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Immunology

Distinct *H-2*-linked regulation of T-cell responses to the pre-S and S regions of the same hepatitis B surface antigen polypeptide allows circumvention of nonresponsiveness to the S region

(hepatitis B virus/vaccine/cellular immunity)

DAVID R. MILICH*, MARY K. MCNAMARA*, ALAN MCLACHLAN*, GEORGE B. THORNTON†, AND FRANCIS V. CHISARI*

*Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, CA 92037; and †Johnson & Johnson Biotechnology Center, Inc., San Diego, CA 92121

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ABSTRACT **Recently, additional polypeptide components of the surface envelope of hepatitis B virus (HBV) have been identified. The *pre-S(1)* and *pre-S(2)* regions of the HBV genome encode NH_2-terminal amino acid residues that together with the *S*-gene product (25 kDa) comprise polypeptides of 33 kDa and 39 kDa. The possible immunopathologic significance of these larger polypeptides and their relevance to vaccine development prompted us to examine the murine immune response to *pre-S(2)*-encoded determinants as compared to *S*-encoded determinants on the same polypeptide. Previous work showed that the pre-S(2) region elicits greater antibody production *in vivo* than does the S region of hepatitis B surface antigen. In this study, we examined immunogenicity of the pre-S(2) region at the T-cell level, *H-2*- and non-*H-2*-linked genetic influences on the pre-S(2) response, and the effect of the immune response to one region on the immune response to the other region. The results indicate that (*i*) the pre-S(2) region is significantly more immunogenic than the S region at the T-cell level; (*ii*) pre-S(2)-region-specific T-cell activation is regulated by *H-2*-linked genes and correlates with the *H-2* restriction of *in vivo* antibody production to the pre-S(2) region; (*iii*) the *H-2* restriction of the T-cell response to the pre-S(2) region is distinct from the *H-2* restriction of the T-cell response to S-region determinants; (*iv*) non-*H-2*-linked and non-*Igh*-linked genes also influence the humoral immune response to the pre-S(2) region; and (*v*) immunization of an S-region-nonresponder, pre-S(2)-region T-cell-responder strain with HBV envelope particles containing both the pre-S(2) and S regions can circumvent nonresponsiveness to the S region through pre-S(2)-specific T-cell helper function.**

The hepatitis B surface antigen (HBsAg) is composed of a major polypeptide, p25, and its glycosylated form, gp28 (1). However, p25 begins at the third possible translational initiation site of a larger open reading frame (ORF) and is preceded in-phase by either 163 or 174 codons (subtype-dependent) designated as the pre-*S* region (5). A hepatitis B virus (HBV)-associated 33/36-kDa glycoprotein has been identified (6), and it was suggested that the sequence of gp33 starts at the second translational initiation signal of the ORF, which is 55 codons upstream of the third signal (7). It has been shown that gp33 consists of the p25 sequence and an NH_2-terminal 55 amino acids encoded in this *pre-S(2)* region (8, 9). In support of this, Neurath *et al.* (10) synthesized a peptide encompassing the 26 NH_2-terminal amino acids of the pre-S(2) region and found that anti-peptide antibodies reacted with gp33. Recently, the products encoded from the first translational initiation site have been identified as p39/gp42, consisting of the gp33 sequence and an NH_2-terminal 108–119 amino acids encoded in the *pre-S(1)* region (11).

That the *pre-S* region is conserved in all the described HBV DNA sequences and throughout evolution (12) suggests a functional role for this region. This interpretation is enhanced by the observation that both the gp42 and gp33 polypeptides appear to be preferentially expressed in viremic carriers as opposed to carriers with minimal or no infectious virions in the blood (8, 11). This suggests a correlation between viral replication and synthesis of the higher molecular weight polypeptides of HBsAg. For the above reasons, it has been suggested that higher molecular weight polypeptides containing *pre-S*-encoded amino acids be included in future second-generation HBsAg vaccines (10, 14).

Previous examination of quantitative and qualitative aspects of the murine humoral and cellular immune response to HBsAg demonstrated the influence of at least two *H-2*-linked immune-response (*Ir*) genes (15–17). HBsAg high-responder ($H\text{-}2^{d,q}$), and nonresponder ($H\text{-}2^{f,s}$) haplotypes have been identified (15). The HBsAg used in those studies contained the p25/gp28 polypeptides but lacked the *pre-S*- and *S*-encoded higher molecular weight polypeptides and is designated here HBsAg/p25. The pre-S(2) region of HBsAg is more immunogenic, in terms of antibody production *in vivo*, than is the S region (2). Based on the quantity and kinetics of *in vivo* anti-pre-S(2) antibody production in a series of *H-2*-congeneic strains, responsiveness of the *H-2* haplotypes has been ranked in the following order: $H\text{-}2^b$, $H\text{-}2^q$, $H\text{-}2^d$ (high responders); $H\text{-}2^s$ (intermediate); $H\text{-}2^k$ (low); $H\text{-}2^f$ (nonresponder) (2).

In this study we examined immunogenicity at the T-cell level, *H-2*- and non-*H-2*-linked genetic influences on the pre-S(2)-specific immune response, and possible overlapping regulatory mechanisms [i.e., would T-cell helper function generated against the pre-S(2) region influence the anti-S response]. For this purpose we utilized recombinant HBsAg particles designated HBsAg/p34 (14). HBsAg/p34 particles are composed of the *S*-encoded p25/gp28 polypeptides plus the *pre-S(2)*- and *S*-encoded gp34 polypeptide. Groups of *Igh*- and *H-2*-congeneic murine strains were immunized with HBsAg/p34 and *in vivo* antibody production and *in vitro* T-cell interleukin 2 (IL-2) production induced by pre-S(2)- and S-region determinants were examined.

MATERIALS AND METHODS

Mice. C57BL/10 (B10), B10.D2, B10.S, B10.BR, B10.M, C_3H, and $C_3H.Q$ strains were obtained from the breeding colony at the Research Institute of Scripps Clinic. Female

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Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IL-2, interleukin 2; PLN, popliteal lymph node.

mice 6–8 weeks old at the initiation of the experiments were used in cell studies.

Antigen Preparations. A purified preparation of HBsAg/*ay* was provided by R. Louie (Cutter Laboratories, Berkeley, CA). This preparation was treated with pepsin during purification and is composed exclusively of the 25-kDa polypeptide and its glycosylated form and is designated herein as HBsAg/p25. 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 (14) were provided by P. Tiollais (Pasteur Institute, Paris). The CHO-derived particles are composed of the *S*-encoded p25 (65%) plus the *pre-S(2)*- and *S*-encoded p34 (35%) (14) and are designated herein as HBsAg/p34. A synthetic pre-S peptide encompassing the NH_2-terminal 26 residues of the pre-S(2) region (10) was provided by A. R. Neurath (New York Blood Center, New York).

Immunizations. For study of *in vivo* antibody production, groups of five or six mice were immunized by i.p. injection of 1.0 μg of HBsAg/p34 in 0.2 ml of complete Freund's adjuvant (CFA). Since the relative amount of p34 in the HBsAg/p34 was 35% and the pre-S(2) region accounts for ≈25% (by weight) of p34, a 1-μg dose of HBsAg/p34 is equivalent to 913 ng of S-region protein and 87 ng of pre-S(2)-region protein. Mice were given a booster injection of 0.5 μg of HBsAg/p34 in incomplete adjuvant. Mice were bled from the retroorbitral plexus on days 10 and 24 after primary immunization and 2 weeks following secondary immunization. *In vivo* priming for the T-cell activation assay was accomplished by injection of a total of either 16 μg of HBsAg/p25 or 4 μg of HBsAg/p34 in CFA (80 μl) into both hind footpads of recipient mice.

Measurement of *in Vivo* Anti-HBs Production. Pooled murine sera were evaluated for anti-S-region antibody in an indirect, HBsAg subtype-specific, immunoglobulin class-specific RIA using solid-phase HBsAg/p25 (0.1 μg per well) and goat anti-mouse IgG and developed with an ^{125}I-labeled, affinity-isolated swine anti-goat Ig as described (15). Anti-pre-S(2)-region antibody was detected by an indirect RIA using solid-phase, reduced and denatured HBsAg/p34 particles (0.1 μg per well) as described (2).

HBsAg-Specific T-Cell Activation. T-cell activation was measured by antigen-induced IL-2 production. Groups of five mice were primed with HBsAg/p25 (16 μg) or HBsAg/p34 (4 μg) in the hind footpads, and draining popliteal lymph node (PLN) cells were harvested and pooled 8 days after immunization. Next, 5×10^5 viable PLN cells in 0.1 ml of Click's medium (3) were placed in flat-bottom microtiter wells with 0.1 ml of medium containing HBsAg/p25 or HBsAg/p34 or of medium alone. Cultures were incubated for 24 hr at 37°C in a humidified 5% CO_2 atmosphere, and supernatants were collected. Cells of an IL-2-dependent cytotoxic T-lymphocyte line (CTL-L2) (4) were cultured in the supernatant to be tested in flat-bottom microwells (10^4 cells per well) for 24 hr. During the final 4 hr, the cultures were incubated with 1 μCi (1 Ci = 37 GBq) of [*methyl*-^{3}H]thymidine; the cells then were harvested onto filter strips for determination of incorporated ^3H. IL-2 production was expressed as IL-2-dependent [^{3}H]thymidine incorporation by CTL-L2 cells in supernatant from HBsAg-treated cultures minus incorporation that occurred in supernatant from control cultures not treated with HBsAg. This assay is HBsAg-specific and correlates with HBsAg-induced T-cell proliferation (17).

RESULTS

Immunization of an S-Region Nonresponder, Pre-S(2)-Region Responder Strain with HBsAg/p34 Can Circumvent Nonresponsiveness to the S Region. The HBsAg preparations used in these studies were assayed for pre-S(2) antigen by quantitative inhibition of an anti-pre-S(2)-specific antiserum produced in mice as described (2). Based on concentrations necessary to yield 50% inhibition, HBsAg/p34 inhibited ≈28-fold more efficiently than an HBsAg preparation composed primarily of HBsAg/p25 but containing a trace amount (PAGE analysis) of p34 (HBsAg/p25/p34). The HBsAg/p25 preparation was totally nonreactive. Inhibition by a synthetic pre-S peptide (10) is included for comparison (Fig. 1).

The B10.S strain is a total nonresponder to S-region determinants even after secondary immunization with 4–20 μg of HBsAg/p25 (16). However, the B10.S strain produced a primary and secondary IgG response to the pre-S(2) region upon immunization with 1 μg of HBsAg/p34 (87 ng of pre-S(2)-region protein) (2). Upon secondary immunization with HBsAg/p34, this "S-region-nonresponder" strain produced an anti-S specific response in addition to the anti-pre-S(2) response (Table 1). The anti-S antibody produced by the B10.S strain following HBsAg/p34 immunization is subtype-specific (anti-HBs/*y*), whereas that produced by the B10, B10.D2, and B10.BR strains contain both group- and subtype-specific anti-S (Table 1). Therefore, the B10.S strain is a pre-S(2)-region responder and an S-region nonresponder, yet secondary immunization with HBsAg/p34 induces both anti-S and anti-pre-S(2) antibody. This indicates that the anti-pre-S(2) response can positively influence the anti-S response in this strain.

Influence of Non-*H-2*-Linked Genes on the Humoral Anti-pre-S(2) Response. To examine the influence of *Igh*-linked genes on the humoral response to the pre-S(2) region, BALB/c and *Igh*-congeneic CB.20 mice were compared. The BALB/c strain demonstrated 4-fold greater anti-pre-S(2) region responses at days 10 and 24 and a 2-fold greater anti-S region response at day 24 following primary immunization (Table 2). This illustrates the influence of *Igh*-linked genes on at least the primary pre-S(2)-specific response. Additionally, when the B10.S and SJL/J strains (both $H\text{-}2^s$, Igh^b) were compared, the SJL/J strain demonstrated significantly greater responses specific for the pre-S(2) and S regions, indicating the influence of non-*H-2*-, non-*Igh*-linked genes (Table 2). Since both these strains are S-region nonresponders when immunized with HBsAg/p25, the non-*H-2*- and non-*Igh*-linked genetic influence most likely operates at the level of the pre-S(2)-region response.

The T-Cell Response Elicited by the Pre-S(2) Region of HBsAg Is Significantly Greater Than the T-Cell Response Elicited by the S Region. Several factors relative to *in vivo* antibody production to the pre-S(2) region of HBsAg sug-

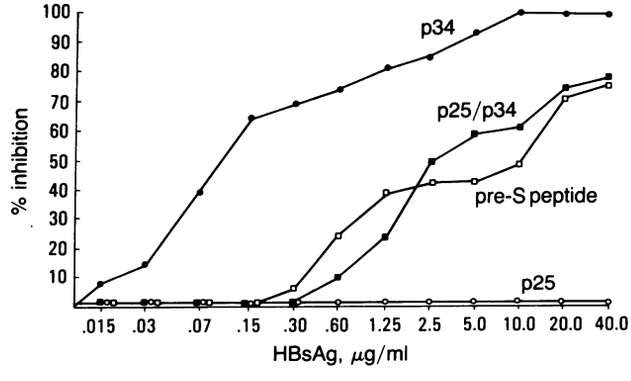

FIG. 1. Detection of pre-S(2) region determinants on HBsAg particles. Various concentrations of HBsAg/p34, HBsAg/p25, HBsAg/p25/p34 (a serum-derived HBsAg with a trace amount of gp34) were preincubated overnight with a 1:500 dilution of mouse (B10) pre-S(2)-specific antiserum. The antibody–antigen mixtures were then added to solid-phase HBsAg/p34 (0.1 μg per well), and the inhibition was determined. Inhibition by a synthetic pre-S(2) peptide (26 NH_2-terminal residues) (10) is included for comparison.

Table 1. Specificity of anti-S-region antibody obtained after immunization of S-region-responder and -"nonresponder" strains with HBsAg/p34

	Antibody titer			
	Anti-*a* (group)		Anti-*y* (subtype)	
Strain	Primary	Secondary	Primary	Secondary
B10	1:40	1:1280	1:160	1:5120
B10.D2	1:20	1:1280	1:160	1:10,240
B10.S	0	0	0	1:640
B10.BR	0	1:20	0	1:640

Groups of five mice of the indicated strains were immunized with 1 μg of HBsAg/p34 and sera were analyzed for group-specific (anti-*a*) and subtype-specific (anti-*y*) anti-S-region antibody (15) 24 days after primary immunization and 2 weeks after secondary immunization.

gested the influence of pre-S(2)-specific T-cell help as opposed to T-cell help derived through recognition of S-region determinants (i.e., pre-S(2) as hapten). First, *in vivo* anti-pre-S(2) antibody production is *H-2*-linked in a manner distinct from anti-S antibody production (2). Second, immunization with HBsAg/p34 induces significant amounts of anti-pre-S(2) antibody of the IgG class as early as 10 days following i.p. immunization with 87 ng of pre-S(2) protein (2). Finally, HBsAg/p25-nonresponder status could be circumvented by immunization with HBsAg/p34 (Tables 1 and 2).

To directly examine T-cell recognition of the pre-S(2) region of HBsAg, antigen-dependent IL-2 production by T cells in HBsAg/p34- and HBsAg/p25-primed mice was determined. $C_3H.Q$ mice were immunized with 16 μg of HBsAg/p25 [amount required to elicit an optimal 8-day, S-region-specific, T-cell proliferative response (13)], and 8-day PLN T cells were challenged with various concentrations of HBsAg/p34 or HBsAg/p25 preparations. Immunization with HBsAg/p25 primed S-region-specific T cells, which produced equivalent levels of IL-2 upon challenge *in vitro* with the two HBsAg preparations (Fig. 2*a*). Since HBsAg/p34 and HBsAg/p25 contained equivalent amounts of S-region protein, it was expected that they would elicit comparable T-cell responses in PLN T cells primed with HBsAg/p25. In contrast, immunization of $C_3H.Q$ mice with 4 μg of HBsAg/p34 (3.65 μg S; 0.35 μg pre-S) induced significantly greater pre-S(2)-specific IL-2 production, with a left-shifted dose–response curve as compared to the S-region-specific T-cell response (Fig. 2*b*). The minimal S-region-specific T-cell response observed after HBsAg/p34 immunization can be attributed to a suboptimal S-region priming dose (3.65 μg). Note that the pre-S(2)-region priming dose (0.35 μg) is 1/10th the S-region dose and 1/45th the optimal S-region (HBsAg/p25) priming dose (16 μg), yet induced a significantly greater T-cell response than either dose of the S-region antigen (Fig. 2 *a* and *b*). These data indicate that the pre-S(2) region is more immunogenic than the S region of HBsAg at the T-cell level in terms of the effective priming dose, the magnitude of the T-cell response, and the quantitative *in vitro* dose–response curve.

The Pre-S(2)-Specific T-Cell Response Is Regulated by *H-2*-Linked Genes and Correlates with *in Vivo* Anti-pre-S(2) Antibody Production. To assess both the influence of *H-2*-linked genes on the pre-S(2)-specific T-cell response and the relevance of this response to *in vivo* anti-pre-S(2) antibody production, we examined the T-cell responses to HBsAg/p34 in a series of *H-2*-congeneic strains. The strains studied, in order of magnitude of anti-pre-S(2) antibody production, were B10 > B10.D2 > B10.S > B10.BR. Similar to the $C_3H.Q$ strain, the B10 strain demonstrated a pre-S(2)-specific T-cell response, and significant production of IL-2 was observed at an HBsAg/p34 concentration as low as 1.5 ng/ml (Fig. 3*a*). The minimal S-region-specific (HBsAg/p25) T-cell response in this strain indicates that the preponderance of the response induced by HBsAg/p34 was pre-S(2)-specific. The B10.D2 strain also produced a pre-S(2)-specific T-cell response, with a minimal HBsAg/p34 dose of 7 ng/ml (4.7-fold difference between B10 and B10.D2) (Fig. 3*b*). The greater S-region-specific response in this strain, as compared to the minimal response of the B10 strain, is consistent with the HBsAg/p25-responder status of these strains (B10.D2 > B10) (17). The S-region-nonresponder strain B10.S also exhibited a pre-S(2)-specific T-cell response after priming with HBsAg/p34 (Fig. 4*a*). However, the dose–response curve was right-shifted relative to the B10 and B10.D2 strains, with a minimal HBsAg/p34 dose of 30 ng/ml (20-fold difference between B10 and B10.S). HBsAg/p25 did not elicit a response in this strain, which is consistent with its S-region-nonresponder status. The B10.S strain is nonresponsive at the T-cell level even to an optimal 16-μg dose of HBsAg/p25, therefore anti-S-region antibody production after HBsAg/p34 immunization in this strain most likely reflects functional pre-S(2)-region-specific T-cell help for B-cell clones that recognize the S region. This "cross-regional" T-cell help appears to be limited to S-region subtype determinants, since group-specific anti-S-region antibody is not produced (Table 1).

The strain that exhibits the lowest response to pre-S(2) in terms of antibody production is B10.BR, which is also a low responder in terms of T-cell IL-2 production (Fig. 4*b*). The minimal HBsAg/p34 concentration to elicit a T-cell response was 60 ng/ml (40-fold difference between B10 and B10.BR), and the pre-S(2) specificity was again demonstrated by the absence of T-cell activation by the alternative HBsAg preparation. The B10.M strain, which does not produce anti-pre-S(2) antibody *in vivo*, showed a minimal T-cell response, requiring HBsAg/p34 at 500 ng/ml (333-fold difference between B10 and B10.M) (Fig. 4*c*). A difference between S-region and pre-S(2)-region T-cell responses is that strain variation relative to the pre-S(2) region is characterized by shifts in dose–response curves with minimal differences in IL-2 production at maximal antigen concentrations. In contrast, S-region-specific T-cell proliferative responses among

Table 2. Influence of non-*H-2*-linked genes on the anti-pre-S(2) response

			Anti-pre-S(2) titer			Anti-S titer		
	Haplotype		Primary			Primary		
Strain	*H-2*	*Igh**	10 days	24 days	Secondary	10 days	24 days	Secondary
BALB/c	*d*	*a*	1:160	1:5120	ND	0	1:640	ND
CB.20	*d*	*b*	1:40	1:1280	ND	0	1:320	ND
SJL/J†	*s*	*b*	1:80	1:2560	1:20,480	0	1:320	1:5120
B10.S†	*s*	*b*	0	1:160	1:2560	0	0	1:640

Groups of five mice of the indicated strains were immunized with 1 μg of HBsAg/p34 and sera were assayed for pre-S(2)- and S-region antibody 10 and 24 days after primary immunization and 2 weeks after secondary immunization. ND, not done.
**Igh-1* locus.
†S-region nonresponders when immunized with 1–20 μg of HBsAg/p25 (13).

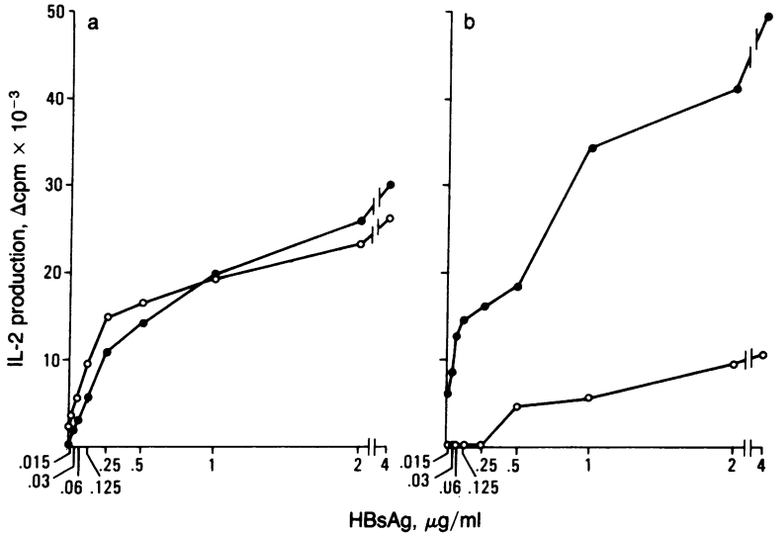

FIG. 2. T-cell IL-2 production induced by HBsAg/p25 and HBsAg/p34 priming. $C_3H.Q$ mice were immunized in the hind footpads with 16 μg of HBsAg/p25 (*a*) or 4 μg of HBsAg/p34 (*b*). Eight days after immunization, PLN cells were harvested and incubated in culture with medium containing various concentrations of HBsAg/p34 (●) or HBsAg/p25 (○) or with medium alone for 24 hr. IL-2 production was assessed by culturing the IL-2 dependent CTL-L2 cell line with supernatants derived from the above cultures. CTL-L2 proliferation was determined by incorporation of [^{3}H]thymidine and expressed as cpm adjusted for background (Δcpm). Background was [^{3}H]thymidine incorporation of CTL-L2 cells cultured in supernatant derived from HBsAg-primed PLN cells cultured with medium alone.

high to low responder strains differ in both respects (17). This observation suggests possible qualitative differences in T-cell recognition of the pre-S(2) as opposed to the S region. Based on the *in vitro* T-cell antigen dose–response curves, the hierarchy of pre-S(2)-specific T-cell responsiveness B10 > B10.D2 > B10.S > B10.BR correlates with *in vivo* anti-pre-S(2) antibody production in these *H-2*-congeneic strains. Note also that this order of pre-S(2)-specific T-cell responsiveness differs from that of S-region-specific T-cell responsiveness (B10.D2 > B10 > B10.BR > B10.S) (17). These results indicate that *H-2*-linked genes influence pre-S(2)-specific T-cell activation as well as *in vivo* antibody production in a manner distinct from the *H-2*-linked-gene influence observed for the response to the S region.

DISCUSSION

Previous work showed that the pre-S(2) region is significantly more immunogenic than the S region in terms of antibody production *in vivo* (2). In the present study, we examined the murine humoral and cellular responses to the pre-S(2) region as compared to the S region in terms of immunogenicity at the T-cell level, the influence of *H-2*- and non-*H-2*-linked genes, and possible overlapping regulatory mechanisms. The results demonstrate that (*i*) the pre-S(2) region of HBsAg is significantly more immunogenic than the S region in terms of *in vitro* T-cell activation; (*ii*) pre-S(2)-region-specific T-cell activation is regulated by *H-2*-linked genes and correlates with the *H-2* restriction of *in vivo* antibody production to the pre-S(2) region; (*iii*) *H-2* restriction of the T-cell response to the pre-S(2) region is distinct from the *H-2* restriction of the T-cell response to the S region; (*iv*) non-*H-2*-linked and non-*Igh*-linked genes also influence the pre-S(2) response; and (*v*) overlapping regulatory mechanisms exist at the helper T-cell level since immunization of an S-region-nonresponder, pre-S(2)-region T-cell-responder strain with HBsAg/p34 can circumvent nonresponsiveness to the S region.

The pre-S(2) region elicted a greater *in vivo* antibody response as compared to the S region (2). This difference was reflected by a lower effective immunization dose and a higher quantity of antibody production, and the onset of the pre-S(2) response preceded the S-region-specific antibody response in all strains tested. The predominance of anti-pre-S(2) IgG as early as 10 days after primary immunization suggested efficient helper T-cell influence. Investigation of T-cell activation specific for the pre-S(2) region has revealed differences between pre-S(2) and S immunogenicity. The effective priming dose required to induce a pre-S(2)-specific T-cell response was lower than that required to induce an S-region response, the magnitudes of pre-S(2)-specific responses were greater, and the minimal antigen dose required to elicit IL-2 production by T cells *in vitro* was lower for the pre-S(2) region than for the S region. These results suggest that the enhanced humoral immunogenicity of the pre-S(2) region may reflect very efficient T-cell activation by pre-S(2)-region determinant(s).

The S region of HBsAg is composed of 226 residues and the pre-S(2) region is composed of an additional NH_2-terminal 55 amino acids. This system allowed us to examine whether the antibody response to the pre-S(2) region would appear "hapten-like" and be regulated through T-cell recognition of

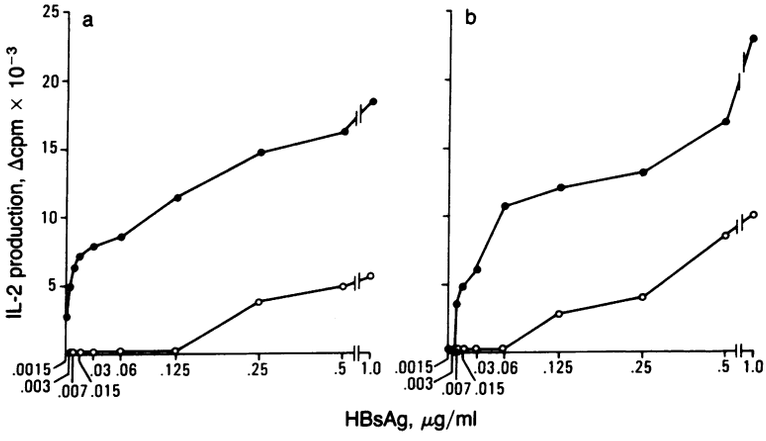

FIG. 3. Comparison of S-region- and pre-S(2)-region-specific T-cell IL-2 production in *H-2*-congeneic strains. B10 (*a*) and B10.D2 (*b*) mice were immunized with 4 μg of HBsAg/p34. Eight days later, PLN cells were harvested and cultured in medium containing HBsAg/p34 (●) or HBsAg/p25 (○) or in medium alone for 24 hr. IL-2 production was determined as described for Fig. 2.

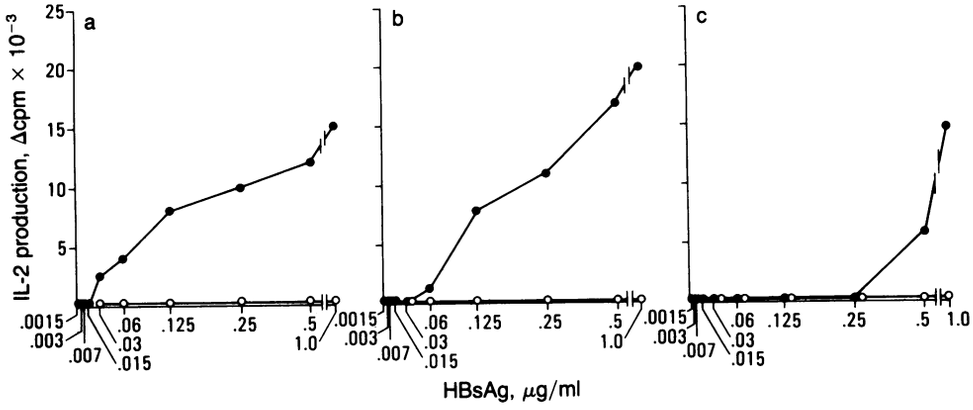

FIG. 4. Comparison of S-region-and pre-S(2)-region-specific T-cell IL-2 production in *H-2*-congeneic strains. B10.S (*a*), B10.BR (*b*), and B10.M (*c*) mice were immunized and T-cell antigen (●, HBsAg/p34; ○, HBsAg/p25)-dependent IL-2 production was determined as described for Fig. 2.

S-region determinants or whether it would be regulated independently. Further, would an immune response to one region influence the immune response to the other (i.e., carrier effect)? Since Milich *et al.* (16) had mapped and determined the influence of the *H-2*-linked *Ir* genes that regulate the immune response to the S region, we were able to examine these questions with regard to the pre-S(2) region. It was shown that both *in vivo* antibody production and T-cell activation specific for the pre-S(2) region are influenced by *H-2*-linked genes. High- ($H\text{-}2^{b,d,q}$), intermediate- ($H\text{-}2^{s}$), low- ($H\text{-}2^{k}$), and nonresponder ($H\text{-}2^{f}$) haplotypes were identified. The *H-2*-linked variation in T-cell IL-2 production correlated with *in vivo* antibody production. This hierarchy of response status to the pre-S(2) region of HBsAg differs from that observed for the S region (16). This indicates that distinct *H-2*-linked genes can influence S- and pre-S(2)-specific immune responses. However, since S- and pre-S(2)-region determinants exist on the same polypeptide (p34), it is conceivable that helper T cells specific for a determinant(s) on one region may be capable of providing functional help to B-cell clones recognizing a determinant(s) on the other region. The responses of the B10.S strain seem to confirm this possibility. The B10.S strain is nonresponsive to HBsAg/p25 but, when immunized and boosted with HBsAg/p34, produced an S-region antibody response as well as a pre-S(2)-region response. This suggests that a pre-S(2) determinant(s) is acting as a "carrier" for S-region "hapten" in this strain. The nonresponder status of the B10.S strain can be bypassed by immunization with an erythrocyte-HBsAg/p25 conjugate (13). The hypothesis that the pre-S(2) region can act as carrier for S-region determinant(s) predicts that the pre-S(2) region possesses determinant(s) capable of activating T cells, which was shown to be the case. Whereas immunization of the B10.S strain with HBsAg/p25 does not elicit a T-cell response (17), immunization with HBsAg/p34 induces significant pre-S(2)-specific T-cell activation. It appears that these pre-S(2)-specific T cells are capable of helping B-cell clones that recognize S as well as pre-S(2) determinants. In this regard it is interesting that the anti-S antibody produced by S-region-nonresponder strains (B10.S, SJL/J) immunized with HBsAg/p34 was limited to subtype specificity in contrast to strains responsive to both the S and pre-S(2) regions (B10, B10.D2, B10.BR), which produced both group- and subtype-specific anti-S-region antibody. This indicates that the T-cell help for S-region-specific B-cell clones, which appears to be mediated through T-cell recognition of pre-S(2) determinants, is qualitatively or at least quantitatively different from the T-cell help resulting from recognition of S-region determinants at both the T- and the B-cell level.

In addition to the influences of *H-2*-linked genes on the immune response to the pre-S(2) region, the differences observed between the BALB/c and CB.20 strains indicate that *Igh*-linked genes also affect the quantitative response. Further, the significantly superior anti-pre-S(2) response of the SJL/J strain as compared to the B10.S strain (*H-2* and *Igh* identical) indicates that non-*H-2*- and non-*Igh*-linked genes can amplify the pre-S(2)-region humoral response. This is in contrast to the anti-S-region response, since non-*H-2*-linked genes were shown to have only a minimal effect on this response (15).

The implications of these results relative to development of alternative HBsAg vaccines are clear. The pre-S(2) region is a superior immunogen as compared to the S region at both the T-cell and the B-cell level. *H-2*-linked genes regulate the T-cell responses to pre-S(2)- and S-region determinants independently, and therefore the likelihood of genetic nonresponsiveness to the entire particle is decreased. Finally, the pre-S(2) region appears capable of generating helper T-cell activity that can induce S-region as well as pre-S(2)-region antibody production, thereby circumventing S-region nonresponsiveness. These characteristics argue that inclusion of the pre-S(2) region may augment the effectiveness of future HBV vaccines.

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1. Peterson, D. L., Roberts, I. M. & Vyas, G. N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1530–1534.
2. Milich, D. R., Thornton, G. B., Neurath, A., Kent, S., Michel, M., Tiollais, P. & Chisari, F. V. (1985) *Science* **228**, 1195–1199.
3. Click, R. & Alter, B. (1972) *Cell. Immunol.* **3**, 264–268.
4. Smith, K. & Ruscetti, F. (1981) *Adv. Immunol.* **31**, 137–150.
5. Tiollais, P., Charnay, P. & Vyas, G. N. (1981) *Science* **213**, 406–411.
6. Stibbe, W. & Gerlich, W. H. (1982) *Virology* **123**, 436–442.
7. Cattaneo, R., Will, H., Hernandez, N. & Schaller, H. (1983) *Nature (London)* **305**, 336–338.
8. Stibbe, W. & Gerlich, W. H. (1983) *J. Virol.* **46**, 626–628.
9. Machida, A., Kishimoto, S., Ohnuma, H., Baba, K., Ito, Y., Miyamoto, H., Funatau, G., Oda, K., Usuda, S., Togami, S., Nakamura, T., Miyakawa, Y. & Mayumi, M. (1984) *Gastroenterology* **86**, 910–918.
10. Neurath, A. R., Kent, S. B. H. & Strick, N. (1984) *Science* **224**, 392–395.
11. Heermann, K. H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52**, 396–402.
12. Laub, O., Rall, L., Truett, B. M., Shaul, Y., Standring, D. N., Valenzuela, P. & Rutter, W. J. (1983) *J. Virol.* **48**, 271–280.
13. Milich, D. R., Alexander, H. & Chisari, F. V. (1983) *J. Immunol.* **130**, 1401–1407.
14. Michel, M. L., Pontisso, P., Sobczak, E., Malpiece, Y., Streeck, R. E. & Tiollais, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7708–7712.
15. Milich, D. R. & Chisari, F. V. (1982) *J. Immunol.* **129**, 320–325.
16. Milich, D. R., Leroux-Roels, G. G., Louie, R. & Chisari, F. V. (1984) *J. Exp. Med.* **159**, 41–56.
17. Milich, D. R., Louie, R. & Chisari, F. V. (1985) *J. Immunol.* **134**, 4194–4202.