GENETIC REGULATION OF THE IMMUNE RESPONSE
TO HEPATITIS B SURFACE ANTIGEN (HBsAg)

IV. Distinct H-2-linked Ir Genes Control Antibody Responses to
Different HBsAg Determinants on the Same Molecule and Map to the
I-A and I-C Subregions

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Immune response (Ir)[1] genes associated with the murine major histocompatibility complex (H-2) have been shown to control cellular and humoral responses to determinants on numerous T cell-dependent antigens including simple synthetic amino acid copolymers (1–2) and complex natural proteins (3–6). In view of these findings and the relevance of the immune response to the pathogenesis of hepatitis B virus (HBV) infection and current vaccine programs, our laboratory has been investigating the role of Ir genes in regulating the immune responses to antigenic determinants of hepatitis B surface antigen (HBsAg) particles. HBsAg is a large (2.5–3.5×10^6 mol wt) complex antigen possessing a common group-specific determinant, designated a, and two sets of subtype-specific determinants, d/y and w/r. The protein component is primarily composed of 25,000 D (25 kD) protein and 30 kD glycoprotein polypeptide subunits with identical amino acid sequences (7).

We have previously demonstrated that the humoral immune responses to the group-specific a and subtype-specific d and y determinants of HBsAg are controlled by H-2-linked Ir genes (8). High responder ($H\text{-}2^{q,d}$), intermediate to low responder ($H\text{-}2^{a>b>k}$), and nonresponder ($H\text{-}2^{s,f}$) haplotypes have been identified (9). The influence of an Ir gene(s) mapping in the I-A subregion on the primary antibody responses to both the a and d determinants of HBsAg and an additional influence of a gene(s) mapping to the right of the I-E subregion on at least the secondary anti-d response have been previously reported (8). In this paper, we have investigated the kinetics and specificity of in vivo antibody production in

[1] Abbreviations used in this paper: CFA, complete Freund's adjuvant; HA, hemagglutination; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HEL, hen egg lysozyme; Ir, immune response; Mb, sperm whale myoglobin; PLN, popliteal lymph node; RIA, radioimmunoassay; Th, T helper; Ts, T suppressor.

additional congeneic, H-2-recombinant strains immunized with HBsAg of the *ad* and *ay* subtypes in order to further delineate the map positions of the relevant Ir genes and their influence on the immune response to distinct antigenic determinants on HBsAg.

This current analysis indicates that the humoral anti-HBs response is regulated by at least two H-2-linked, Ir genes designated Ir-HBs-1, mapping in the I-A subregion, and Ir-HBs-2, mapping in the I-C subregion (right of I-E and left of H-2D). A gene(s) mapping in the I-A subregion regulates the primary responses to all HBsAg determinants, whereas the influence of the I-C subregional gene(s) appears to be determinant specific, primarily affecting the response to the *d* and *y* subtype-specific determinants of HBsAg. The anti-*a* response is regulated exclusively by Ir-HBs-1. Strains possessing only the Ir-HBs-2 gene produce no anti-*a* response, and a subtype-specific antibody response is detected only after secondary or tertiary immunization. In contrast, the influence of Ir-HBs-2 in the presence of Ir-HBs-1 is detected upon primary immunization and is additive rather than exclusive. There is also suggestive evidence that the presence of the E^k molecule, at least in the context of I-A^k, may have a suppressive influence on the anti-HBs response. These data indicate that, although the I-A subregion exerts a dominant influence, distinct Ir genes, mapping in separate I subregions, control immune responses to alternate HBsAg determinants on the same protein molecule.

In addition, the previous finding that HBsAg nonresponsiveness can be circumvented by conjugation of HBsAg to a carrier moiety suggested the expression of Ir-HBs genes in helper T cell function (10). Herein, we have initiated analysis of the proliferative T cell responses to HBsAg in responder, nonresponder, and (responder × nonresponder)F_1, H-2 congeneic strains and confirmed H-2 restriction of HBsAg-specific T cell proliferation. The kinetics and specificity of T cell proliferative responses paralleled in vivo antibody production.

Materials and Methods

Mice.   C57BL/10 Sn (B10), B10.S(7R), B10.S(9R), B10.A(2R), B10.A(4R), B10.A(5R), B10.BR, D2.GD, A/J, A.TH, and A.TL murine strains were obtained from the breeding colony at the Research Institute of Scripps Clinic. B10.T(6R) and B10.HTT mice were provided by Dr. Hugh McDevitt of Stanford University, Palo Alto, CA and B10.S(8R), B10.RSF1, and A.AL mice were provided by Dr. Chella David of the Mayo Clinic, Rochester, MN. Female mice between 6 and 8 wk of age at the initiation of the experiments were used in all studies.

HBsAg Preparations.   Preparations of HBsAg pooled from (*ad*) or (*ay*) donors were purified by a combination of ultracentrifugation, ammonium sulfate precipitation, pepsin digestion, and gel chromatography. Since these are pooled HBsAg preparations, other subtype-specific determinants (ie., *w/r*) would be expected to be present. However, upon immunization, these preparations have elicited anti-*a* and anti-*d* or -*y* responses only. This may result from the immunization dose, although the absence of anti-*w* or -*r* responses in guinea pigs immunized with HBsAg subtypes has previously been described (11). The HBsAg preparations were free of contaminating human serum proteins as tested by Ouchterlony analysis and immunoelectrophoresis against goat anti-human serum.

Immunizations.   To study in vivo antibody production, groups of six mice were immunized by intraperitoneal injection of 4.0 μg of HBsAg in 0.2 ml complete Freund's adjuvant (CFA) on day 0. They were bled from the retroorbital plexus on days 10 and 24, boosted identically on day 30, and bled again 14 d after secondary immunization (2°).

In vivo priming for the lymph node proliferative assay was accomplished by injection of a total of 16.0 μg of HBsAg in CFA in a volume of 80 μl into the two hind footpads of recipient mice.

Measurement of In Vivo Anti-HBs Production.   Serum antibody responses to HBsAg were measured by two methods. Pooled murine sera were evaluated for anti-HBs in an indirect, HBsAg subtype-specific, immunoglobulin class-specific, radioimmunoassay (RIA) using solid-phase HBsAg (*ad* or *ay* subtype) goat anti-mouse IgG and developed with a ^{125}I-labeled, affinity-isolated swine anti-goat Ig (SAGG-^{125}I). The methodology, specificity, and sensitivity of this solid-phase RIA have been previously described (8, 9). Titers are expressed as the reciprocal of $\log_2$ of the highest serum dilution to yield 2.5 times the counts of preimmunization sera. To analyze single murine serum samples for anti-HBs, a hemagglutination (HA) system was used. Human type O, Rh-negative erythrocytes were coated with HBsAg (*ad* or *ay* subtype) by the CrCl$_3$ method (12) and added to 0.025 ml of serially diluted test sera in microtiter V-bottom plates. Results were expressed as the reciprocal of $\log_2$ of the highest serum dilution to yield agglutination. All anti-HBs assays were performed in 5–10% normal human serum to neutralize any possible antibodies to contaminating human plasma proteins that may not have been removed from the HBsAg preparation by the purification procedures.

Pooled serum samples of strains demonstrating a fourfold difference in anti-HBs titer as measured by RIA were uniformly found to be significantly different ($P < 0.01$) when individual serum HA titers of these strains were compared using the Student t test. Therefore, a fourfold difference in RIA anti-HBs titer was considered statistically significant.

Lymph Node Proliferation Assay.   An HBsAg subtype-specific proliferation assay using draining popliteal lymph node cells was modified from the method of Corradin and Chiller (13) as described previously (10). Briefly, mice were immunized in the hind footpads with 16 μg of HBsAg in CFA and draining popliteal lymph node (PLN) cells were harvested 8 d postimmunization. 4×10^5 viable unfractionated PLN cells in 0.1 ml of Click's media (14) modified by the addition of Hepes (10 mM), gentamycin (10 μg/ml), and 0.5% syngeneic mouse serum were placed in flat-bottom microtiter wells (Falcon 3072; Falcon Labware, Oxnard, CA) with 0.1 ml of HBsAg of the *ad* or *ay* sybtype (1.0–0.025 μg/ml) or culture media. Cultures were incubated for 5 d at 37°C in a humidified atmosphere of 5% CO$_2$ in air and pulsed with 1 μCi [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) 16–18 h before harvesting; proliferation was assessed by incorporation of ^{3}H into DNA. The data are expressed as: stimulation index (SI) = cpm test antigen/cpm media control. The HBsAg-specific proliferative response of unfractionated PLN cells harvested from 5 to 12 d postimmunization was previously shown to represent antigen-specific T cell proliferation (10).

To determine the effect of monoclonal anti-I-A reagents on T cell proliferation, HBsAg-primed, nylon column-passed PLN T cells (4×10^5) were cultured with HBsAg-pulsed, syngeneic, irradiated (2,500 rad) spleen cells (antigen-presenting cells) in the presence of various dilutions of anti-I-A reagents. MK-D6 (anti-I-A^d) and MK-S4 (anti-I-A^s), originally from Kappler and Marrack (15) were generously provided by Dr. J. A. Berzofsky, National Institutes of Health.

Results

Complex Region Gene Control of the Anti-HBs Response.   Regulation of the immune response to the group-specific *a* determinant of HBsAg has been tentatively mapped to the I-A/I-B subregion of the H-2 complex, and evidence has been suggested that additional I subregional loci may influence the secondary subtype-specific *d* response (8). To further delineate I region gene control, the kinetics and specificity of in vivo antibody production after HBsAg immunization has been analyzed in a number of congeneic, H-2-recombinant strains. Groups of six mice of each strain were immunized intraperitoneally with either 4.0 μg of

HBsAg/ad or HBsAg/ay and individual and pooled serum anti-HBs/ad or anti-HBs/ay, and anti-HBs/a titers were determined at days 10 and 24 (1°), and after secondary immunization (2°). In some instances, tertiary (3°) immunizations with HBsAg/ay were given due to the reduced immunogenicity of HBsAg/ay as compared with the ad subtype.

The IgG anti-HBs titers of B10 congeneic, H-2 recombinant strains immunized with HBsAg/ad or HBsAg/ay are presented in Figs. 1 and 2, respectively. Relevant H-2 region and subregion alleles are shown for convenience. The B10 strain responded to the a, d, and y determinants of HBsAg after primary immunization and IgG class antibodies were detected by day 24. The B10.T(6R) strain, likewise, responded to all three determinants of HBsAg; however, IgG class anti-a- and anti-d- or anti-y-specific antibodies were detected as early as day 10. This early IgG response pattern is exclusively characteristic of the high responder H-2^q and H-2^d haplotypes (9). Secondary anti-HBs titers were not significantly different between mice of the B10 and B10.T(6R) strains. The B10.S(7R) strain was totally nonresponsive to all HBsAg determinants even after secondary or tertiary immunization. Since the B10.T(6R) and B10.S(7R) strains share H-2D^d, the D region is not involved in regulation of the anti-HBs response. The B10.S(9R) strain produced no primary or secondary anti-a response; however, a secondary anti-d and a tertiary anti-y response in 50% of mice immunized
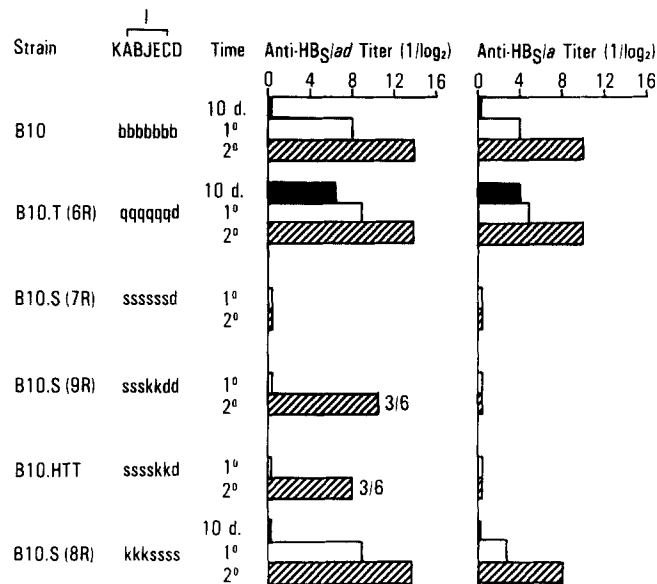

FIGURE 1.   In vivo anti-HBs production in congeneic, H-2 recombinant strains of the B10 series after HBsAg/ad immunization. Groups of six mice of the indicated strains were immunized and boosted with 4.0 µg i.p. of HBsAg/ad. Sera from primary, 10-d (closed bars), 24-d (1°; open bars), and secondary (2°; hatched bars) bleedings were analyzed for anti-ad- (left) and anti-a- (right) specific antibody by RIA. Titers are expressed as the reciprocal of the log₂ of the highest serum dilution to yield 2.5 times the counts of preimmunization sera. Only positive sera were pooled for RIA determination; unless otherwise noted (i.e., ⅗), 100% of individual sera were positive by HA before pooling. The relevant major histocompatibility complex region and subregion alleles of each strain are shown.
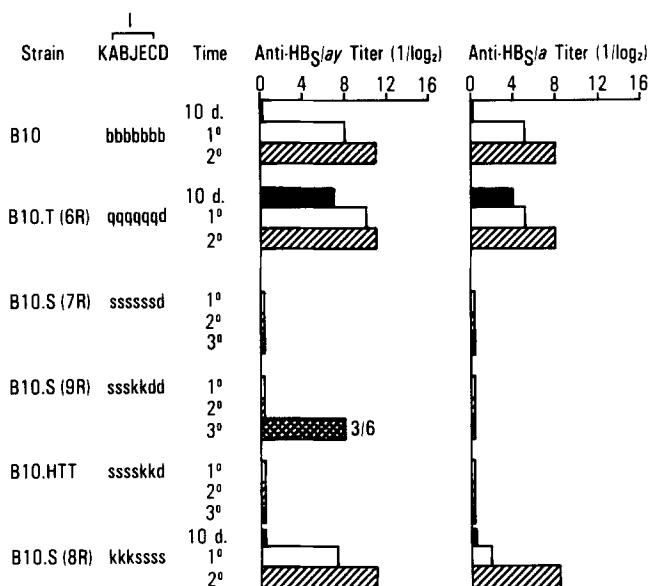
Strain	KABJECD	Time	Anti-HBs/ay Titer (1/log₂)	Anti-HBs/a Titer (1/log₂)
			0 4 8 12 16	0 4 8 12 16
B10	bbbbbbb	10 d. / 1° / 2°		
B10.T (6R)	qqqqqqd	10 d. / 1° / 2°		
B10.S (7R)	ssssssd	1° / 2° / 3°		
B10.S (9R)	ssskkdd	1° / 2° / 3°	3/6	
B10.HTT	sssskkd	1° / 2° / 3°		
B10.S (8R)	kkkssss	10 d. / 1° / 2°		

FIGURE 2. In vivo anti-HBs production in congeneic, H-2 recombinant strains of the B10 series after HBsAg/ay immunization. Groups of six mice of the indicated strains were immunized and boosted either once or twice with 4.0 μg i.p. of HBsAg/ay. Sera from primary, 10-d (closed bars), 24-d (1°; open bars), secondary (2°; hatched bars) or tertiary (3°; cross-hatched bars) bleedings were analyzed for anti-ay- (left) and anti-a- (right) specific antibody by RIA. Titers are expressed as the reciprocal of the log₂ of the highest serum dilution to yield 2.5 times the counts of preimmunization sera. Only positive sera were pooled for RIA determination; unless otherwise noted (i.e., ³⁄₆), 100% of individual sera were positive by HA before pooling. The relevant major histocompatibility complex region and subregion allele of each strain are shown.

with HBsAg/ad or HBsAg/ay, respectively, was observed. In response to HBsAg/ad immunization, the B10.HTT strain differed only slightly from the B10.S(9R) strain, with a fourfold lower secondary anti-d response in 50% of the recipients (Fig. 1). However, with respect to the anti-y response, the B10.HTT strain was totally nonresponsive after three HBsAg/ay immunizations, whereas 50% of the B10.S(9R) strain responded to the third immunization with an anti-y response (Fig. 2). Similar to the B10.S(9R) strain, the B10.HTT strain produced no anti-a response even after tertiary immunization with HBsAg of either subtype.

Comparison of the responses of the B10.S(9R) and B10.S(7R) strains indicates that control of the anti-a response maps to I-A/I-B (H-2K irrelevant; see below), since the presence of the nonresponder s allele in I-A/I-B is sufficient to preclude an anti-a-specific antibody response. The responses of the B10.S(9R) and B10.HTT strains suggest that a gene(s) to the right of the I-B subregion can influence the secondary anti-d/y responses. Therefore, it was of interest to determine if the presence of nonresponder alleles to the right of I-B would affect the anti-d or anti-y responses in the context of a responder allele in I-A/I-B. The B10.S(8R) strain was analyzed and positive primary anti-a, -d, and -y responses were observed at day 24 (Figs. 1 and 2). Note also the B10.S(8R) strain, similar to all strains that produce an a-specific response, demonstrated an antisubtype response of greater magnitude than the group-specific anti-a response at all time

points. The B10.S(8R) strain results indicate that a responder allele in I-A/I-B is sufficient to confer responsiveness to all HBsAg determinants after primary immunization. Similarly, the D2.GD strain (not congeneic to B10) possesses the same I-E^b, I-C^b subregional loci as the nonresponding B10.RSF1 strain. However, the presence of the I-A^d/I-B^d subregion produced a characteristic high responder phenotype (10 d, IgG, anti-a and anti-d) indicating the dominant influence of the I-A/I-B subregion on the anti-HBs response (Fig. 3). Most likely the relevant Ir gene(s) maps to the I-A rather than the I-B subregion since monoclonal anti-I-A of the appropriate specificity significantly inhibits the y- and a-specific T cell proliferative responses in BALB/c mice (Table I). Note that the anti-I-A^d monoclonal supernatant (MK-D6) quantitatively inhibited both the group-specific a and the subtype-specific y proliferative responses, but that a-specific proliferation was inhibited to a greater extent. Therefore, in the case of the BALB/c strain (H-2^d), an I-A^d-encoded molecule(s) is relevant to both a- and y-specific T cell responses.

Influence of the I-A and I-C Subregions on the Anti-HBs/ad Responses in H-2 Recombinant Strains on an A Genetic Background. The B10.S(9R) and B10.HTT H-2 congeneic strains differ within the I region only at the I-J and I-C subregions, yet demonstrated quantitative differences in anti-d responses and qualitative differences with respect to anti-y responsiveness (B10.HTT was nonresponsive to the y determinant). To examine the influence of the I-C subregion, we compared the in vivo antibody responses to HBsAg/ad immunization in A/J (I-C^d) and A.AL (I-C^k) H-2 congeneic strains, which express an I region difference
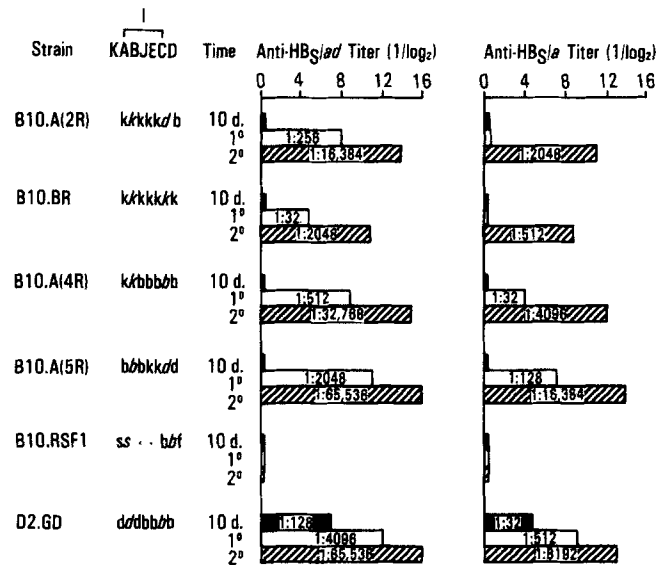

FIGURE 3. In vivo anti-HBs production in congeneic, H-2 recombinant strains of the B10 series afer HBsAg/ad immunization. Groups of six mice of the indicated strains were immunized and boosted with 4.0 µg of HBsAg/ad. Sera from primary, 10-d (closed bars), 24-d (1°; open bars), and secondary (2°; hatched bars) bleedings were analyzed for anti-ad- (*left*) and anti-a- (*right*) specific antibody by RIA. Titers are expressed as the reciprocal of the log₂ of the highest serum dilution to yield 2.5 times the counts of preimmunization sera. The relevant major histocompatibility complex region and subregion alleles of each strain are shown.

TABLE I

Effect of Monoclonal Anti-I-A Reagents on the HBsAg-specific Proliferative Responses of BALB/c T Cells Reconstituted with BALB/c Antigen-presenting Cells

T cell	Anti-Ia	Dilution	In vitro antigen-presenting cell pulse* (cpm)		
			HBsAg/ay	HBsAg/ad	Media
HBsAg/ay-primed	0	—	24,000	14,000	3,000
				Percent suppression	
	Anti-I-A^{d‡}	1:10	99	100	—
		1:80	62	100	—
		1:320	43	82.3	—
	Anti-I-A^{s§}	1:10	0	0	—
		1:80	0	0	—

* HBsAg-ay-primed, nylon column-passed PLN T cells (4 × 10^5) were cultured with (2 × 10^5) HBsAg/ay- or HBsAg/ad-pulsed syngeneic, irradiated (2,500 rad) spleen cells (antigen-presenting cells) in the presence of the indicated dilutions of anti-I-A reagents, and the proliferative responses were determined after 5 d in culture.

$\ddagger$ MK-D6 (monoclonal supernatant; private specificity).

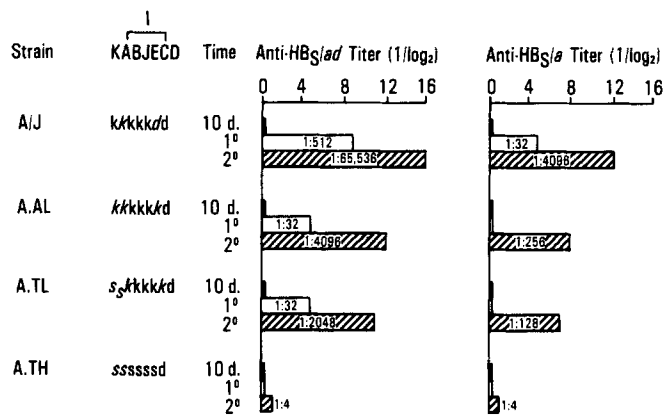$\S$ MK-S4 (monoclonal supernatant; cross-reacts with I-A^f only).

FIGURE 4. In vivo anti-HBs production of congeneic, H-2 recombinant strains of the A series after HBsAg/ad immunization. Groups of six mice of the indicated strains were immunized and boosted with 4.0 μg of HBsAg/ad. Sera from primary, 10-d (closed bars), 24-d (1°; open bars), and secondary (2°; hatched bars) bleedings were analyzed for anti-ad- (left) and anti-a- (right) specific antibody by RIA. Titers are expressed as the reciprocal of the log$_2$ of the highest serum dilution to yield 2.5 times the counts of preimmunization sera. The relevant major histocompatibility complex region and subregion alleles of each strain are shown. Note the A.TL strain I-A subregion is depicted as $_s$k, which refers to the H-2^s and H-2^k origins of the A$_\alpha$ polypeptide chain of this strain.

only in the I-C subregion. As shown in Fig. 4, the primary and secondary anti-d responses of the A/J strain were 16-fold greater than the anti-d responses of the A.AL strain. The anti-a response also appeared to be significantly influenced by the I-C^d subregion allele, although this is not a consistent finding (discussed below). Comparison of the A/J and A.AL strain responses illustrates the significance of the I-C subregion to the anti-HBs response and demonstrates a difference between the I-C^d and I-C^k alleles. Additionally, these results demonstrate

that the influence of the I-C^d allele on the anti-d response in the context of a responder allele in I-A (I-A^k) is additive and can be detected after primary immunization. In contrast, the influence of the I-C^d allele in the context of a nonresponder allele in I-A [i.e., B10.S(9R)] is exclusive and can only be detected after secondary immunization.

Comparison of the A.TH and A.TL strains illustrates the irrelevance of the H-2K region since both strains possess the H-2K^s region and the A.TL strain is an HBsAg responder, whereas the A.TH strain is a nonresponder (Fig. 4). The A.AL and A.TL strains were compared because part of the A$_\alpha$ polypeptide chain of the A molecule in the A.TL strain is of H-2^s haplotype (nonresponder) origin rather than H-2^k haplotype origin due to a possible intragenic recombinant event (16), and it was of interest to determine if the derivation of the A$_\alpha$ polypeptide component of the I-A-encoded A molecule influenced the anti-HBs response. As shown in Fig. 4, the A.AL and A.TL strains were not significantly different in their responses to the a or d determinants of HBsAg, suggesting that a determinant on the A$_\beta$ or nonrecombinant part of the A$_\alpha$ polypeptide chain is associated with the recognition of HBsAg.

Relevance of the I-C Subregion on Anti-HBs/ad Responses in H-2 Recombinant Strains on a B10 Genetic Background and Possible Negative Influence of the I-E^k/I-C^k Subregion. Comparison of in vivo anti-HBs/ad production between B10.BR and B10.A(2R) strains, which differ significantly only in the I-C subregion (H-2D irrelevant), confirmed that a gene(s) influencing the anti-d response maps to the I-C subregion (Fig. 3). The B10.A(2R) strain (I-C^d) produced an eightfold greater 24-d primary anti-d response than the B10.BR strain (I-C^k). The secondary anti-d response of the B10.A(2R) strain was also eightfold higher than that of the B10.BR strain. The primary anti-a response was not influenced by the I-C^d allele, but the B10.A(2R) secondary anti-a response was fourfold greater than that of the B10.BR strain.

Previous studies of in vivo anti-HBs production in the C$_3$H and CBA strains (9) and the response of the B10.BR H-2 congeneic strain indicate that the H-2^k haplotype confers low responsiveness to HBsAg. However, the B10.S(8R) and B10.A(4R) strains produced 16-fold greater primary anti-d responses than the B10.BR strain (Figs. 1 and 3). Like B10.BR, these strains possess the I-A^k subregion, but unlike B10.BR, they do not express an E^k molecule. These results suggest the possibility that I-E^k, at least in the context of I-A^k, may be suppressive. If expression of an E^k molecule is suppressive, it appears that an E$_\beta$^{k} chain is required, since the B10.A(5R) strain (I-A^b, I-E^k) produces a vigorous primary and secondary anti-HBs response (Fig. 3). It should be noted that the B10.S(8R) and B10.A(4R) strains also lack the I-C^k subregion as compared with the B10.BR strain; possession of I-C^k cannot be ruled out as possibly relevant to the "inferior" B10.BR response.

H-2 Restriction of T Lymphocyte Proliferative Responses to the a and d Determinants of HBsAg. To examine the role of H-2-linked Ir genes in regulating the response to HBsAg determinants at the T cell level, the HBsAg-specific T cell proliferative responses of H-2 congeneic B10.T(6R) responder, B10.S(7R) nonresponder, and (responder × nonresponder)F$_1$ mice were analyzed. Groups of four mice were primed with HBsAg/ad (16 μg) in vivo and pooled PLN cells harvested 8 d

postimmunization and challenged in vitro with HBsAg/*ad* or HBsAg/*ay* to determine the *ad*- and *a*-specific T cell proliferative responses, respectively. B10.T(6R) mice demonstrated significant *d*-specific proliferation (SI, 3.0) at HBAg/*ad* concentrations from 0.05 to 1.0 µg/ml and required 10-fold higher HBsAg/*ay* doses to induce significant *a*-specific proliferation, which was consistently of lower magnitude (Fig. 5). In contrast, the B10.S(7R) strain demonstrated no HBsAg-specific T cell proliferative responses at 8 d postimmunization, and the 12-d response was also negative (data not shown). The (R × NR)F_1 mice produced *ad*- and *a*-specific proliferative responses equivalent to the B10.T(6R) responder parent.

Discussion

The analysis of H-2 recombinant strains immunized with HBsAg of *ad* and *ay* subtypes indicates that at least two distinct Ir genes influence antibody production to determinants on the same HBsAg molecule. An Ir gene(s) mapping in the I-A subregion regulates responsiveness to all antigenic determinants of HBsAg (group-specific *a* and subtype-specific *d* or *y*) after primary immunization; we have designated it Ir-HBs-1. The presence of a responder allele in I-A conferred responsiveness to the *a*, *d*, and *y* determinant after primary immunization, whereas the presence of a nonresponder allele in I-A conferred nonresponsiveness to all three HBsAg determinants after primary immunization. Mapping the regulation of the anti-*a* response to the I-A subregion was confirmed by the fact that all H-2 recombinant strains possessing the I-A^s allele were nonresponsive to the *a* determinant even after hyperimmunization and regardless of the presence of a responder haplotype at other subregional loci. In contrast, secondary immunization of recombinant strains lacking Ir-HBs-1 revealed the influence of an Ir gene(s) on the subtype-specific anti-*d* or anti-*y* responses mapping in the I-C subregion; we have designated this gene Ir-HBs-2. For example, B10.S(9R) mice produce an exclusive antisubtype-specific response after secondary or tertiary immunization with HBsAg of the *ad* or *ay* subtype without concomitant anti-*a*
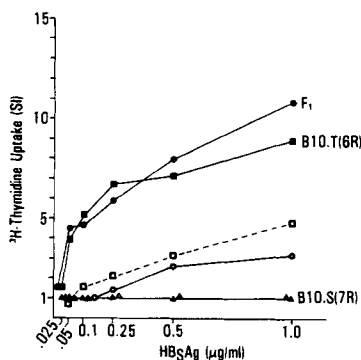

FIGURE 5.   HBsAg-specific T cell-proliferative responses of B10.T(6R) responder, B10.S(7R) nonresponder, and F_1 H-2 congeneic, strains. Groups of four mice of the indicated strains were immunized with 16 µg of HBsAg/*ad* in CFA, and 8 d later PLN cells were harvested and stimulated in vitro with HBsAg/*ad* (closed symbols) or HBsAg/*ay* (open symbols). The HBsAg-specific proliferative responses are expressed as a stimulation index (SI = cpm test antigen/cpm media control. Media controls ranged from 1,000 to 5,000 cpm.

production. The significantly different primary anti-d responses of the H-2 recombinant strains possessing the same responder allele in the I-A (I-A^k) subregion and differing only in the I-C subregion [B10.A(2R) vs. B10.BR and A/J vs. A.AL] demonstrated that the influence of Ir-HBs-2 in the context of a responder allele in I-A (Ir-HBS-1) is additive rather than exclusive and can be detected after primary immunization. Quantitative differences are observed between the I-C^d and I-C^k alleles when these strains were compared after primary and secondary immunization with HBsAg/ad. The I-C^d allele conferred superior anti-d responsiveness as compared with the I-C^k allele in both strain combinations. In addition, quantitative differences were observed between the I-C^d and I-C^k alleles when the B10.S(9R) and B10.HTT strains were immunized with HBsAg/ad. Qualitative differences were seen when these strains were immunized with HBsAg/ay: the B10.HTT strain was totally nonresponsive, whereas 50% of the B10.S(9R) mice produced anti-y-specific antibody. The observation that the B10.HTT strain (I-C^k) responded to the d determinant and not the y determinant upon hyperimmunization is also of interest. It has been suggested that the d and y determinants of HBsAg may differ by as few as two amino acid residues (17, 18), yet the I-C^k allele permitted responsiveness to the d but not the y determinant. This may provide insight into mechanisms of possible interaction between I-C-encoded molecules and restricted domains on HBsAg. The greater responses of the B10.A(4R) strain (I-A^k, I-C^b) and the B10.S(8R) strain (I-A^k, I-C^s) compared with the B10.BR strain (I-A^k, I-C^k) may relate to the I-E subregional differences expressed by these strains. Therefore, while the comparisons of A/J vs. A.AL and B10.A(2R) vs. B10.BR strains, which share the I-E^k allele, illustrate the relevance of the I-C subregion, it cannot be assumed that additional mechanisms are not operative in strains differing at I-E. For example, the presence of I-E^k-restricted T suppressor cells in the lactate dehydrogenase B system was recently demonstrated (19).

The observations of ~50% responsiveness in strains possessing a responder allele in I-C in the context of a nonresponder allele in I-A [B10.S(9R), B10.HTT] is difficult to assess. We have observed this heterogeneity in two different H-2-recombinant strains involving a total of 33 mice in response to two separate antigens (d and y) and suggest it may be more than artifactual. Evidence for classifying these strains as responsive to HBsAg comes from the fact that upon further hyperimmunization, at least minor antisubtype responses are observed in virtually 100% of mice as well as low titer anti-a responses in some mice. A similar observation has been made in the sperm whale myoglobin (Mb) system, involving the B10.HTT strain, wherein it was suggested that nonresponse was more likely an artifact than response and that the strain was considered responsive (20). We are currently conducting genetic analysis of (9R) responder and (9R) nonresponder mice to resolve this issue. In any event, the validity of the concept of dual Ir gene control does not rely solely on the response status of the B10.S(9R) and B10.HTT strains, as discussed previously.

Cumulatively, these genetic data clearly indicate the existence of at least two H-2-linked Ir genes regulating the response to HBsAg determinants, one mapping in the I-A subregion and the other in the I-C subregion. It should be noted that we provide no formal evidence that gene(s) to the right of I-C and to the

left of H-2D are not involved in regulation of the response to HBsAg, and use the term "I-C subregion" in this broad sense. However, a question arises as to whether the gene(s) mapping in the I-C subregion (Ir-HBs-2) effects the anti-*a* response as well as the anti-*d/y* responses, since the I-C subregion allele appeared to influence the anti-*a* response in certain recombinant strains. We suggest that Ir-HBs-2 does not directly regulate the anti-*a* response because: (*a*) no anti-*a* responses are detected in the absence of Ir-HBs-1; (*b*) the presence of the high responder I-C^d allele did not affect the anti-*a* response in all recombinant strains and; (*c*) anti-I-A^d monoclonal antibody incompletely inhibited the *y*-specific T cell proliferative response of BALB/c (H-2^d) mice possessing both Ir-HBs genes, but totally abrogated the anti-*a*-specific proliferative response, suggesting that the anti-*a* response is exclusively restricted by the I-A subregion. The apparent influence of the I-C subregion on the anti-*a* response may reflect T cell helper activity directed to the *d* or *y* determinants that nonspecifically amplifies the anti-*a* response.

Although these genetic experiments were analyzed by measuring in vivo antibody production, the T cell proliferative responses to the *a* and *d* determinants of HBsAg were H-2-restricted and the kinetics and specificity of the T cell responses paralleled those of in vivo antibody production. For example, the *d*-specific proliferative responses preceded and were of greater magnitude than the *a*-specific proliferative responses. In addition, the responses of the (R × NR)F$_1$ mice indicated that HBsAg-specific T cell proliferation is inherited as a dominant trait with no gene dosage effects as observed for antibody production (9). These results, the T dependent nature of HBsAg (21), and the fact that nonresponsiveness to HBsAg can be circumvented by conjugation to a carrier moiety (10) establish that Ir-HBs genes are expressed at the T cell level.

The regulation of the immune response to HBsAg bears some remarkable similarities with other Ir gene-restricted, naturally occurring, protein antigens. The immune response to hen egg lysozyme (HEL) is controlled by two Ir gene loci, one in the I-A and the other believed to be in the I-C subregion (22). Differential effects between I-C^d and I-C^k alleles in the HEL system have been reported as well (23). The murine antibody and T lymphocyte proliferative response to Mb were found to be under the control of two distinct H-2-linked Ir genes, one mapping in the I-A subregion (Ir-MB-1) and the other mapping in the I-C subregion (Ir-Mb-2) (24). Furthermore, H-2-recombinant strains possessing only the Mb responder allele in I-A were responsive to all cyanogen bromide cleavage fragments, whereas strains possessing only the Mb responder allele in I-C responded to the NH$_2$-terminal fragment but not to the COOH-terminal fragment, and these responses were evaluated after secondary or tertiary immunization (24). Therefore, similar to the HBsAg system, the I-A responder allele confers full responsiveness to Mb, and the I-C responder allele in the absence of an I-A responder allele confers responsiveness to fewer or a subset of determinants.

Any hypothesis regarding the relationship of I-A and I-C subregional loci and their influence on the regulation of responsiveness to a multideterminant, protein antigen such as HBsAg must explain the predominant influence of the I-A subregion on responses to all determinants coexistent with the influence of the

I-C subregion on the responses to the subtype-specific determinants. Multiple independent Ir genes mapping in I-A that regulate the a, d, and y responses seem unlikely in view of the fact that H-$2^{s,f}$ haplotype-bearing strains are nonresponsive to all HBsAg determinants. The I-C subregion may encode immune suppressor genes. Such a role for the I-C subregion in the control of the generation of suppressor T cells (Ts) in the mixed lymphocyte reaction has been proposed (25). Additionally, I-C subregion-encoded suppressor factors have been reported to regulate lymphokine production (26) and contact sensitivity (27). Although nonresponders to HBsAg can be induced to produce anti-HBs in vivo by alteration of the route as well as dose of immunization, we have been unable to demonstrate a role for Ts cells in HBsAg nonresponsiveness (10). However, we are continuing to explore this possibility as it may apply to the influence of the I-E/I-C subregions. Regarding the influence of the I-A subregion, an hypothesis consistent with existing data predicts the existence of a T cell "carrier determinant" on HBsAg, recognized by helper T (Th) cells in the context of an Ia molecule encoded within the I-A subregion. Such Th cells may be able to provide functional help to B cell clones specific for the a, d, and y epitopes. There is increasing evidence that T cell determinants on a molecule can be distinct from B cell determinants or antibody-binding sites in a variety of antigen systems including, glucagon (28), insulin (29), β-galactosidase (30), lysozyme (31), myoglobin (32), and the encephalomyelitogenic, myelin basic protein peptide (33). In this regard, immunization of mice (10) and rabbits (18) with keyhole limpet hemocyanin-conjugated synthetic peptide analogues of HBsAg determinants induced significant anti-HBs responses. In contrast, immunization of mice with free synthetic peptide analogues of the d and y determinants of HBsAg induced minimal antibody responses and did not distinguish H-2^q from H-2^s haplotype-bearing strains (10). These data suggest that these synthetic B cell epitopes do not possess T cell determinants, and therefore, Ir-restricted, T cell helper activity is absent. Indeed, evaluation of the immune response to chemically synthesized peptide fragments of HBsAg suggests the existence of distinct T and B cell epitopes on HBsAg (manuscript in preparation). With respect to native HBsAg, a responder allele in I-A may result in carrier-specific help engaging all determinant-specific B cell clones. The concept of a T cell carrier determinant on HBsAg need not exclude the existence of determinant-specific Th or Ts cells specific for d or y determinants and restricted by the I-E/I-C subregion. The presence of a nonresponder allele in I-A would preclude carrier-specific help and may necessitate secondary or tertiary immunization to expand smaller d/y determinant-specific T cell precursor pools, which may selectively cooperate with determinant-specific B cell clones. Strains possessing both Ir-HBs genes would reflect an additive rather than exclusive effect of Ir-HBs-2.

The difficulties in demonstrating I-C-encoded, serologically detectable molecules have prompted questioning of the existence of the I-C subregion (34). However, the recent demonstration of xenoantisera that detect I-C^d- and I-C^b-encoded products (35) and the observation that at least three Ir gene-restricted, multideterminant, naturally occurring protein antigens are influenced by genes mapping in the I-C subregion clearly support the existence of an I-C subregion. The consistent involvement of the I-C subregion and the similarities of the

regulation of the immune response to these protein antigens suggest a unique influence of this subregion on responses to naturally occurring protein antigens that may not be relevant to simple copolymer antigens.

The results of these investigations raise several questions regarding the mechanism of responsiveness to HBsAg. How do distinct Ir genes regulate the response to different determinants on the same molecule? What is the nature of the determinant(s) recognized by T helper cells and B cells? What is the mechanism of I-C subregion influence? In addition, the evidence of multiple I-region gene control of the murine immune responses to HBsAg provides a model to more adequately understand the complex and variable human immune responses to HBsAg after vaccination as well as during the course of HBV infection. In this regard, it is notable that an association between HLA-DR phenotype and non-responsiveness to a trial HBsAg vaccine has been reported (36), and a human HBsAg vaccine recipient was observed to produce an anti-d response in the absence of an anti-a response (37). These preliminary findings suggest that, after immunization, distinct Ir genes may influence regulation of the human immune response to the a and d determinants of HBsAg, as demonstrated herein for the murine immune response.

Summary

We have previously demonstrated that the murine humoral immune responses to the group-specific a and subtype-specific d/y determinants of hepatitis B surface antigen (HBsAg) are controlled by H-2-linked immune response (Ir) genes. High responder (H-$2^{d,q}$), intermediate responder (H-$2^{a > b > k}$) and nonresponder (H-$2^{f,s}$) haplotypes have been identified (8, 9). The kinetics and specificity of in vivo antibody production after HBsAg immunization in congeneic, H-2-recombinant strains was analyzed to further define relevant Ir genes and their influence on the immune response to distinct antigenic determinants.

These studies indicate that the humoral anti-HBs response is regulated by at least two Ir genes, one in the I-A subregion (Ir-HBs-1) and one in the I-C subregion (Ir-HBs-2) of the murine H-2 complex. Ir-HBs-1 regulates the primary responses to all HBsAg determinants, whereas the influence of Ir-HBs-2 is determinant specific, affecting the responses to the d or y determinants. The anti-a response is regulated exclusively by Ir-HBs-1. Strains possessing only the Ir-HBs-2 gene [B10.S(9R) and B10.HTT] produce no anti-a response and a subtype-specific antibody response is detected only after secondary or tertiary immunization. In contrast, the influence of Ir-HBs-2 in the presence of Ir-HBs-1 is detected upon primary immunization and is additive rather than exclusive. There is also suggestive evidence that the presence of the E^k molecule, at least in the context of I-A^k, may have a suppressive influence on the anti-HBs response. Additionally, HBsAg-specific, T cell proliferative responses were H-2 restricted and the kinetics and specificity of T cell proliferative responses paralleled in vivo antibody production. These data indicate that, although the I-A subregion exerts a dominant influence, distinct Ir-HBs genes, mapping in separate I subregions, control immune responses to alternate HBsAg determinants on the same protein molecule.

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