). The pre-S(1) 27–53 sequence inserted between HBcAg amino acids 75 and 83 induced an extremely high antibody response against the pre-S(1) insert that is comparable to anti-HBc titers achieved after immunization with native HBcAg particles. The anti-pre-S(1) peptide responses after immunization with HBc-(27–53)-HBc-(133–

TABLE 2. Anti-HBc monoclonal antibody analysis of hybrid HBcAg particles[a]

Anti-HBc MAb	OD_{492} of hybrid particles		
	HBc-(27–53)-HBc-(133–143)	(12–47)-PC-HBc	HBcAg
3105	0	0.63	0.61
3120	0.32	0.54	0.90
440	0	0.23	0.45
442	0	0.35	0.21
C1-5	0	0.48	0.84
C3-1	0	0.49	0.15

[a] Microtiter plates were coated with hybrid HBcAg (50 ng per well), and the indicated anti-HBc monoclonal antibodies (MAb) (16 ng) were added. The bound monoclonal antibodies were determined by the addition of a goat anti-mouse Ig second antibody followed by a peroxidase-labeled swine anti-goat Ig as described previously (23).

TABLE 3. Immunogenicities of hybrid HBcAg particles[a]

Mouse strain, immunogen, and antiserum day	Antibody titer (reciprocal of dilution)				
	HBcAg	pre-S(1) (12–47)	pre-S(1) (32–53)	pre-S(2) (133–143)	HBsAg/P39
BALB/c (*H-2*^{d})					
(27–53)-HBc					
10	10,240		0	0	0
24	163,840		160	0	0
(12–47)-PC-HBc					
10	10,240	2,560	2,560	0	0
24	655,360	20,480	20,480	0	1,280
HBc-(27–53)-HBc-(133–143)					
10	160	10,240	10,240	160	0
24	10,240	655,360	655,360	160	2,560
2°	163,840		2.6×10^6	640	10,240
HBc-(133–143)					
10	10,240	0	0	0	0
24	655,360	0	0	160	40
2°	10.4×10^6	0	0	640	160
B10 (*H-2*^{b})					
HBc-(27–53)-HBc-(133–143)					
10	640		10,240		0
24	2,560		81,920		640
B10.S (*H-2*^{s})					
HBc-(27–53)-HBc-(133–143)					
10	2,560		10,240		0
24	10,240		655,360		2,560
BALB/c					
pre-S(1) 12–47					
10	0	160	0	0	0
24	0	160	0	0	0
pre-S(1) 32–53					
10	0	0	0	0	0
24	0	0	0	0	0
pre-S(2) 133–143					
10	0	0	0	0	0
24	0	0	0	0	0

[a] Mice were immunized with 20 μg of hybrid particles or 100 μg of synthetic peptides, and 10- and 24-day primary antisera or secondary antisera (2°) were analyzed for reactivity with the indicated solid-phase antigens by ELISA.

143) were 4-fold greater at day 10 and 32-fold greater at day 24 than the responses ellicited by (12–47)-PC-HBc particles. Antibodies detected on the pre-S(1) and pre-S(2) peptides were also reactive with native envelope particles (HBsAg/P39). The enhanced immunogenicity of the internally inserted sequence is not unique to BALB/c mice; it was verified in three inbred mouse strains of different major histocompatibility complex backgrounds (Table 3) and confirmed in outbred rabbits (results not shown). The HBc-(27–53)-HBc-(133–143) particles induced only very low antibody titers to the carboxy-terminal-fused pre-S(2) sequence, similar to the HBc-(133–143) particles (Table 3).

To control for potential intrinsic immunogenicity of the pre-S sequences inserted into HBcAg, we immunized BALB/c mice with synthetic peptides representing the inserted sequences. The pre-S sequences represented by synthetic peptides without a carrier molecule induced little or no antibody in BALB/c mice (Table 3). To investigate the fine specificity of the humoral immune responses to pre-S(1) elicited by immunization with the hybrid HBc/pre-S(1) particles, murine sera were analyzed with a panel of synthetic peptides (Table 4). The predominant anti-pre-S(1) antibody response elicited by either the amino-terminal or internal pre-S(1) insertion was directed against the carboxy terminus (amino acids 41 to 53) of the inserted sequence, as suggested by earlier epitope mapping studies (23).

The amino- and carboxy-terminal-modified HBcAg particles induced high-titered anti-HBc antibody production (Table 3). To further study the impact of the deletion of HBcAg residues 76 to 82 (sequence 76–82) on native HBcAg immunogenicity, we analyzed the anti-HBc titer in several mice strains immunized with HBc-(27–53)-HBc-(133–143) particles. Particles with the internal pre-S(1) insertion elicited relatively low primary anti-HBc titers compared with the other chimeric HBcAg particles (Table 3). This indicates that a major immunogenic region of HBcAg was removed by the deletion of residues 76 to 82.

HBcAg is a T-cell-independent as well as T-cell-dependent immunogen (22). Insertion of sequences at the amino terminus of HBcAg directly or through the precore linker sequence did not abrogate the T-cell independence of HBcAg (Table 5). However, only the pre-S(1) 12–47 sequence fused to the precore linker sequence also elicited a low-titered anti-pre-S(1) response in BALB/c (*nu/nu*) athymic mice (Table 5). The internal pre-S(1) insert abrogated the T-cell independence of HBcAg, most likely by virtue of the deletion of critical HBcAg residues.

(ii) T-cell immunogenicity. Immunization of BALB/c mice

TABLE 4. Fine specificity of anti-pre-S(1) antibodies[a]

Mouse strain	Immunogen	Antiserum day	Anti-pre-S(1) titer (reciprocal of dilution) with peptide:			
			12–21	22–32	32–41	41–53
BALB/c	(12–47)-PC-HBc	10	0	0	0	160
		24	160	640	0	2,560
BALB/c	HBc-(27–53)-HBc-(133–143)	10	0	0	0	1,280
		24	0	0	0	40,960
B10	HBc-(27–53)-HBc-(133–143)	10	0	0	0	1,280
		24	0	0	0	10,240
B10.S	HBc-(27–53)-HBc-(133–143)	10	0	0	0	2,560
		24	0	0	0	40,960

[a] Mice were immunized as described for Table 3, and the sera were assayed by ELISA on four different synthetic peptides representing the pre-S(1) sequences (subtype *adw*).

with (12–47)-PC-HBc and HBc-(27–53)-HBc-(133–143) particles elicited antibody production specific for HBcAg and the inserted pre-S(1) sequences. However, the source of T-cell help for anti-HBc and anti-pre-S(1) antibody production is not apparent from the serological analysis. To address this issue, BALB/c ($H\text{-}2^d$) and SJL/J ($H\text{-}2^s$) mice were immunized with (12–47)-PC-HBc particles or the free peptide 12–47 and the specificities of the T-cell proliferative responses were determined. As shown in Fig. 3A, immunization of BALB/c mice with (12–47)-PC-HBc resulted in the priming of T cells specific for HBcAg and the HBcAg peptide 85–140 but not pre-S(1) 12–47-specific T cells. This was expected, because the pre-S(1) 12–47 sequence does not contain a T-cell site recognized by BALB/c mice, as illustrated by nonresponsiveness to immunization with peptide 12–47 (Fig. 3C). In contrast, in SJL mice, the pre-S(1) 12–32 sequence represents an efficient T-cell site, as shown after immunization with peptide 12–47 (Fig. 3D). Nevertheless, immunization of SJL/J mice with (12–47)-PC-HBc resulted in the priming of T cells specific for HBcAg and HBcAg peptides 85–140 and 120–131, but not 12–47-specific T cells (Fig. 3B).

Therefore, in both mouse strains tested, the source of Th cell function for anti-HBc and anti-pre-S(1) antibody production emanated from HBcAg-specific T cells. Similar results were obtained after immunization with HBc-(27–53)-HBc-(133–143) particles (41), which indicates that the deletion in HBcAg did not affect T-cell recognition, at least in the three major histocompatibility complex haplotypes studied.

DISCUSSION

The enhanced immunogenicity of the particulate HBcAg (22, 26–28) has led to its use as a carrier molecule for intrinsically less-immunogenic heterologous peptidic epitopes (3, 5–7, 25, 29, 38, 39, 42, 45). Although this approach has led to the induction of antibodies against various sequences as diverse as foot-and-mouth disease virus capsid proteins and human immunodeficiency virus Gag, the more practical approach of using HBcAg as a carrier for hepatitis B virus surface antigen epitopes has been attempted only recently (3, 38, 39, 42, 45). The advantage of using HBcAg as a carrier for HBV surface antigen epitopes is that immunization with HBcAg alone has been reported to partially protect chimpanzees from HBV-induced disease (18, 30, 31). Furthermore, T cells primed against HBcAg can be expected to react with HBcAg after an exposure to HBV, thus potentially providing Th cell recall memory for antibody production to envelope proteins (28) as well as HBcAg epitopes and/or cytotoxic T-lymphocyte induction. Prompted by these considerations, we have examined the potential of HBcAg to act as a carrier as well as the importance of the location of the inserted epitopes. Epitopes from the pre-S region of the HBV envelope, which have previously been shown to induce protective immune responses in chimpanzees, were chosen for insertion (17, 23, 24, 47). Epitopes derived from the pre-S(2) and the pre-S(1) regions were (i) fused to precore sequences similar to previous amino-terminal fusions to HBcAg, (ii) fused to the authentic HBcAg amino terminus, (iii) inserted internally between HBcAg amino acids 75 and 83 (thereby deleting HBcAg residues 76 to 82), or (iv) fused to the carboxy-terminal amino acid 156 of a truncated HBcAg. A pre-S(1) sequence fused to the authentic HBcAg amino terminus was not surface accessible and not immunogenic. However, a similar pre-S(1) sequence was exposed on the surface of particles and immunogenic when fused to the carboxy-terminal four precore amino acids. This suggests that the amino terminus of native HBcAg may not be exposed on the particle surface, and heterologous epitopes are exposed at the exterior of HBcAg particles by virtue of the precore and/or linker amino acids. The fact that a variety of different epitopes have been exposed at the HBcAg particle surface and rendered immunogenic by fusion to precore residues (5, 7, 29) argues that this is not a peculiarity of the pre-S(1) sequence that we used. Unless this phenomenon is specific for the pre-S(1) sequence, it may be misleading to suggest that amino-terminal fusion of foreign epitopes to HBcAg is a method of enhancing their immunogenicity (5–7, 29). Our data suggest that to become exposed on the surface, foreign epitopes should be fused to a precore linker sequence or

TABLE 5. T-cell independence of hybrid HBcAg particles[a]

Immunogen	Antibody titer (reciprocal of dilution)		
	HBcAg	pre-S(1) (32–53)	pre-S(2) (133–143)
(27–53)-HBc	640	0	0
(12–47)-PC-HBc	2,560	160	0
HBc-(27–53)-HBc-(133–143)	0	0	0
HBcAg	5,120	0	0

[a] BALB/c (*nu/nu*) athymic mice were immunized with 20 μg of hybrid HBc/pre-S particles, and antibody titers at day 10 were determined by ELISA as described for Tables 3 and 4.

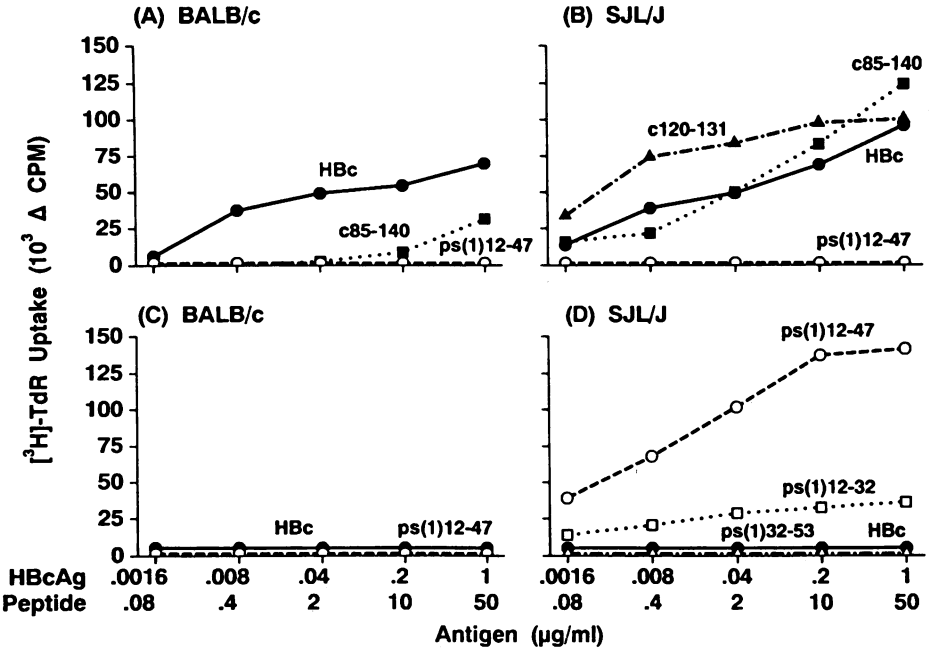

FIG. 3. T-cell recognition of hybrid (12–47)-HBc particles. Groups of four BALB/c and SJL/J mice were immunized with 10 μg of (12–47)-HBc (A and B) or 100 μg of the synthetic pre-S(1) peptide 12–47 (C and D), and draining popliteal lymph node cells were analyzed for in vitro T-cell proliferation elicited by the indicated antigens. T-cell proliferation is expressed as [^{3}H]TdR uptake corrected for background proliferation in the absence of antigen (Δcpm). Background proliferation ranged from 2,200 to 8,900 cpm.

internally inserted within HBcAg for even greater immunogenicity.

Fusion of foreign epitopes at the amino or carboxy terminus of HBcAg did not change the antigenicity and immunogenicity of native HBcAg. However, insertion of a pre-S(1) sequence internally within HBcAg, at a position which had previously been suggested to represent a dominant antibody-binding region (35), drastically changed the antigenicity and immunogenicity of HBcAg. In fact, only one of six anti-HBc monoclonal antibodies was reactive with HBc-(27–53)-HBc-(133–143) particles. This defines the 76–82 region as important in terms of HBcAg antigenicity and distinguishes among the epitopes defined by these monoclonal antibodies. Monoclonal antibodies against native HBc and human anti-HBc-positive patient sera do not react significantly with overlapping HBcAg synthetic peptides as solid-phase reagents in ELISA-type assays, which argues that the major HBcAg epitopes are conformation dependent (2, 20). This contradicts a single report suggesting an immunodominant sequential epitope within HBcAg (10). In contrast, epitope mapping of anti-HBc monoclonal antibodies with overlapping fusion proteins leads to the identification of HBcAg amino acids 74 to 89 as critical residues (35) but not sufficient for native HBcAg antigenicity (20). Our data support the importance of HBcAg amino acids 76 to 82 to the antigenicity of HBcAg. Previous cross-inhibition studies using native HBcAg demonstrated that monoclonal anti-HBc antibodies and polyclonal anti-HBc-positive patient sera evidenced a high degree of cross-inhibition (12) and could not fully discriminate between anti-HBc monoclonal antibodies 3105 and 3120. However, monoclonal antibody 3105 is nonreactive and monoclonal antibody 3120 is reactive with HBc-(27–53)-HBc-(133–143) particles. Therefore, reactivity with this mutant HBcAg particle can distinguish between the dominant HBcAg epitopes defined by these reciprocally inhibiting monoclonal antibodies. HBc-(27–53)-HBc-(133–143) particles induce anti-HBc antibodies but to a much lower titer than native HBcAg, indicating that at least one B-cell epitope of HBcAg is conserved on these particles (i.e., defined by monoclonal antibody 3120) and that this epitope does not depend on residues 76 to 82.

While the amino- and carboxy-terminal-modified hybrid HBcAg particles maintained the T-cell independence of native HBcAg (27), only the (12–47)-PC-HBc fusion protein induced a low but detectable anti-pre-S(1) response in BALB/c (*nu/nu*) athymic mice. Interestingly, the internal pre-S(1) insertion abrogated the T-cell independence of the anti-HBcAg response and the anti-pre-S(1) response was not T-cell independent; however, the HBc-(27–53)-HBc-(133–143) particles were the most immunogenic in terms of the anti-pre-S(1) response. Therefore, factors other than T-cell independence appear to play the major role in the efficient carrier effects of HBcAg.

Amino-terminal fusion, carboxy-terminal deletion and fusion, and insertion of foreign epitopes internally within HBcAg did not prevent particle formation. Furthermore, with the exception of the pre-S(1) sequence fused to the amino terminus of authentic HBcAg, all foreign sequences inserted or fused were surface accessible. The carboxy-terminal-fused pre-S(2) epitopes induced the least antibody response to the foreign sequence, in accordance with previous results (3, 38, 45). Amino-terminal fusion of pre-S(1) residues 12 to 47 to a precore linker exposed this sequence on the particle surface and rendered it immunogenic. The highest antibody response against a heterologous epitope was elicited when a pre-S(1) epitope was inserted between HBcAg amino acids 75 and 83 (at least 32-fold higher in a primary immune response compared with the amino-terminal fusion to a precore linker). Another group has recently inserted a rhinovirus epitope and a human immunodeficiency

virus epitope between HBcAg amino acids 80 and 81 and reported enhanced immunogenicity for the internally inserted rhinovirus epitope compared with the same epitope fused to an amino-terminal precore linker sequence (6).

The extraordinary immunogenicity of internally inserted heterologous sequences within HBcAg remains to be explained. Surface accessibility on the particles is certainly a prerequisite, but it is not sufficient, as evidenced by the lack of immunogenicity of carboxy-terminal-fused pre-S(2) sequences, which are exposed on the surface of HBc-(133–143) and HBc-(27–53)-HBc-(133–143) hybrid particles. The stability of the hybrid particles is most likely an important consideration for immunogenicity. However, it is highly unlikely that hybrid HBc/pre-S particles with a deletion and insertion and a carboxy-terminal deletion and fusion are more stable than those that only have a carboxy-terminal deletion and fusion. In a particle composed of identical subunits, it cannot be assumed that amino termini or carboxy termini of all molecules are identically oriented. Indeed, as we have reasoned previously (39), the fact that HBcAg particles incorporate RNA in *E. coli* by virtue of their protaminelike carboxy terminus implies that at least 1 in 3 or 2 in 3 carboxy termini, assuming a symmetry of the HBcAg particles in which T = 3 (1), are oriented to the particle interior. The amino termini of native HBcAg may not be surface oriented at all, and the number of epitopes fused to a precore linker that become surface accessible per single particle is unknown. Thus the density of epitopes on a particle is another consideration and may, in part, account for their immunogenicity. Finally, the orientation between the T- and B-cell sites within the hybrid HBcAg particle may be relevant to the differential immunogenicity described herein. It has been previously reported that the orientation of T- and B-cell sites relative to each other on synthetic immunogens can dramatically affect immunogenicity (15, 21). The predominant T-cell sites within HBcAg have been mapped to the 85–140 region (26). Therefore, the T-to-B-cell site orientation of the more-immunogenic hybrid particles is carboxy to amino; it is amino to carboxy for the less immunogenic HBc-(133–143) particles.

A question that has not been previously addressed is the source of T-cell help for antibody production to the foreign epitopes after immunization with hybrid HBcAg particles. This question has potentially important implications, especially for non-HBV vaccine development. If T cells predominantly recognize the HBcAg carrier moiety, they will not be primed against a potential T-cell site relevant to the pathogen and included in the inserted sequence. Very efficient T-cell responses were elicited by the hybrid HBc/pre-S particles that were exclusively specific for HBcAg. Although (12–47)-PC-HBc contains a pre-S(1) T-cell site recognized in the context of major histocompatibility complex class II molecules of SJL/J mice (23), immunization with (12–47)-PC-HBc failed to activate pre-S(1)-specific T cells. One possible explanation is that this fusion protein may be processed differently than the native antigen containing the same T-cell site. Therefore, this result suggests that the mere inclusion of a T-cell site in the inserted sequence does not guarantee that it will be functional in the context of the hybrid particle. If this is a general phenomenon, it may be a concern when HBcAg particles are used to deliver non-HBV epitopes representing both T- and B-cell sites. Nevertheless, it appears that the ability of HBcAg to elicit a strong HBcAg-specific Th cell response in the context of multimeric B-cell epitopes may explain the efficient carrier effects of particulate HBcAg.

An additional aspect of hybrid HBcAg particles is that they can be constitutively expressed to high levels in vaccine strains of various *Salmonella* spp. (38, 39, 42) and probably other prokaryotes. These recombinant *Salmonella* carrier strains constitute potential oral-route combination vaccines (37). We have demonstrated that attenuated *Salmonella typhimurium* strains expressing HBc-(27–53)-HBc-(133–143) can induce high-titered serum anti-pre-S(1) antibodies after oral immunization in mice (39).

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