

NONOVERLAPPING T AND B CELL DETERMINANTS ON
AN HEPATITIS B SURFACE ANTIGEN PRE-S(2) REGION
SYNTHETIC PEPTIDE

BY DAVID R. MILICH,* ALAN McLACHLAN,* FRANCIS V. CHISARI,* AND
GEORGE B. THORNTON[‡]

*From the *Department of Basic and Clinical Research, Scripps Clinic and Research
Foundation, La Jolla, California 92037; and [‡]Johnson and Johnson Biotechnology Center,
San Diego, California 92121*

The hepatitis B surface antigen (HBsAg)¹ is present both on the envelope of the intact virion and as free, circulating, 22-nm particles in the serum of infected patients. The two major polypeptides of HBsAg, p25 and its glycosylated form gp28, have identical amino acid sequences encoded by the S gene of the hepatitis B virus (HBV) genome. Recently (1), an additional higher mol wt glycoprotein component of HBsAg was identified as gp33/gp36. The p33 polypeptide contains the sequence of p25 and has 55 additional amino acids at the amino terminus, encoded by the pre-S(2) region of the HBV genome (1, 2). In support of this, Neurath et al. (3) synthesized a peptide encompassing the 26 NH₂-terminal amino acids of the pre-S(2) region, and antibodies to this peptide (p120-145) reacted with p33-containing particles (HBsAg/p33) but not with particles lacking the pre-S(2) region (HBsAg/p25). It has also been shown (4) that antibodies to p120-145 compete with anti-native pre-S(2)-specific antibodies for the same or closely related binding site(s) on native HBsAg/p33 particles. Additionally the native pre-S(2) region, expressed on recombinant HBsAg/p33 particles, has been shown (4, 5) to be significantly more immunogenic (at the B and T cell levels) than the S region of HBsAg. Moreover, independent H-2-linked regulation of the pre-S(2) and S region immune responses decreases the probability of genetic nonresponsiveness after HBsAg/p33 immunization (5). For these and other reasons, the inclusion of pre-S(2)-bearing polypeptides in future HBsAg particle vaccines has been suggested (3-7). These observations, and the fact that the synthetic peptide p120-145 was also reported (3) to be highly immunogenic in animals, prompted us to examine T cell recognition of this pre-S(2)-specific synthetic peptide in terms of fine specificity, H-2-linked genetic influences,

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¹ *Abbreviations used in this paper:* HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; CHO, Chinese hamster ovary; PLN, peripheral lymph node.

comparison to antibody binding, and relevance to T cell recognition of the native protein.

The results of this study indicate that the predominant T and B cell recognition sites on the synthetic pre-S(2) peptide are nonoverlapping. T cell recognition is focused on the NH₂-terminal sequence, and antibody (B cell) recognition is focused on the COOH-terminal sequence. The fine specificity of T cell recognition of p120-145 was defined by a single amino acid substitution. The fine specificity of B cell recognition has also been investigated and is the subject of another study.² The immune response to the pre-S(2) synthetic peptide p120-145 is regulated by H-2-linked genes, but the pattern of H-2 restriction differed from that of the native pre-S(2) region response. This finding, and the inability of p120-145-primed T cells to recognize native pre-S(2) region protein indicate that, in contrast to the B cell response, p120-145 does not represent a native pre-S(2) region T cell recognition site. However, p120-145 does represent a totally synthetic immunogen possessing distinct T and B cell determinants, and the T cell recognition site is capable of priming T cell help for antibody production, which is crossreactive with the native pre-S(2) region on HBsAg/p33 particles. This system offers evidence that a synthetic T cell site coupled to a synthetic B cell site can comprise a functional immunogen (vaccine) without the necessity of a heterologous protein carrier to elicit T cell helper function.

Materials and Methods

Mice. C57BL/10 (B10), B10.D2, B10.BR, B10.S, B10.M, C₃H.Q, C₃H, and SJL/J murine strains were obtained from the breeding colony at the Research Institute of Scripps Clinic. Female mice, 6–8 wk of age at the start of the experiments, were used in all studies.

HBsAg Particles and Synthetic Peptide Analogs of the Pre-S(2) Region. Recombinant HBsAg particles derived from Chinese hamster ovary (CHO) cells transfected with a plasmid containing the S gene and the pre-S region of HBV (7) were provided by P. Tiollais (Pasteur Institute, Paris). The CHO-derived particles are composed of the S-encoded p25 plus the pre-S(2) and S-encoded p33 in a ratio of ~3:1 (7), and are herein designated as HBsAg/p33. The peptides were synthesized by the Merrifield solid-phase method, and were subjected to HPLC on a C18 reverse-phase column. All the peptides used eluted as a single major peak (>90%). Synthetic peptides were provided by the peptide laboratory of the Biotechnology Center, Inc. (Sorrento Valley, CA).

The amino acid sequence of the pre-S(2) region from residues 120–145 is illustrated in Fig. 1. Sequence variation relative to three HBsAg subtypes *ayw* (8), *adw₂* (9), and *adw* (10) are shown. The peptides synthesized and used in this study are also depicted in Fig. 1. The first methionine, which represents the NH₂-terminus of p33, is labelled 120, since it can be preceded by 119 residues comprising the pre-S(1) region yielding a polypeptide of 39 kD (11).

Measurement of In Vivo Antibody Production. Pooled murine sera were evaluated for antibody in an indirect, solid-phase RIA using solid-phase HBsAg/p33 (0.1 µg/well) or synthetic peptides (1–2 µg/well), and goat anti-mouse IgG, and developed with an ¹²⁵I-labelled, affinity-purified swine anti-goat Ig as described (12). Data are expressed as antibody titer representing the highest dilution to yield three times the counts of preimmunization sera. Mice were immunized for determination of in vivo antibody production by i.p. injection of either 1.0 µg of HBsAg/p33 or 100 µg of synthetic peptide emulsified

² Milich, D. R., A. McLachlan, F. V. Chisari, T. Nakamura, G. B. Thornton. Two distinct but overlapping antibody binding sites in the pre-S(2) region of HBsAg localized within 11 continuous residues. Manuscript submitted for publication.

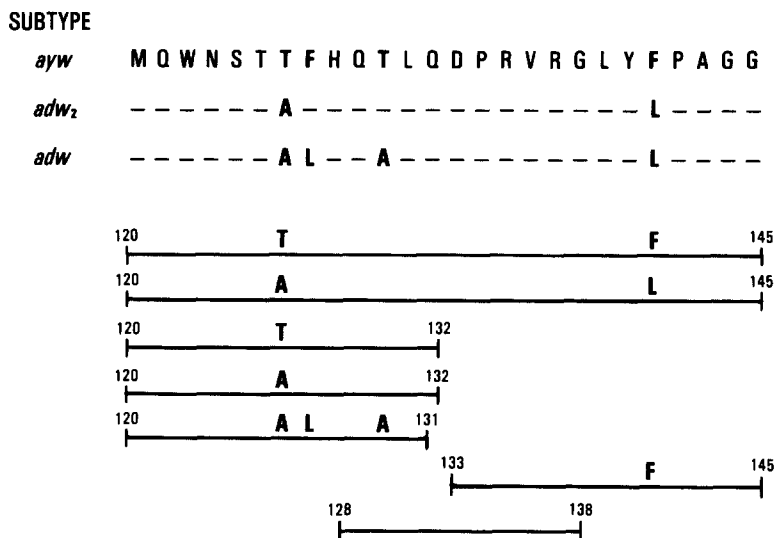


FIGURE 1. The amino acid sequence of residues 120–145 of the pre-S(2) region of HBsAg. Sequences of the *ayw*, *adw₂*, and *adw* subtypes of HBV are shown. The sequences are presented in the one-letter code system, a dashed line represents a residue unchanged from the *ayw* sequence. Also depicted are the synthetic peptide analogs used in this study.

in CFA. Sera was collected 24 d after primary immunization, and 2 wk after secondary immunization with a half dose of antigen in IFA.

T Cell Proliferative Assay. Groups of mice were primed with either 4 μ g of HBsAg/p33 or 100 μ g of synthetic peptide in CFA by hind footpad injection. 8–10 d after immunization, draining popliteal lymph node (PLN) cells were harvested, and 5×10^5 cells in 0.1 ml of Click's medium (13) were cultured with 0.1 ml of medium containing HBsAg/p33, various synthetic peptides, or medium alone. Cells were cultured for 96 h at 37°C in a humidified 5% CO₂ atmosphere, and during the final 16 h, 1 μ Ci of [³H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added. The cells were then harvested onto filter strips for determination of [³H]TdR incorporation. The data are expressed as cpm corrected for background proliferation in the absence of antigen (Δ cpm). The T cell nature of the proliferation was confirmed in selected experiments by concurrently analyzing antigen-specific, dose-dependent, IL-2 production in 24-h supernatants as described (14). Since the PLN proliferative responses always correlated with IL-2 production, only the proliferative responses are reported.

Results

The Anti-Peptide (p120-145) Pre-S(2) Response Is H-2 Restricted, But in a Manner Distinct from H-2 Restriction of Response to Native Pre-S(2) Region. Since we had previously shown (5) that the immune response to the native pre-S(2) region was regulated by H-2-linked genes and was mediated at the T cell level, we wished to determine whether the response to the synthetic pre-S(2) peptide was similarly regulated. A panel of B10 H-2-congenic strains was immunized i.p. with either HBsAg/p33 particles or with the free pre-S(2)-specific synthetic peptide p120-145, both of the *ayw* subtype (Fig. 2). The anti-pre-S(2) region responses were analyzed by RIA using p120-145 as the solid-phase ligand. Anti-HBs/p33 sera were tested 24 d after primary immunization (pre-S(2) response predominates over the S response at this time [4]), and anti-peptide sera were tested after

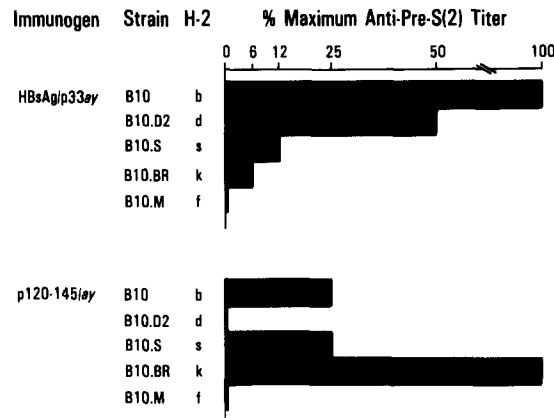


FIGURE 2. Distinct H-2 restriction of the immune responses to the native pre-S(2) region on HBsAg/p33 particles and the pre-S(2) region synthetic peptide p120-145. Groups of five mice from a series of H-2-congenic strains were immunized with either HBsAg/p33 *ay* (*top*), or p120-145/*ay* (*bottom*). Serum samples were pooled and pre-S(2)-specific antibody production, determined by RIA using p120-145/*ay* as the solid-phase ligand, was analyzed after primary immunization with HBsAg and secondary immunization with p120-145. The results are expressed as a percent of the highest responder strains response. (i.e., B10 immunized with HBsAg/p33, 1:4,096; B10.BR immunized with p120-145, 1:80,000). The H-2 haplotype of each strain is shown.

secondary immunization. As illustrated in Fig. 2, HBsAg/p33 immunization elicited pre-S(2)-specific antibody in all strains except B10.M, and the hierarchy of responsiveness was as follows B10 > B10.D2 > B10.S > B10.BR, indicating that the anti-pre-S(2) response is influenced by H-2-linked genes. The results of immunization with p120-145 also indicated the influence of H-2-linked genes on the antibody response, however, the order of responsiveness B10.BR > B10, B10.S > B10.D2, B10.M (nonresponders) was significantly different as compared with HBsAg/p33 immunization. For example, the B10.D2 strain produced anti-p120-145-specific antibody after HBsAg/p33 immunization, and yet is a total nonresponder to the p120-145 peptide upon immunization with p120-145. In contrast, the B10.BR strain is a low responder to p120-145 after HBsAg/p33 immunization, whereas this strain is the highest responder after p120-145 immunization (i.e., 1:80,000 titer).

The C₃H.Q (H-2^q) strain showed the highest response to p120-145 immunization, with a secondary titer of $1:1.2 \times 10^6$ (see Fig. 4). Therefore, the C₃H.Q strain was used in many of the experiments described below.

Since H-2 restriction is determined at the T cell level, these results argue very strongly that T cell recognition sites on the native pre-S(2) region (i.e., residues 120-174) need not be identical to those recognized when the synthetic peptide p120-145 is the immunogen. In the case of the B10.D2 strain, which does show a pre-S(2)-specific, T cell proliferative response after HBsAg/p33 immunization (5) and possesses p120-145-specific B cell clones, it is clear that the antibody response to the pre-S(2) region is not mediated by T cell recognition of the p120-145 sequence, since this strain is nonresponsive to immunization with p120-145. In other words, p120-145 does not contain a T cell determinant recognizable in the context of the H-2^d haplotype (BALB/c mice [H-2^d]) are also nonresponsive

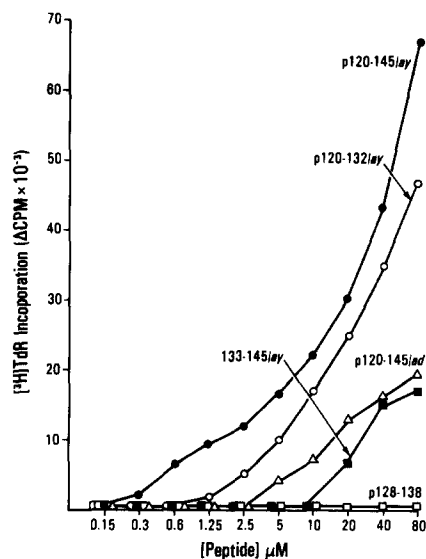


FIGURE 3. The C₃H.Q T cell proliferative response after p120-145/ay immunization. A group of C₃H.Q mice were immunized with 100 μg of p120-145/ayw, and T cell proliferation specific for the indicated peptides was determined. T cell proliferation was determined by incorporation of [³H]thymidine and expressed as cpm adjusted for background (Δ cpm). Background proliferation in the absence of antigen ranged from 1,000 to 4,000 cpm.

to this peptide). Consistent with this is the finding that the B10.D2 and BALB/c strains primed with p120-145 did not show a p120-145-specific T cell proliferative response (data not shown).

T Cell Recognition of p120-145 Focuses on the NH₂ Terminus, Whereas B Cell (antibody) Recognition Focuses on the COOH-terminal Sequence. To examine the fine specificity of T and B cell (antibody) recognition of the pre-S(2) region synthetic peptide p120-145, C₃H.Q mice were immunized with p120-145/ayw, and the specificity of the in vitro proliferative T cell response and the in vivo antibody response were determined. For this purpose an NH₂-terminal peptide (p120-132), a COOH-terminal peptide (p133-145), and an overlapping peptide (p128-138) were synthesized (Fig. 1). To determine possible subtype-specific influences, p120-145/adw₂ was also synthesized. Note that the adw₂ subtype differs from the ayw subtype at amino acid positions 126 and 141.

After p120-145/ay priming, p120-145/ay and p120-132/ay were capable of eliciting significant proliferative T cell responses, whereas p133-145/ay was minimally reactive, and p128-138 was totally nonstimulatory (Fig. 3). The fact that there was a less than twofold difference between the proliferative response of p120-145/ay-primed T cells induced by p120-145 and p120-132 through most of the dose-response curve pinpoints a predominant T cell recognition site to the NH₂-terminal 120-132 residues. Furthermore, the minimum degree of crossreactivity between p120-145 of the ayw vs. the adw₂ subtype indicated that residue 126 was critical to T cell activation. Peptide p120-145-primed T cells were also challenged in vitro with native HBsAg/p33 particles, which did not elicit a proliferative response. The reciprocal experiment, priming with HBsAg/p33 and challenging with p120-145, was also negative (data not shown).

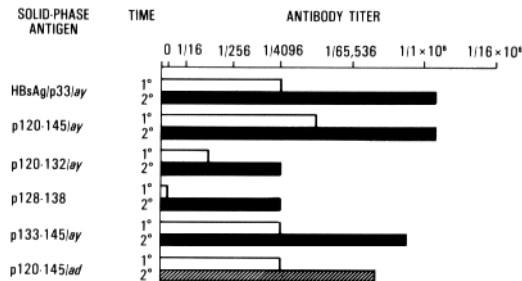


FIGURE 4. In vivo antibody production after p120-145/ay immunization in C₃H.Q mice. A group of 5 C₃H.Q mice were immunized with 100 μ g of p120-145/ayw, and sera were collected 24 d after primary immunization (1°) and 2 wk after secondary immunization (2°) with a half-dose of antigen. Sera were pooled and analyzed by RIA for binding to the indicated solid-phase ligands. The results are expressed as the maximum sera dilution to yield three times the counts of preimmunization sera (titer).

This observation confirms the notion that p120-145 need not represent the T cell recognition site on the native pre-S(2) region, as suggested by the H-2 restriction data.

The specificity of the antibody response after p120-145/ay immunization is presented in Fig. 4. Sera were tested 2 wk after primary and secondary immunizations. The antibody response to p120-145 differed from the T cell response in several significant ways. First, anti-p120-145 bound native HBsAg/p33 almost to the same extent as the homologous peptide (anti-native HBs/p33 also binds p120-145 very efficiently [4]). Second, the majority of antibody bound to the COOH-terminal p133-145 sequence (1:409,600), as opposed to p120-132 (1:4096), which elicited the greatest T cell proliferative response. Third, a small percentage of antibody bound p128-138, whereas this peptide did not activate p120-145-primed T cells. Finally, anti-p120-145/ayw antibody showed a high degree of crossreactivity for the p120-145/adw₂ subtype peptide, in contrast to the T cell response. These data indicate that p133-145 represents the dominant antibody binding site on p120-145 as well as a major binding site on native HBsAg/p33, and p120-132 represents the dominant T cell recognition site on the synthetic peptide p120-145, but not with respect to the native protein, at least in the C₃H.Q strain.

Since we had identified dominant, independent T and B cell epitopes on p120-145, we also wished to compare the relative immunogenicities of the T cell epitope (p120-132) and the B cell epitope (p133-145) with the intact immunogen (p120-145). Although, p120-132 induced a primary (4-wk) antibody response reactive with its own sequence, it did not induce a response crossreactive on native HBsAg/p33 (Table I). Peptide 133-151 was used to represent the B cell epitope in this experiment, and elicited a primary antibody response that reacted equivalently with native HBsAg/p33 and the p133-145 sequence (i.e., 1:10,240). However, these responses were quite low as compared with the response elicited by p120-145, which possesses both T and B cell determinants (Table I).

A Single Amino Acid Substitution Defines T Cell Recognition of Synthetic Peptide 120-132. Because p120-132/ay efficiently induced proliferation of p120-145/ay-primed T cells, and because subtype appeared important, C₃H.Q mice

TABLE I
Comparative Immunogenicity of Uncoupled Pre-S(2) Region Synthetic Peptides in C₃H.Q Mice

Immunogen	Antibody titer (reciprocal) and specificity			
	p120-145	p120-132	p133-145	HBsAg/p33
p120-145	512,000	5,120	256,000	160,000
p120-132	160	640	0	0
p133-151	10,240	0	10,240	10,240

Groups of five mice were immunized with 100 μ g i.p. of each peptide of the *ayw* subtype in CFA, and sera were collected and pooled 4 wk after primary immunization.

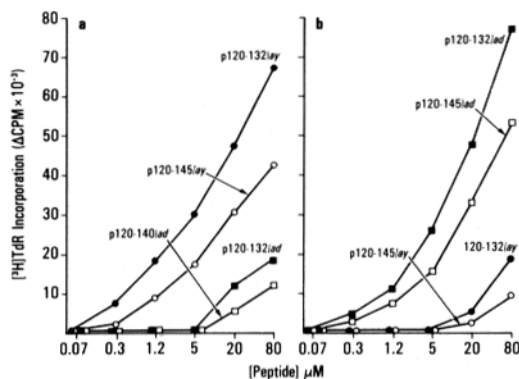


FIGURE 5. The C₃H.Q T cell proliferative response after p120-132 immunization. Groups of 3 C₃H.Q mice were immunized with 100 μ g of either p120-132/*ayw* (a) or p120-132/*adw*₂ (b), and T cell proliferation specific for the indicated peptides determined as described in Fig. 3.

were primed with either the *ayw* or the *adw*₂ subtype sequence of p120-132, and T cells were challenged in vitro with 120-132/*ay*, 120-145/*ay*, 120-132/*ad*, or 120-145/*ad*. The *ayw* and *adw*₂ sequences of 120-132 differ only at residue 126 (threonine, alanine, respectively). T cells primed with the *ayw* sequence of 120-132 proliferated in response to p120-132/*ay* and p120-145/*ay*, but only marginally to the *adw*₂ sequences (66.6-fold difference) (Fig. 5a). In the reciprocal experiment, T cells primed with the *adw*₂ sequence of 120-132 proliferated when challenged with p120-132/*ad* and p120-145/*ad*, and were only minimally reactive to the *ayw* sequences (32-fold difference) (Fig. 5b). These data indicate the importance of residue 126 in the T cell recognition of 120-132, and show that a single amino acid can dramatically affect the fine specificity of T cell recognition, since the *ayw* and *adw*₂ sequences are relatively noncrossreactive. To further define the T cell recognition site, C₃H.Q mice were primed with p120-131 of the *adw* sequence, which differs from both the *ayw* and *adw*₂ sequences at residues 127 and 130, and lacks residue 132 (Fig. 1). As illustrated in Fig. 6, the *adw* and *adw*₂ sequences were quite crossreactive, and the *ayw* sequence was nonstimulatory for p120-131/*adw*-primed T cells. This finding emphasizes the importance of residue 126, and suggests that residues 127, 130, and 132 are not critical to T cell recognition of the p120-132 sequence.

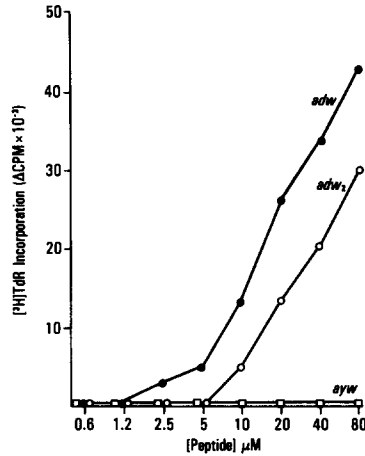


FIGURE 6. The influence of subtype on the $C_3H.Q$ T cell proliferative response after immunization with p120-131/*adw*. A group of three $C_3H.Q$ mice was immunized with $100 \mu\text{g}$ of p120-131/*adw*, and T cell proliferation induced by the homologous peptide (p120-131/*adw*), p120-132/*adw*₂, and p120-132/*ayw* was determined as described in Fig. 3.

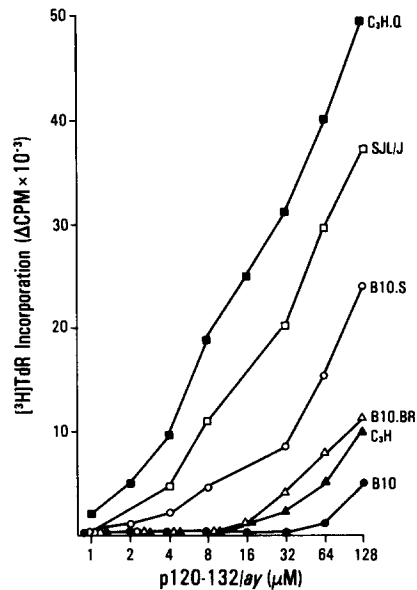


FIGURE 7. Strain variability of the p120-132-specific T cell response after p120-145 immunization. Groups of three mice each of the indicated strains were immunized with $100 \mu\text{g}$ of p120-145/*ayw*, and T cell proliferation induced by p120-132/*ayw* was determined as described in Fig. 3.

To examine whether T cell recognition of p120-132 was unique to the $C_3H.Q$ strain, several other murine strains were immunized with p120-145/*ayw*, and T cell proliferative responses specific for p120-132/*ayw* were determined (Fig. 7). All strains except B10 showed a subtype-dependent T cell proliferative response to the immunogen, p120-145/*ayw* (data not shown). Although the $C_3H.Q$ strain responded best to p120-132, all strains tested except the B10 strain (SJL/J,

TABLE II
Reactivity of Anti-p120-132/ay with Subtype Sequences

Immunogen	Immunization	Antibody titer (reciprocal) and specificity		
		p120-132/ayw	p120-132/adw ₂	p120-131/adw
p120-132/ay	Primary	1,280	640	80
	Secondary	5,120	2,560	40
p120-145/ay	Primary	10,240	5,120	640

Sera were collected 4 wk after primary immunization and 2 wk after secondary immunization.

B10.S, B10.BR, and C₃H) showed a significant response to p120-132 after p120-145 priming. Therefore, it appears that p120-132 can be recognized in the context of the H-2^a, H-2^s, and H-2^k haplotypes, but not the H-2^b haplotype. Interestingly, B10 strain T cells immunized with p120-145/ayw proliferated equivalently to the *adw*₂ and *ayw* subtype sequences of p120-145, and B10 was the only strain that showed at least a marginal response to challenge with native HBsAg/p33 (data not shown). The absence of a subtype-specific response indicates that residue 126 is not critical to T cell recognition of p120-145 in the B10 strain.

We also wished to examine the fine specificity of *in vivo* antibody production to p120-132. Therefore, C₃H.Q mice were immunized with p120-132/ayw and primary and secondary sera were analyzed for reactivity on solid-phase p120-132/ayw, 120-132/adw₂, and 120-131/adw (Table II). In contrast to T cell recognition, antibody to p120-132/ayw crossreacted with the *adw*₂ sequence quite well (twofold difference), indicating that the alanine-for-threonine substitution at residue 126 did not severely affect the antibody binding site on p120-132. Conversely, anti-p120-132/ay did not bind well to p120-131/adw, indicating that the COOH-terminus of p120-132 is involved in antibody recognition. For comparison, primary anti-p120-145/ayw serum was also examined for fine specificity, and gave similar results, except that the more immunogenic p120-145 elicited a greater response to all antigens (Table II). Note that, at the T cell level, the *adw*₂ and *adw* sequences were very crossreactive. These data suggests that, even with respect to a synthetic peptide as small as 13 amino acids, T and B cell recognition sites can be distinct.

NH₂-terminal Peptide 120-132 Can Prime T Cell Help for COOH-terminal Peptide 133-145. Because it appeared that distinct T cell and B cell epitopes existed on p120-145, we examined the ability of the isolated T cell site (p120-132) to prime T helper cell function for the dominant antibody binding site on p120-145 (p133-145). C₃H.Q mice were either primed with 100 µg of p120-132/ay in CFA or injected with CFA alone, and 4 wk later were boosted with either 100 µg of p120-145/ay or 1.0 µg of HBsAg/p33/ay in incomplete adjuvant. Sera were analyzed for an IgG response specific for various determinants 1 wk after the boost. As illustrated in Table III, p120-132 was capable of priming the anti-p133-145 response after the boost with p120-145. Unprimed mice did not produce anti-pre-S(2) of any specificity, however, p120-132-primed mice produced an IgG titer of 1:640 specific for the p133-145 peptide. The other solid-phase antigens all possess the 120-132 sequence, so it is difficult to discriminate

TABLE III
Ability of p120-132 to Prime In Vivo Antibody Production

Priming immunization	Boost	Antibody titer (reciprocal) and specificity			
		p120-145	p120-132	p133-145	HBsAg/p33
p120-132	—	160	640	0	0
—	p120-145	0	0	0	0
p120-132	p120-145	2,560	5,120	640	1,280
—	HBsAg/p33	320	40	160	160
p120-132	HBsAg/p33	640	640	40	320

Groups of four C₃H.Q mice each were primed with 100 μ g of p120-132 in CFA administered i.p., or with CFA alone (—), and 4 wk later, they were boosted with either 100 μ g of p120-145 or 1 μ g of HBsAg/p33 in incomplete adjuvant. Mice were bled 1 wk after the boost, and sera were assayed for IgG specific for the indicated antigens. All antigens were of the *ayw* subtype.

anti-p133-145 from anti-p120-132 on these ligands. Although p120-132 primed the anti-p133-145 response after a p120-145 boost, p120-132 did not prime any pre-S(2)-specific response after the HBsAg/p33 boost (Table III). This finding confirms that p120-132-primed C₃H.Q T cells do not recognize this sequence on the native protein, which is consistent with the lack of a HBsAg/p33-induced proliferative response, and the H-2 restriction data.

Discussion

This study was undertaken to examine T cell recognition of an HBsAg, pre-S(2) region synthetic peptide (p120-145) previously shown (3) to be highly immunogenic and to represent a dominant antibody binding site on the native pre-S(2) region (3, 4). The results of this study indicate the following: (a) the anti-p120-145 response is regulated by H-2-linked genes, but differs from the pattern of H-2 restriction observed for the native anti-pre-S(2) response; (b) nonoverlapping, dominant T and B cell recognition sites were identified on the synthetic peptide 120-145; (c) the fine specificity of T cell recognition of p120-145 is defined by a single amino acid substitution; (d) the synthetic peptide containing both T and B cell determinants is highly immunogenic in responder strains, whereas separate T cell or B cell peptide determinants are minimally immunogenic; and (e) the synthetic T cell recognition site can prime T cell help for antibody production to the synthetic B cell site, which is crossreactive with the native pre-S(2) region.

Previous work (4, 5) showed the influence of H-2-linked genes on antibody production and the T cell proliferative response to the native pre-S(2) region of HBsAg. The order of responsiveness at the antibody level correlated with the T cell proliferative responses and was as follows: B10 > B10.D2 > B10.S > B10.BR > B10.M (nonresponder). Since antibody specific for the native pre-S(2) region and the synthetic peptide are highly crossreactive (4), it was possible to compare the influence of H-2-linked genes on in vitro antibody production to the native pre-S(2) region vs. the synthetic peptide 120-145. Although the pre-S(2) region exists on a larger polypeptide (p33), which includes S region antigenic determinants, the pre-S(2)-specific antibody and T cell responses have been shown (4,

5) to be of greater magnitude, precede in time, occur at a lower HBsAg dose and be independently regulated as opposed to the S region-specific responses. Therefore, genetic and other influences of the immune response to the S region on the pre-S(2) region response can be discriminated and/or negated. The order of responsiveness to p120-145 after immunization with the unconjugated homologous synthetic peptide p120-145 is B10.BR > B10, B10.S > B10.D2, B10.M (nonresponders) was clearly different from that observed after immunization with pre-S(2) region-containing particles (HBsAg/p33). For example, the B10.D2 strain produced anti-p120-145 after immunization with HBsAg/p33, but was totally nonresponsive when immunized with p120-145. Since the B10.D2 and BALB/c (both H-2^d) strains are very responsive at the T cell level to the native pre-S(2) region (5) but not to p120-145, it is obvious that p120-145 does not represent the T cell determinant on the pre-S(2) region recognized by H-2^d bearing strains. Conversely, the B10.BR strain is a low responder when immunized with the intact pre-S(2) region, and a high responder after p120-145 immunization. This finding suggests that suppressor-activating determinants may exist elsewhere on the native protein, which downregulate the p120-145-specific response. Suppressor mechanisms have been implicated (14, 15) in the low-responder status of the B10.BR strain after HBsAg/p25 immunization. These results indicate that the immunogenicity of a constituent peptide, even though that peptide represents a dominant antibody binding site on the native protein, is not necessarily predictive of the immunogenicity of the intact molecule, and vice versa. Furthermore, immunogenicity of a carrier-free peptide is critically dependent on T cell helper function, which is regulated by H-2-linked genes. Since the pattern of H-2 restriction was different after peptide or HBsAg/p33 immunization, it is clear that T cell recognition sites on p120-145 need not be identical to those recognized when the native pre-S(2) region is the immunogen. This was more directly shown by the observation that p120-145-primed T cells from most strains did not recognize the native pre-S(2) region upon in vitro challenge, and did not prime an in vivo anti-pre-S(2) antibody response after a boost with HBsAg/p33. This was true even in the C₃H.Q strain, which is a high responder in terms of anti-p120-145 antibody production whether immunized with p120-145 or HBsAg/p33. Therefore, a constituent peptide may represent an antibody binding site on the native protein, but lack a T cell recognition site relevant to the native protein. We have analyzed numerous nonoverlapping pre-S(2) region synthetic peptides encompassing the entire 55 amino acid region without successfully identifying a synthetic T cell site that activates HBsAg/p33-primed T cells. We are convinced that this is not due to antigen processing characteristics or other unknown influences unique to this particulate antigen, since we have been successful in delineating a native T cell recognition site in the pre-S(1) region of the p39 polypeptide of HBsAg using similar techniques (16). Likewise, numerous investigators (17-19) have localized T cell recognition sites on other native protein antigens using synthetic peptides.

Although the 120-145 sequence of the pre-S(2) region does not appear to have a T cell recognition site relevant to the native protein in most strains, a dominant T cell determinant, relevant to p120-145 immunization, was identified on the NH₂-terminus of p120-145 (p120-132). This T cell determinant does not overlap

with the dominant antibody (B cell)-binding site on the COOH-terminus (p133-145) of p120-145. In the C₃H.Q strain, the p120-132 sequence represents the dominant T cell recognition site on p120-145, as shown by its ability to activate p120-145-primed T cells approximately as well as the immunogen, and by the ability of p120-132 to prime T cell help for antibody production to the p133-145 sequence. The fine specificity of T cell recognition of p120-132 was further refined by examining the influence of subtype. The *ayw* and *adw*₂ subtypes differ by a single amino acid substitution at residue 126 in the 120-132 sequence; nevertheless, p120-132/*ayw*-specific T cells were virtually noncrossreactive on p120-132/*adw*₂, and reciprocally, p120-132/*adw*₂-specific T cells did not cross-react with p120-132/*ayw*. The trimolecular complex model of T cell recognition proposed by Heber-Katz et al. (20) suggests that class II-encoded Ia molecules and antigen physically interact at one subsite on the antigen (agretope), and another subsite on the antigen contacts the T cell receptor (epitope). With respect to the peptide antigen p120-132, a single amino acid substitution at residue 126 drastically reduced T cell activation, and furthermore, immunization with the substituted analog elicited a comparable T cell response specific for the substitution. In the context of the trimolecular model, these results are consistent with residue 126 representing a contact site with the T cell receptor (epitope) (18). The fact that both peptides were equivalently immunogenic in the C₃H.Q (H-2^q) strain, regardless of the threonine to alanine substitution, suggests that the agretope would reside in a shared sequence outside residue 126. If this were true, the two peptides should compete for the same Ia-binding site (21, 22), and p120-132/*adw*₂ would inhibit the activation of p120-132/*ayw*-primed T cells elicited by p120-132/*ayw*. However, no such inhibition occurred (our unpublished observation), suggesting that residue 126 may be involved in the interaction with Ia (i.e., agretope). In this case, both substitutions at 126 must be permissible in the context of Ia^q (i.e., immunogenic), and the threonine vs. alanine Ia^q interactions variably influence the trimolecular complex to the extent that at least two noncrossreactive T cell clones would be generated to a single primary sequence (epitope). Since agretope inhibition experiments have not been universally successful (23), we favor the interpretation that residue 126 represents a contact site with the T cell receptor, however, further experiments with truncated and substituted synthetic peptides in a series of B10 H-2-congenic strains will be required to resolve this question. Examination of the T cell proliferative response to a third subtype sequence, p120-131*adw*, which differs from the *ayw* sequence at residues 126, 127, 130, and 132, and from the *adw*₂ sequence at residues 127, 130, and 132, emphasized the importance of residue 126, and revealed that residues 127, 130, and 132 were not critical to T cell recognition. Therefore, we can tentatively localize a dominant T cell determinant on p120-145 to residues 120-126, although the possible influence of residues 128 and 129 cannot be excluded.

Having identified a dominant site on p120-145 recognized by T cells of the C₃H.Q and other strains, we wished to examine the specificity of antibody produced after p120-145/*ayw* immunization in C₃H.Q mice. In contrast to T cell recognition, the majority of antibody specific for p120-145 bound to the COOH-terminal sequence p133-145, and showed a high degree of crossreactivity

for the *adw*₂ sequence. Furthermore, a large proportion of anti-p120-145 antibody crossreacted with native HBsAg/p33, unlike p120-145-primed T cells. Additionally, antibody raised against the p120-132 sequence appeared to recognize distinct regions of the peptide as compared with T cell recognition. These results indicate that T and B cell recognition sites can be distinct even on a synthetic peptide as small as 13 residues. Physical-chemical characterization of these and other synthetic T and B cell sites within the pre-S region of HBsAg will be the subject of future studies to determine whether a structure-function relationship can be identified. In this regard, it has recently been suggested (24, 25) that T cell recognition sites on a protein may be represented by amphipathic structures displaying periodicity in hydrophobic residues. Note that the sequence comprising residues 121-135 in the pre-S(2) region fits this amphipathicity hypothesis (J. A. Berzofsky, personal communication). The pre-S region of HBsAg may serve as a useful model for comparing T cell and B cell recognition sites because of the largely sequential (as opposed to conformational) requirements for antibody binding to this region (3, 4). This characteristic made it feasible to define two distinct but overlapping antibody binding sites within the p133-143 sequence of the pre-S(2) region of HBsAg using synthetic peptides and mAbs as described elsewhere.²

The results of this study have implications with respect to the development of completely synthetic vaccines. In view of preliminary experiments suggesting that antisera to the pre-S(2) region of HBsAg may be protective against HBV infection, a vaccine exclusively composed of pre-S(2) region determinants could be contemplated. More likely, pre-S(2) and S region determinants may be combined in future HBV vaccines. Since the pre-S(2) and S regions exist on the same polypeptide, this strategy has the additional advantage that a T cell response to the pre-S(2) region circumvents nonresponsiveness to the S region in S region nonresponder mice (5). Since the synthetic T cell determinant p120-132 was capable of priming T cell help for antibody production to the synthetic B cell determinant p133-145, and since this antibody reacted with the native pre-S(2) region, this system provides evidence that synthetic T and B cell recognition sites can be combined to yield a functional immunogen (vaccine). Due to the MHC-dependent restrictions imposed on T cell recognition of antigen, several T cell recognition sites or a combination of free and conjugated peptide may be required to insure responsiveness in a high percentage of vaccine recipients. In this regard, the p120-132 sequence served as a T cell recognition site in mice bearing the H-2^{q.s.k} haplotypes but not the H-2^b haplotype. An ideal synthetic vaccine will be composed of T and B cell recognition sites identical to those recognized on the native molecule. Such a vaccine would induce neutralizing antibody and elicit T and B cell memory relevant to the pathogen in case of a subsequent infection after neutralizing antibody wanes. A synthetic T cell determinant would also obviate the requirement to conjugate synthetic B cell sites to a heterologous protein carrier. Although p120-145 may not represent the ideal synthetic pre-S(2) region immunogen (in mice), it does represent a completely synthetic and functional immunogen, similar to a peptide-carrier conjugate, although not requiring the heterologous protein carrier to elicit T cell helper function. These results, and the identification of T cell recognition sites within

the S (26) and pre-S(1) regions of native HBsAg (16) increase, at least conceptually, the feasibility of a completely synthetic HBV vaccine.

Summary

We have examined T cell recognition of a hepatitis B surface antigen (HBsAg), pre-S(2)-region synthetic peptide, p120-145, in terms of fine specificity, H-2-linked genetic influences, comparison to antibody binding, and relevance to T cell recognition of the native protein. We showed that the immune response to the synthetic peptide is regulated by H-2-linked genes, but that the pattern of H-2 restriction differed from that observed for the native anti-pre-S(2) response. Dominant and nonoverlapping T cell and B cell recognition sites were identified on the synthetic peptide p120-145. T cell recognition is focussed on the NH₂-terminal sequence, and antibody (B cell) recognition is focussed on the COOH-terminal sequence. The fine specificity of T cell recognition of p120-145 was defined by a single, subtype-dependent amino acid substitution. With respect to the immunogenicity of p120-145, the synthetic peptide containing both T and B cell determinants is highly immunogenic in responder strains, whereas separate T or B cell peptide determinants are minimally immunogenic. Furthermore, the synthetic T cell recognition site can prime T cell help for antibody production to the synthetic B cell site, which is crossreactive with the native pre-S(2) region of HBsAg/p33 particles. This system provides evidence that totally synthetic T cell and B cell recognition sites can be combined to yield a functional immunogen.

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