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The Hepatitis B Virus Core and e Antigens Elicit Different Th Cell Subsets: Antigen Structure Can Affect Th Cell Phenotype†

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Secretion of the hepatitis B virus (HBV) e antigen (HBeAg) has been conserved throughout the evolution of hepadnaviruses. However, the function of this secreted form of the viral nucleoprotein remains enigmatic. It has been suggested that HBeAg functions as an immunomodulator. We therefore examined the possibility that the two structural forms of the viral nucleoprotein, the particulate HBV core (HBcAg) and the nonparticulate HBeAg, may preferentially elicit different T helper (Th) cell subsets. For this purpose, mice were immunized with recombinant HBcAg and HBeAg in the presence and absence of adjuvants, and the immunoglobulin G (IgG) isotype profiles of anti-HBc and anti-HBe antibodies were determined. Second, in vitro cytokine production by HBcAg- and HBeAg-primed Th cells was measured. The immunogenicity of HBcAg, in contrast to that of HBeAg, did not require the use of adjuvants. Furthermore, HBcAg elicited primarily IgG2a and IgG2b anti-HBc antibodies, with a low level of IgG3, and no IgG1 anti-HBc antibodies. In contrast, the anti-HBe antibody response was dominated by the IgG1 isotype; low levels of IgG2a or IgG2b anti-HBe antibodies and no IgG3 anti-HBe antibodies were produced. Cytokine production by HBcAg- and HBeAg-primed Th cells was consistent with the IgG isotype profiles. HBcAg-primed Th cells efficiently produced interleukin-2 (IL-2) and gamma interferon (IFN-γ) and low levels of IL-4. Conversely, efficient IL-4 production and lesser amounts of IFN-γ were elicited by HBeAg immunization. The results indicate that HBcAg preferentially, but not exclusively, elicits Th_1-like cells and that HBeAg preferentially, but not exclusively, elicits Th_0 or Th_2-like cells. Because HBcAg and the HBeAg are cross-reactive in terms of Th cell recognition, these findings demonstrate that Th cells with the same specificity can develop into different Th subsets based on the structural form of the immunogen. These results may have relevance to chronic HBV infection. Circulating HBeAg may downregulate antiviral clearance mechanisms by virtue of eliciting anti-inflammatory Th_2-like cytokine production. Last, the influence of antigen structure on Th cell phenotype was not absolute and could be modulated by in vivo cytokine treatment. For example, IFN-α treatment inhibited HBeAg-specific Th_2-mediated antibody production and altered the IgG anti-HBe isotype profile toward the Th_1 phenotype.

The hepatitis B virus (HBV) has a number of unique characteristics. One is the production of a secreted form of the nucleocapsid, HBV e antigen (HBeAg). The particulate nucleocapsid (HBV core antigen [HBcAg]) is an intracellular protein that encapsulates the viral DNA and plays an important role in the maturation of the virus. In contrast, the nonparticulate, secreted HBeAg is not required for replication or infection (5, 37). In fact, no known function for HBeAg in the viral life cycle has been reported. We and others have suggested that HBeAg can modulate the complex interaction between HBV and the immune system (22, 23, 46). For example, studies in a murine transgenic (Tg) model suggested that HBeAg can cross the placenta and establish T helper (Th) cell tolerance in utero specific for HBeAg and HBcAg (23), which are largely cross-reactive at the level of Th cell recognition in mice and humans (24). It was proposed that such a mechanism may be responsible for the high chronicity rates (~90%) observed in babies infected perinatally by their HBeAg-positive mothers (22). Interestingly, babies infected perinatally with an HBeAg-negative mutant form of HBV experience an acute or fulminant acute course of infection rather than a persistent infection (45). Therefore, conservation of expression of HBeAg may be a viral strategy to guarantee persistence during vertical transmission of HBV, which is the major source of chronic infection in areas where hepatitis B is endemic. However, expression of HBeAg is also conserved in the avian hepadnaviruses, in which neonatal tolerance mechanisms are not relevant. Furthermore, adult infection with the HBeAg-negative mutant appears to correlate with an acute fulminant course of infection rather than the relatively benign acute course which characterizes most adult-onset infections (4, 18, 35). It therefore appears that HBeAg may function to modulate the immune response during adult HBV infection in ways unrelated to neonatal tolerance.

To begin to understand potential immunoregulatory functions of circulating HBeAg, we have compared the abilities of HBeAg and HBcAg to elicit the Th_1 and Th_2 cell subsets. For this purpose, mice were immunized with various structural forms of HBcAg and HBeAg, including particulate HBcAg; nonparticulate HBeAg; P16, the polypeptide subunit of HBcAg; and synthetic peptides representing Th cell recognition sites within HBcAg and HBeAg. Induction of Th cell subsets was determined by directly measuring Th_1-type (interleukin-2 [IL-2] and gamma interferon [IFNγ]) and Th_2-type (IL-4) cytokine production elicited by immunization and in vitro culture with the various antigens and by analyzing the immunoglobulin G

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(IgG) isotype profile of antibodies produced in vivo. In the mouse, IgG1 and IgE production is regulated by IL-4 released from Th_2 cells and IgG_{2a} production is regulated by IFN-γ, a Th_1 cell-derived cytokine (10). The Th_0 cell subset produces cytokines characteristic of both subsets. The results indicate that the structural form of the immunogen and/or antigen can influence the Th cell phenotype of the response and may have implications for viral persistence in HBV infection.

MATERIALS AND METHODS

Mice. C57BL/10 (B10) (*H-2*^{b}) and H-2 congenic B10.S (*H-2*^{s}) and (B10 × B10.S)F_1 mice were obtained from the breeding colony of The Scripps Research Institute. The Tg lineages designated Tg31e and Tg10c, which express HBeAg (9 to 13 ng/ml) in the serum and HBcAg intracellularly, respectively, were produced as described previously (23, 30).

Recombinant proteins and synthetic peptides. The HBV core (HBc) gene encodes two polypeptides. Initiation of translation at the first start codon (AUG) results in a 25-kDa precore protein that is secreted as HBeAg after removal of 19 residues of the leader sequence and 34 COOH-terminal amino acids. Initiation of translation at the second AUG leads to the synthesis of a 183-amino-acid 21-kDa protein that assembles to form 27-nm particles that comprise the virion nucleocapsid (HBcAg). Although HBeAg and HBcAg are serologically distinct, they are cross-reactive at the level of Th cell recognition because of the fact that they are colinear throughout most of their primary sequence. Recombinant HBcAg of the *ayw* subtype was produced in *Escherichia coli* and purified as described previously (39). A recombinant HBeAg corresponding in sequence to serum-derived HBeAg encompassing the 10 precore amino acids remaining after cleavage of the precursor and residues 1 to 149 of HBcAg was produced as described previously (40) and is designated PC-HBeAg. The presence of the 10 precore amino acids prevents particle assembly and PC-HBeAg is recognized efficiently by HBeAg-specific monoclonal antibodies (MAbs) but displays little HBc antigenicity (40). An aliquot of truncated HBcAg was reduced and denatured by boiling in sodium dodecyl sulfate–2-mercaptoethanol (1.0%) and alkylated, the sodium dodecyl sulfate was removed by dialysis, and the material was resolubilized in 3 M urea. This preparation consisted predominantly of monomers (16 kDa), with some dimer formation upon nonreducing polyacrylamide gel electrophoresis, and was designated P16. P16 does not bind HBcAg- or HBeAg-specific MAbs.

Peptides were synthesized by the simultaneous multiple peptide synthesis method (14) and were kindly provided by Richard Houghton (Torrey Pines Institute for Molecular Studies, La Jolla, Calif.). The following HBcAg-derived synthetic peptides representing Th cell recognition sites were used and designated by amino acid position from the N terminus of HBcAg: p120-131 (*IA*^{s}), VSFGVWIRTPPA; p129-140 (*IA*^{b}), PPAYRPPNAPIL; and p120-140, a 21-mer comprising both T-cell sites. Synthetic IL-4 corresponding to amino acids 24 to 140 of murine IL-4 was generously provided by Ian Clark-Lewis (Biomedical Research Center, University of British Columbia, Victoria, British Columbia, Canada).

Serology. HBeAg was measured in diluted Tg mouse sera by a commercial enzyme-linked immunosorbent assay (ELISA) (HBe EIA; Abbott, Chicago, Ill.), and PC-HBeAg was used as a standard. Anti-HBc or anti-HBe IgG antibody was measured in murine sera by an indirect solid-phase ELISA using HBcAg or PC-HBeAg as the solid-phase ligand as described previously (26). The data are expressed as antibody titer representing the reciprocal of the highest dilution of pooled sera required to yield an optical density at 492 (OD_{492}) three times the preimmunization value. IgG isotype-specific ELISAs were performed with IgG1-, IgG2a-, IgG2b-, and IgG3-specific second antibodies (Southern Biotechnology, Birmingham, Ala.).

In vivo Th cell assay. Th cell activity for antibody production was determined by the ability of peptide T-cell sites to prime mice to produce anti-HBc or anti-HBe antibody in vivo after challenge with a suboptimal dose of HBcAg or PC-HBcAg, respectively. Groups of five mice were primed intraperitoneally (i.p.) with 100 μg of peptide in complete Freund's adjuvant (CFA). Control mice were primed with CFA alone. Four weeks after priming, the mice were challenged with a suboptimal concentration of HBcAg (0.05 μg) or of PC-HBeAg (0.5 μg) in incomplete Freund's adjuvant (IFA), and sera were collected for measurement of IgG anti-HBc or anti-HBe 7 and 14 days after challenge.

Adoptive transfer of autoantibody production in HBeAg and HBcAg Tg mice. Non-Tg donor mice were immunized in the hind footpads with 100 μg of p129-140 emulsified in CFA or CFA alone, and draining popliteal lymph node (PLN) cells were harvested after 10 days and cultured with p120-140 (0.2 μg/ml) or purified protein derivative for an additional 3 days. In vitro-activated PLN cells (2×10^7) were then transferred into sublethally irradiated (500 R) HBeAg-expressing or HBcAg-expressing Tg (HBeAg Tg or HBcAg Tg) recipient mice. Sera were collected before and at various times after adoptive transfer and analyzed for anti-HBe and anti-HBc antibodies by ELISA.

In selected experiments, T cells were enriched from the PLN preparation before adoptive transfer, which produced similar results. For convenience and because donor mice were primed with and PLN cells were cultured with peptides that represent T-cell recognition sites but not B-cell sites (26), unselected PLN cells were routinely used to transfer HBc/HBe-specific T-cell help in adoptive transfer experiments.

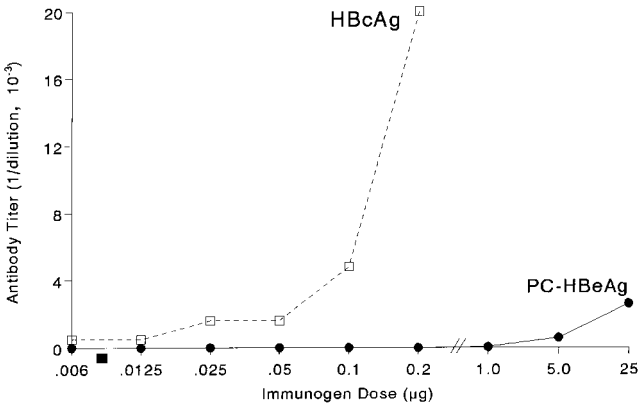

FIG. 1. The particulate HBcAg does not require an adjuvant for immunogenicity. Groups of three (B10 × B10.S)F_1 mice were injected i.p. with various doses of either recombinant HBcAg or PC-HBeAg in saline. Two weeks after immunization, sera were collected, pooled, and analyzed for either IgG anti-HBc or anti-HBe antibodies by ELISA. Antibody titer is expressed as a reciprocal of the highest serum dilution required to yield an OD_{492} value three times that of preimmunization sera. Each data point represents the titer of pooled sera. This experiment was performed on at least three occasions, and the results are representative.

Determination of antigen-specific cytokine production. Groups of mice were immunized i.p. with various antigen preparations either without adjuvant or emulsified in IFA, and 10 to 14 days later, spleen cells were harvested, pooled, and cultured (8×10^6/ml) with various concentrations of a series of antigens. Culture supernatants (SN) were harvested at 24 h for IL-2 determination and at 48 h for IL-4 and IFN-γ determinations. IL-2 was measured by the ability of SN to stimulate proliferation of the IL-2- and IL-4-sensitive NK-A cell line in the presence of MAb 11B11, specific for IL-4 (32). IL-4 was measured by the ability of SN to stimulate proliferation of the IL-4-sensitive CT.4S cell line (generously provided by William Paul, National Institutes of Health, Bethesda, Md.) (16). IFN-γ was measured by two-site ELISA using MAb HB170 and a polyclonal goat anti-mouse IFN-γ (Genzyme Corp., Boston, Mass.). All cytokine experiments were performed at least five times due to variability, and representative data are shown.

Tg autoantibody model and IFN-α treatment. Because HBeAg Tg mice on a B10 (*H-2*^{b}) background (B10-Tg31e) are not completely T-cell tolerant, injection of the synthetic Th cell site p129-140 results in anti-HBe or autoantibody production (25). This Tg model is useful for screening immunomodulatory drugs or therapies. Groups of B10-Tg31e mice were treated either with saline as a control or with various doses of human recombinant IFNα/A.D., a hybrid molecule (specific activity, 7.9×10^7 U/mg) which is fully active in mice. IFN-α/A.D. was obtained from Hoffmann-La Roche (Nutley, N.J.) and was kindly provided by M. J. Brunda. Mice were injected with either 7.5×10^5 or 5.0×10^5 U of IFN-α in 0.2 ml of sterile saline i.p. on days −1, 0, 2, 4, and 6. p129-140 (50 μg in IFA) was injected on day 0. The B10-Tg31e mice were bled on day 14, and total IgG anti-HBe as well as isotype-specific anti-HBe antibody levels were determined by ELISA.

RESULTS

HBcAg is immunogenic in the absence of adjuvants. Previous studies demonstrated that the particulate HBcAg is significantly more immunogenic than the nonparticulate HBeAg in terms of both Th cell recognition and antibody production (26). However, the previous comparisons used either CFA or IFA, and the precore-containing PC-HBeAg was not available for these earlier studies. Therefore, groups of 3 (B10 × B10.S)F_1 mice were immunized with various concentrations (0.006 to 25 μg) of either HBcAg or PC-HBeAg suspended only in saline, and IgG anti-HBc and anti-HBe antibodies were measured by ELISA. As shown in Fig. 1, HBcAg does not require an adjuvant to elicit efficient anti-HBc antibody production. Immunization with as little as 0.006 μg of HBcAg in saline resulted in detectable (1:100) anti-HBc production 2

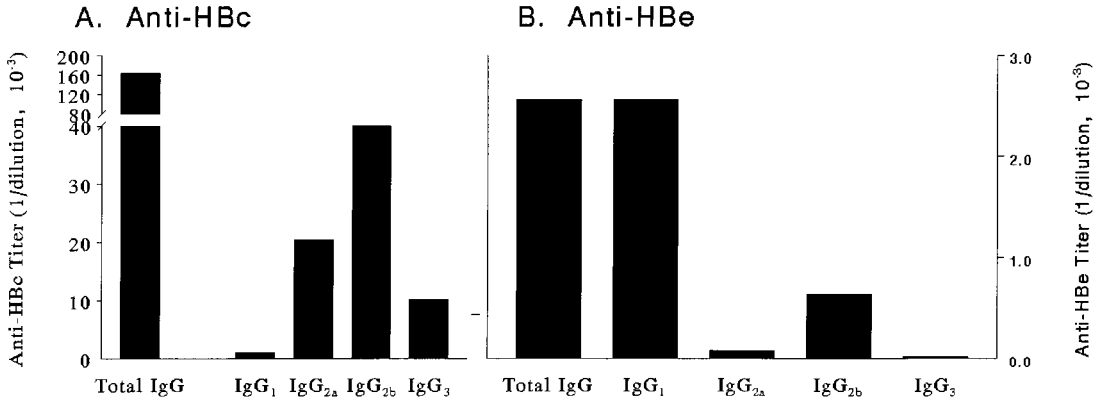

FIG. 2. HBcAg and PC-HBeAg elicit different IgG isotype profiles. Groups of five B10.S mice were injected i.p. with 10 μg of either HBcAg (A) or PC-HBeAg (B) in saline. Two weeks after immunization, sera were collected, pooled, and analyzed for IgG isotype-specific anti-HBc or anti-HBe antibodies by ELISA. IgG1, IgG2a, IgG2b, and IgG3-specific second antibodies were used in the ELISA. Antibody titer is expressed as a reciprocal of the highest serum dilution required to yield an OD_{492} value three times that of preimmunization sera. This experiment was performed twice in B10.S mice and at least once in B10 and (B10 × B10.S)F_1 mice, with similar results.

weeks after a single injection, and an IgG anti-HBc titer of 1:1,600 was achieved with a .025-μg dose of HBcAg (Fig. 1). Increasing the HBcAg dose elicited correspondingly higher anti-HBc titers; for example, 10 μg of HBcAg in saline resulted in an anti-HBc titer of 1:163,840 (data not shown). In contrast, 25 μg of PC-HBeAg in saline was required to elicit significant (1:2,600) anti-HBe antibody production. By this comparison, there is an approximate 1,000-fold difference between the immunogenicities of HBcAg and the PC-HBeAg in terms of antibody production.

Immunizations with HBcAg and PC-HBeAg elicit different IgG isotype profiles. Because injection of HBcAg and HBeAg in saline was immunogenic, it was possible to determine the IgG isotype distribution of anti-HBc and anti-HBe antibodies in the absence of nonspecific adjuvant effects. For this purpose, groups of five B10.S ($H\text{-}2^s$) mice were injected with 10 μg of either HBcAg or PC-HBeAg (i.e., the amount of PC-HBeAg required for immunogenicity) suspended in saline, and total IgG and IgG isotype-specific anti-HBc and anti-HBe antibodies were measured by ELISA (Fig. 2). As expected, injection of identical amounts of HBcAg and HBeAg resulted in significantly greater total IgG anti-HBc compared to anti-HBe antibody production (note the different scales used in Fig. 2A and B). Nonetheless, HBeAg actually elicited more anti-HBe antibody of the IgG1 isotype compared to HBcAg, which elicited no anti-HBc antibodies of the IgG1 isotype. In fact, the IgG1 isotype was predominant in the anti-HBe antibody response, whereas the IgG2b and IgG2a isotypes were predominant in the anti-HBc response. An additional difference was the production of significant IgG3 isotype anti-HBc antibodies and the complete absence of the IgG3 isotype in the anti-HBe response. This experiment was repeated in mice of a different *H-2* genotype (B10, $H\text{-}2^b$), with similar results (data not shown).

To determine if immunization using an adjuvant would alter the signature IgG isotype profiles, groups of five B10.S mice were immunized with either HBcAg (5.0 μg) or PC-HBeAg (10 μg) emulsified in IFA, and total IgG and IgG isotype-specific anti-HBc and anti-HBe antibodies were determined by ELISA (Fig. 3). Use of this oil adjuvant certainly increased the quantities (i.e., titers) of antibodies produced, especially in the anti-HBe response (compare Fig. 2B and 3B). However, the unique IgG isotype patterns which characterize the HBcAg-specific and HBeAg-specific antibody responses were un-

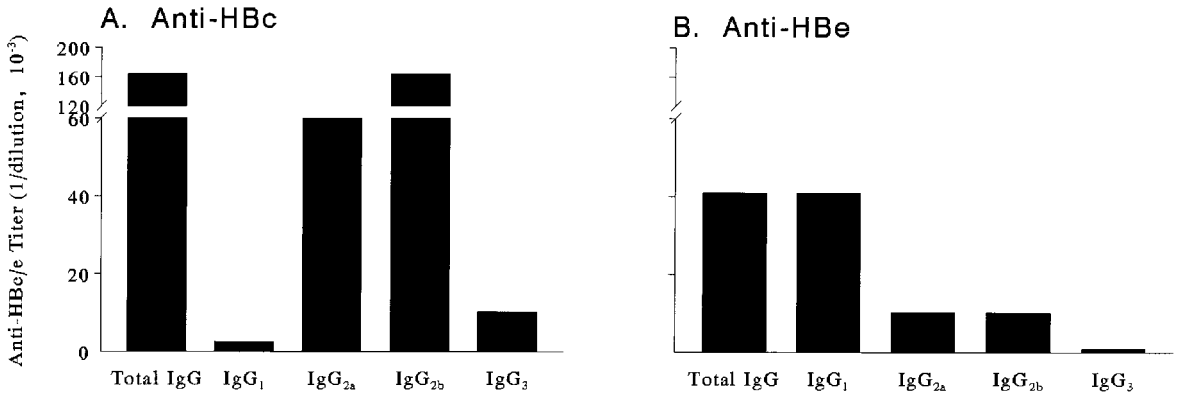

FIG. 3. The use of an oil adjuvant does not alter the IgG isotype profiles elicited by HBcAg and PC-HBeAg. Groups of five B10.S mice were immunized i.p. with either 5 μg of HBcAg emulsified in IFA (A) or 10 μg of PC-HBeAg emulsified in IFA (B). Four weeks after immunization, sera were collected, pooled, and analyzed for IgG isotype-specific anti-HBc or anti-HBe antibodies by ELISA. Antibody titer is expressed as a reciprocal of the highest serum dilution required to yield an OD_{492} value three times that of preimmunization sera. This experiment was performed twice in B10.S mice and at least once in B10 and (B10 × B10.S)F_1 mice, with similar results.

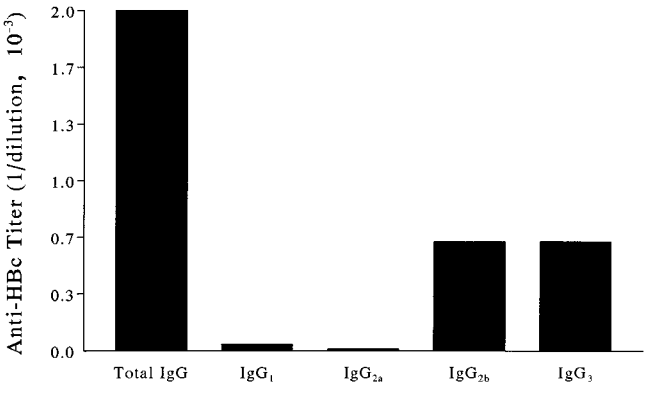

FIG. 4. The HBcAg elicits T-cell-independent IgG isotype production. A group of five BALB/c athymic (*nu/nu*) mice was injected i.p. with 10 μg of HBcAg in saline. Two weeks after immunization, sera were collected, pooled, and analyzed for IgG isotype-specific anti-HBc antibodies by ELISA. Similar injection of BALB/c athymic mice with PC-HBeAg elicited no IgG anti-HBe antibodies (data not shown). Antibody titer is expressed as a reciprocal of the highest serum dilution to yield an OD_{492} value three times that of preimmunization sera. The antibody titers represent pooled sera from five mice. The results are representative of experiments performed on two separate occasions.

changed in the presence or absence of an adjuvant. The IgG isotype hierarchy of the anti-HBc response remained: IgG2b, IgG2a, IgG3, IgG1. Low-level anti-HBc antibody (1:2,560) of the IgG1 isotype was elicited by HBcAg in oil, as opposed to no IgG1 anti-HBc production after immunization with HBcAg in saline. Similarly, the IgG isotype profile of the anti-HBe response was not altered by the use of an oil adjuvant. The IgG1 isotype still dominated the anti-HBe antibody response. The largest effect of the oil adjuvant on the anti-HBe IgG isotype response was the induction of greater amounts of the IgG2a isotype (compare Fig. 3B and 2B).

In summary, the predominance of the IgG1 isotype in the anti-HBe response and the absence of the IgG1 isotype in the anti-HBc response suggests an important influence of IL-4, secreted by the Th_2 cell subset, on the HBeAg-specific antibody response and not on the HBcAg-specific response. Conversely, the anti-HBc response is characterized by efficient IgG2a isotype production, suggesting the prominent influence of IFN-γ, secreted by the Th_1 cell subset, on the HBcAg-specific antibody response.

HBcAg elicits T-cell-independent IgG isotype production. HBcAg, but not HBeAg, can behave as a T-cell-independent antigen (27). Therefore, one method to confirm the impact of T-cell-derived cytokines on the anti-HBc IgG isotype response was to immunize athymic nude mice with HBcAg and measure the IgG isotypes produced. Immunization with 10 μg of HBcAg in saline elicited IgG anti-HBc antibody production in a group of five BALB/c (*nu/nu*) mice (Fig. 4). It is notable that the IgG2b and IgG3 isotypes predominated the anti-HBc antibody response, and virtually no IgG1 or IgG2a anti-HBc antibodies were produced. This result confirms the requirement for HBcAg-specific and, presumably, Th_1 cells in the production of anti-HBc of the IgG2a isotype and suggests T-cell-independent pathways for HBcAg-specific B-cell Ig switching to the IgG2b and IgG3 isotypes.

Direct measurement of Th_1 and Th_2 cytokines produced by HBcAg- and PC-HBeAg-primed T cells. Because the IgG isotype profiles of anti-HBc and anti-HBe antibodies suggested a preferential induction of the Th_2 cell subset by HBeAg and a preferential induction of the Th_1 cell subset by HBcAg, direct measurements of the Th_1 cell cytokines IL-2 and IFN-γ and of the Th_2 cell-derived cytokine IL-4 were performed. Groups of three $(B10 \times B10.S)F_1$ mice were injected with 10 μg of either HBcAg or PC-HBeAg in saline, splenic T cells were cultured with a panel of HBcAg/HBeAg-related antigens in vitro, and culture SN were harvested and assayed for the cytokines IL-2, IFN-γ, and IL-4 (Fig. 5).

Previous studies of comparative murine T-cell proliferative responses elicited by HBcAg and HBeAg revealed that HBcAg was approximately 100-fold more immunogenic than HBeAg (26). Consistent with these earlier results, the most efficient IL-2 and IFN-γ production occurred in HBcAg-primed T cells recalled in vitro with HBcAg. In fact, HBcAg-primed T cells produced approximately three to four times more IFN-γ than PC-HBeAg-primed T cells regardless of the in vitro recall antigen (Fig. 5, middle panels). Immunization with PC-HBeAg elicited antigen-specific IL-2 and IFN-γ production but at levels significantly lower than those elicited by HBcAg immunization. In contrast, PC-HBeAg immunization elicited greater or equal levels of IL-4 production regardless of the in vitro recall antigen compared to immunization with HBcAg (Fig. 5, right panels). Furthermore, when IFN-γ present in the in vitro cultures was neutralized with anti-IFN-γ (1 μg/ml), the amount of detectable IL-4 was significantly greater, especially in the cultures containing T cells derived from PC-HBeAg-primed mice (IL-4 levels in PC-HBeAg-primed T-cell cultures were two- to sixfold higher than in HBcAg-primed T-cell cultures regardless of the in vitro recall antigen). In summary, although both HBcAg and PC-HBeAg are capable of priming in vivo and recalling in vitro a mixture of Th_1-like and Th_2-like cytokine responses, HBcAg preferentially primes Th_1-like cells and PC-HBeAg preferentially primes Th_2-like cells, as determined by in vitro cytokine production. Although the ability of HBcAg or PC-HBeAg to prime a given cytokine response in vivo generally correlated with its ability to recall that same cytokine response in vitro, the final balance between Th_1- and Th_2-like activity may be influenced by cross-regulation. For example, note that PC-HBeAg-primed T cells actually produced higher levels of IL-4 when cultured with HBcAg than when cultured with PC-HBeAg when the IFN-γ was neutralized in vitro by anti-IFN-γ.

It is notable that of the related structural forms of HBcAg and HBeAg (i.e., particulate, HBcAg; nonparticulate, PC-HBeAg; the subunit polypeptide, P16; and the peptide, p120-140) only HBcAg elicited vigorous IFN-γ production from primed splenic Th cells. To determine the effect of this preferential IFN-γ induction on IgG isotype, $(B10 \times B10.S)F_1$ mice were immunized with the various structural forms of HBcAg and HBeAg, and antibody IgG isotype production was determined for each antigen (Table 1). When the nonparticulate structural forms of HBcAg and HBeAg, including PC-HBeAg, P16, and p120-140, were used as immunogens, the predominant IgG isotype produced was IgG1. Lesser amounts of IgG2b and IgG2a antibodies were elicited by PC-HBeAg and P16, and no IgG3 antibodies were produced. The peptide immunogen elicited only IgG1 anti-p120-140 antibodies. As noted previously, HBcAg elicited significant levels of IgG2b and IgG2a antibodies, a low IgG3 anti-HBc titer, and absolutely no IgG1 anti-HBc antibodies (Table 1). The production of high levels of anti-HBc antibodies of the IgG2a isotype is consistent with the secretion of IFN-γ by Th_1-like cells induced by HBcAg immunization (Fig. 5). Furthermore, the absence of the IgG1 isotype in the anti-HBc antibody response suggests that in vivo levels of IFN-γ are sufficient to inhibit IL-4 secretion necessary for IgG1 anti-HBc antibody production.

Distinguishing between Th cell priming and Th-B cell interaction as the primary site of IgG isotype regulation. Th cells

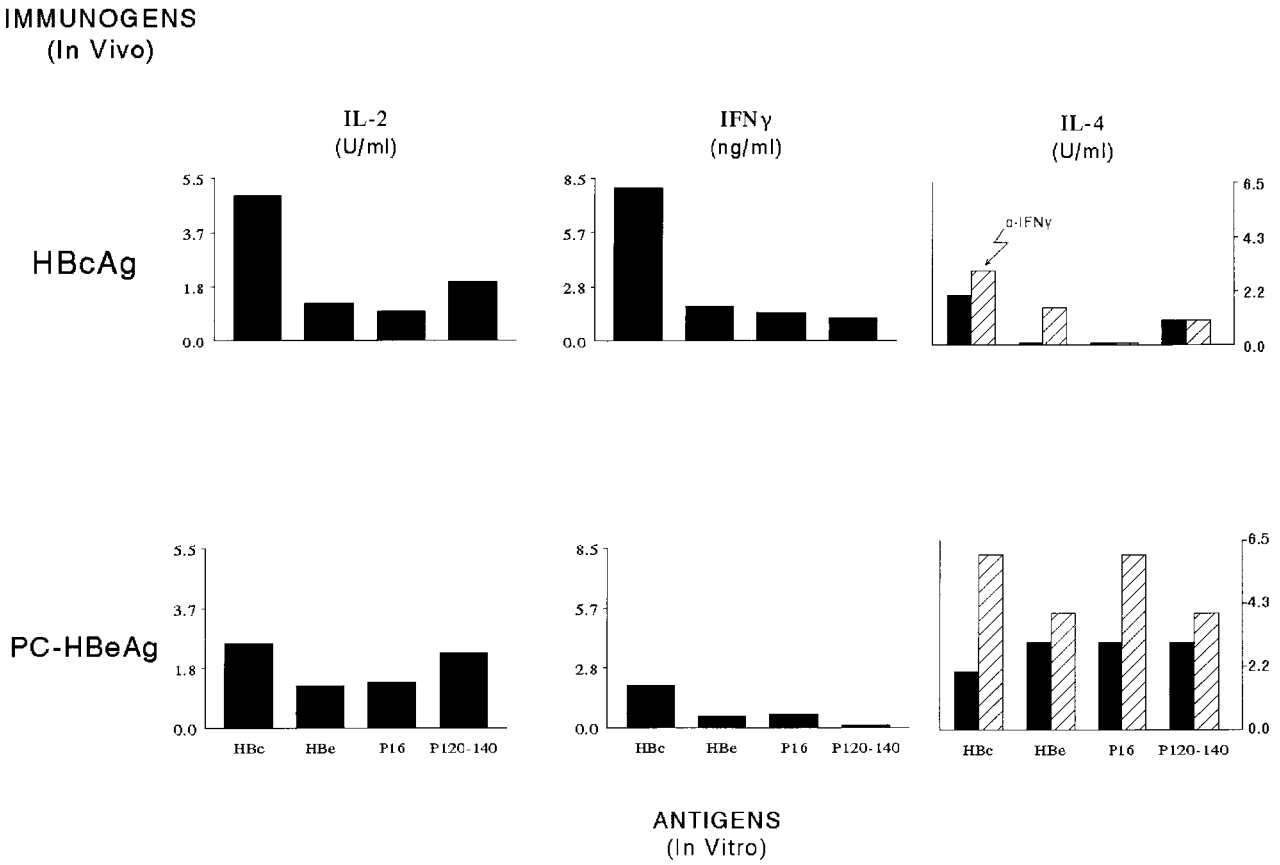

FIG. 5. HBcAg and PC-HBeAg elicit different patterns of T-cell cytokine production. Groups of three (B10 × B10.S)F_1 mice were injected i.p. with 10 μg of either HBcAg or PC-HBeAg in saline. Two weeks later, spleen cells were harvested, pooled, and cultured (8×10^6/ml) in vitro with various concentrations of the indicated antigens (i.e., HBcAg [HBc], PC-HBeAg [HBe], the HBc/HBe structural subunit polypeptide P16, and the synthetic peptide p120-140). Culture SN were collected at 24 h for IL-2 determinations and at 48 h for IFN-γ and IL-4 determination. Absolute levels of cytokine are shown in units per milliliter for IL-2 and IL-4 and in nanograms per milliliter for IFN-γ. Although various concentrations of antigens were used in vitro, only the results of assays using the optimal concentrations of 5 μg of HBcAg per ml and 25 μg of the other antigens per ml are shown. Anti-IFN-γ antibodies (1 μg/ml) were added during the in vitro culture for IL-4 determinations (hatched bars). Similar addition of anti-IL-4 antibodies to the IFN-γ cultures had no effect on IFN-γ production (data not shown). This analysis was performed on at least three occasions in (B10 × B10.S)F_1 mice and at least once in B10 and B10.S mice, and the data shown are representative.

regulate Ig isotype switching by virtue of the cytokines that they release (10). However, at least two levels of Th cell regulation may exist. For example, does the Th cell priming event determine the Th_1-Th_2 cell subset balance, which in turn will determine the IgG isotype pattern, or does regulation of the IgG isotype profile occur at the level of Th_1 or Th_2 cell interaction with an antigen-specific B cell? This second level of regulation could be mediated through preferential pairing of a given specificity of B cell with either Th_1- or Th_2-like cells. To address this question, we attempted to separate the Th cell priming event from Th-B cell interaction in vivo. This was accomplished by priming with either a peptide Th cell recognition site, p120-131, which has previously been defined as a Th_1-like epitope, or a peptide which preferentially elicits HBcAg/HBeAg-specific Th_2-like cells, p129-140 (28). Because p120-131 is IA^s restricted and p129-140 is IA^b restricted, (B10 × B10.S)F_1 mice were used for this experiment. After Th cell priming with peptide, mice were rested for 4 weeks and then challenged with either HBcAg (0.05 μg) or PC-HBeAg (0.5 μg) emulsified in IFA and the primary (7-day) anti-HBc or anti-HBe, respectively; IgG isotype profiles were determined by ELISA. As shown in Fig. 6, priming with the Th_1-like peptide p120-131 did, indeed, result in a Th_1-like IgG isotype profile after HBcAg challenge (Fig. 6, upper panel). For example, no IgG1 anti-HBc was induced, and significant IgG2a anti-HBc antibody was produced. However, priming with the Th_2-like peptide, p129-140, elicited the same Th_1-like IgG isotype profile after challenge with HBcAg as did p120-131. Therefore, in this instance HBcAg used as the challenge antigen appeared to influence the IgG isotype pattern to a greater extent than the peptide used for Th_1 or Th_2 cell priming. Similarly, when PC-HBeAg was used as the challenge antigen,

TABLE 1. Influence of antigen structure on IgG isotype[a]

Immunogen	Dose	Antibody titer (1/dilution)			
		IgG1	IgG2a	IgG2b	IgG3
HBcAg	5 μg, 1°	0	40,960	81,920	640
PC-HBeAg	8 μg, 2°	40,960	2,560	10,240	0
P16	20 μg, 2°	5,120	80	1,280	0
p120-140	50 μg, 1°	2,560	0	0	0

[a] Groups of three (B10 × B10.S)F_1 mice were immunized with a single optimum dose (1°) of HBcAg (5 μg) or p120-140 (50 μg) emulsified in IFA or immunized and boosted (2°) with PC-HBeAg (8 μg) or P16 (20 μg) emulsified in IFA. Two weeks after the last injection, sera were collected and pooled, and the IgG isotype-specific antibody titer specific for the immunizing antigen was determined by ELISA.

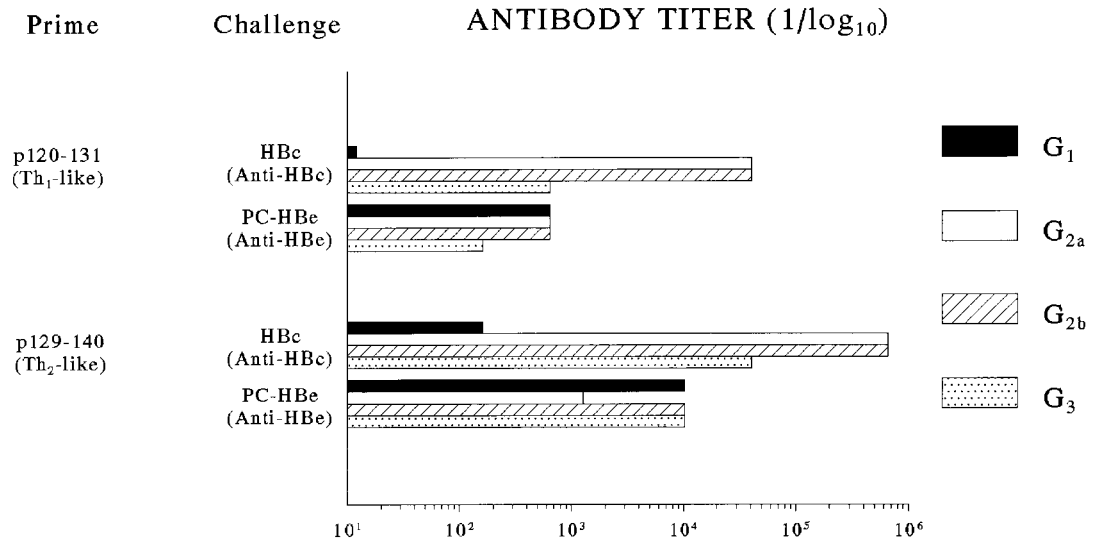

FIG. 6. Effect of Th subset peptide priming on the HBcAg- and PC-HBeAg-specific humoral immune responses. Groups of five (B10 × B10.S)F_1 mice were primed i.p. with 100 μg of either p120-131 or p129-140 in CFA or with CFA alone (not shown). Four weeks later, primed mice were challenged with a suboptimal concentration of HBcAg (0.05 μg) or PC-HBeAg (0.5 μg) in IFA, and sera were collected, pooled, and analyzed for IgG isotype-specific anti-HBc or anti-HBe antibodies, respectively, 7 days after the challenge. Antibody titers, expressed as a reciprocal of the highest serum dilution from pooled sera to yield three times the OD_{492} value of prechallenge sera, were determined by ELISA and corrected for background antibody produced in the absence of peptide priming.

significant levels of the IgG1 isotype of anti-HBe antibodies were produced regardless of the peptide used for priming the Th cell subset. Again, this result indicated that the IgG isotype pattern was being regulated at the level of Th-B cell interaction, suggesting that HBcAg-specific B cells may preferentially pair with Th_1-like cells and PC-HBeAg-specific B cells may preferentially pair with Th_2-like cells. However, only a preference and not an absolute pairing of B cell-Th cell subsets is indicated by these data, since all IgG isotypes are produced to some extent with the exception of anti-HBc of the IgG1 isotype in the context of p120-131 priming and HBcAg challenge. In fact, the heterogeneity of IgG isotypes is much greater in the context of the Th cell peptide priming experiment than when HBcAg or PC-HBeAg is used alone as the immunogen (Fig. 2). This result suggests that priming Th cells with peptide T-cell recognition sites prior to challenge with intact native antigen may broaden the spectrum of Th cell subsets involved in the response, which may have useful clinical applications.

HBcAg and HBeAg Tg mice produce different IgG isotype patterns of autoantibody when adoptively transferred with the same Th cell population. Another method for examining the site of IgG isotype regulation for HBcAg and HBeAg was to adoptively transfer the same Th cell population (i.e., p129-140-primed Th cells) into either HBcAg or HBeAg Tg mice and determine the IgG isotype distribution of anti-HBc or anti-HBe antibodies, respectively. One advantage of this system is that recombinant HBcAg or PC-HBeAg was not necessary since endogenous HBcAg or HBeAg serves as the only source of antigen. This eliminates any possibility that bacterial contaminants (e.g., endotoxin) may influence the IgG isotype profile. Furthermore, the anti-HBc and anti-HBe antibodies produced after Th cell transfer can be considered autoantibody at least in terms of the B-cell compartment. As shown in Fig. 7, HBeAg Tg recipients (F_1-Tg31e) adoptively transferred with p129-140-primed Th cells from a non-Tg donor produced high-titer anti-HBe autoantibody (1:40,960), which was almost exclusively of the IgG1 isotype. In contrast, adoptive transfer of p129-140-primed Th cells from the same non-Tg donor into HBcAg Tg recipients (F_1-Tg10c) resulted in anti-HBc autoantibody production predominantly of the IgG2b and IgG2a isotypes, with only low-level IgG1 anti-HBc antibody production (Fig. 7). Adoptive transfer of CFA-primed Th cells elicited no autoantibody production in F_1-Tg31e or F_1-Tg10c recipients (data not shown).

As previously stated, the p129-140 T-cell site primes predominantly, but not exclusively, Th_2-like cells (28). Therefore, it is especially notable that p129-140-primed Th cells transferred into HBcAg Tg mice elicited a Th_1-like pattern of anti-HBc antibodies. Interestingly, the HBcAg present in Tg10c mice does degrade to some extent and can convert to nonparticulate HBeAg in vivo (30). The IgG isotype analysis of anti-HBe autoantibody produced in F_1-Tg10c mice after adoptive transfer of p129-140-primed Th cells revealed a predominant IgG1 anti-HBe antibody response (data not shown). These results emphasize the strong influence of antigen structure on IgG isotype regulation and suggest that this regulation may occur at the level of Th-B cell interaction.

Effect of IFN-α on the anti-HBe IgG isotype profile in HBeAg Tg mice. Because immunization with HBeAg preferentially elicits a Th_2-like response, which may be relevant to viral persistence in chronic HBV infection, we examined the ability of IFN-α to modulate Th cell subset selection during HBeAg/anti-HBe seroconversion in B10-Tg31e mice, which can serve as an immunologic model of HBeAg-positive, chronic HBV infection. IFN-α was chosen because it currently represents the most effective treatment for chronic HBV infection.

Due to incomplete Th cell tolerance to HBeAg in Tg31e mice on an *H-2*^{b} background, B10-Tg31e mice injected with p129-140 seroconvert from HBeAg-positive to HBeAg-negative status and produce high levels of anti-HBe autoantibody (reference 25 and Fig. 8). As noted previously, the anti-HBe autoantibody is almost exclusively comprised of the IgG1 isotype, consistent with an HBeAg-specific Th_2 cell-mediated response (25, 31). However, in vivo treatment with IFN-α significantly inhibited anti-HBe seroconversion and broadened the IgG isotype profile to include IgG2a and IgG2b as well as IgG1 anti-HBe antibody production (Fig. 8). Further studies will be

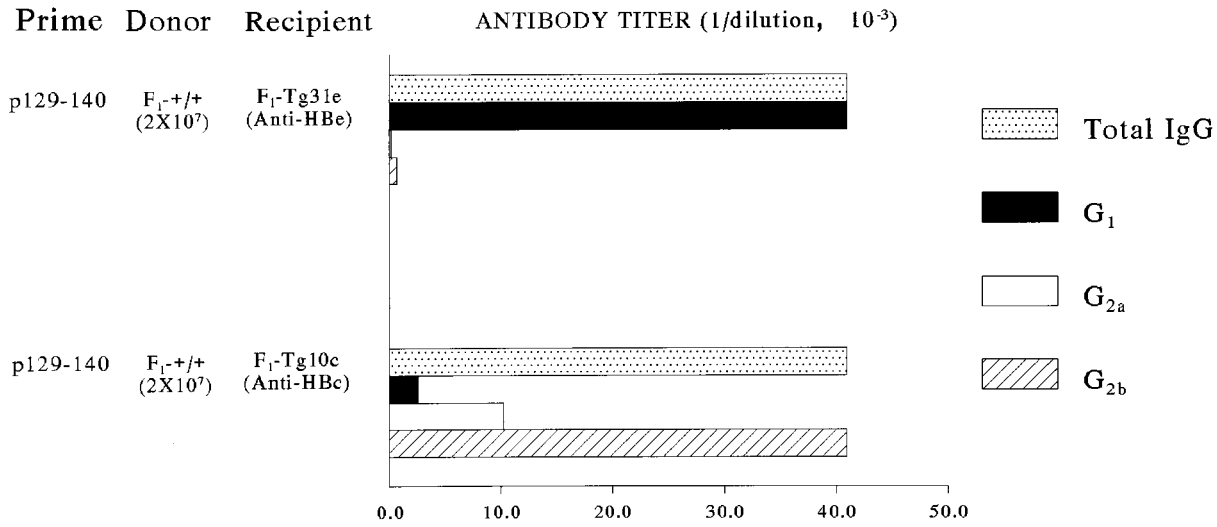

FIG. 7. Adoptive transfers of the same Th cell population yield different IgG isotype profiles in HBeAg and HBcAg Tg recipients. Nontransgenic (B10 × B10.S)F_1 donor mice (F_1 − +/+) were primed in the hind footpads with 100 μg of the synthetic Th cell site p129-140 emulsified in CFA or with CFA alone. Draining PLN cells were harvested and cultured in vitro with either purified protein derivative as a CFA control or the HBc/HBeAg-derived peptide p120-140 (0.2 μg/ml) for 3 days. In vitro-activated Th cells (2×10^7) were then transferred into either five sublethally irradiated (500 R) HBeAg Tg (F_1-Tg31e) or HBcAg Tg (F_1-Tg10c) recipient mice. Sera were collected before transfer and 6 weeks after adoptive transfer and analyzed for IgG isotype-specific anti-HBe or anti-HBc antibodies by ELISA. Antibody titers are expressed as a reciprocal of the highest serum dilution from pooled sera to yield an OD_{492} value three times that of preadoptive transfer sera. Transfer of CFA-only-primed Th cells into F_1-Tg31e or F_1-Tg10c recipient mice did not elicit antibody production (not shown). This experiment was performed on two separate occasions, and the results are representative.

required to confirm that the IFN-α therapy affected HBeAg-specific Th cells independently of a direct effect on HBeAg-specific B cells. However, the serologic analysis certainly suggests that IFN-α treatment is capable of shifting the HBeAg-specific Th_1-Th_2 cell balance from Th_2 cell predominance to an approximately equally mixed Th_1-Th_2 cell response.

DISCUSSION

The purpose of this study was to determine if the structure of the HBV nucleoproteins could influence Th cell subset development. The particulate HBcAg and the nonparticulate PC-HBeAg were compared in terms of immunogenicity, requirement for adjuvant, IgG isotype production, and Th cell

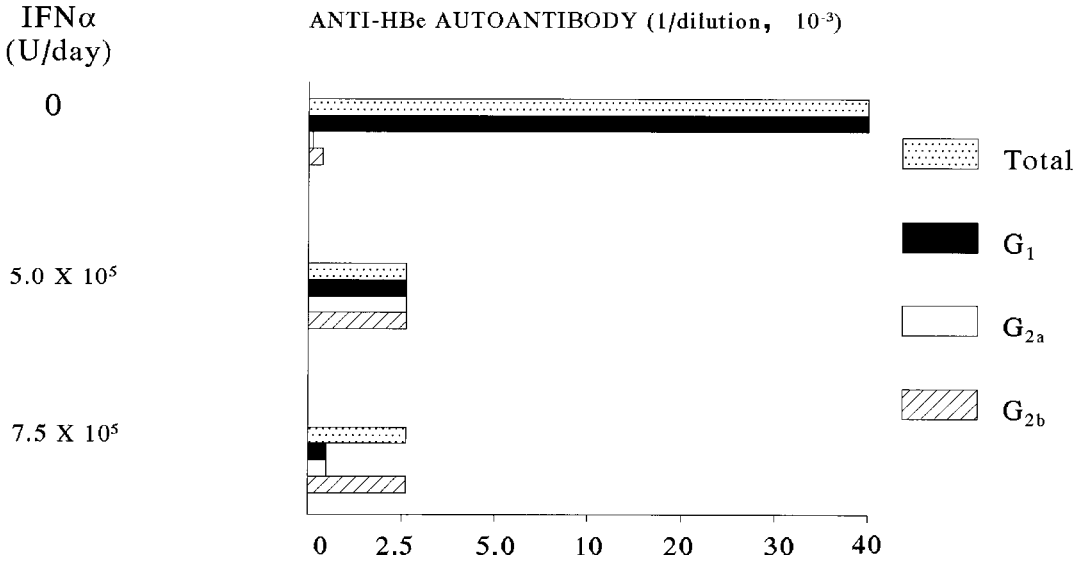

FIG. 8. In vivo treatment with IFN-α suppresses anti-HBe autoantibody production in B10-Tg31e mice and modulates the IgG isotype profile. Groups of five B10-Tg31e mice each were treated with either sterile saline, 7.5×10^5 U of recombinant IFN-α/A.D. per day, or 5.0×10^5 U of IFN-α/A.D. per day injected i.p. in 0.2 ml of sterile saline on days −1, 0, 2, 4, and 6. The HBc/HBeAg-derived T-cell site p129-140 (50 μg in IFA) was injected on day 0. The B10-Tg31e mice were bled on day 14, and isotype-specific anti-HBe autoantibody was determined by ELISA. Autoantibody titer is expressed as a reciprocal of the highest serum dilution from pooled sera required to yield an OD_{492} value three times that of preimmunization sera.

TABLE 2. Comparison of immune responses to HBcAg and PC-HBeAg

Feature	HBcAg	PC-HBeAg
Structure	Particle	Nonparticle
Antibody response	High	Low
Adjuvant required	No	Yes
T-cell dependent	No	Yes (27)
Dominant IgG isotype		
T-cell dependent	IgG2a, IgG2b	IgG1
T-cell independent	IgG2b, IgG3	None
Th cell response		
T-cell proliferation	High (100×)	Low (26)
Dominant cytokine	IFN-γ	IL-4
Dominant Th subset	Th_1-like	TH_0/Th_2-like

cytokine induction. The results of this comparison are summarized in Table 2. Previous studies demonstrated that HBcAg as an immunogen was superior to PC-HBeAg in terms of both antibody production and Th cell induction (26) and that HBcAg could function as a T-cell-independent antigen (27). The current study elucidated additional differences between the immune responses to HBcAg and PC-HBeAg. For example, immunization with HBcAg, in contrast to immunization with PC-HBeAg, did not require an adjuvant to yield a significant antibody response. More notably, immunizations with HBcAg and PC-HBeAg elicited anti-HBc and anti-HBe antibodies which differed characteristically in terms of IgG isotype. Immunization with PC-HBeAg in saline resulted in a predominantly IgG1-restricted primary anti-HBe response, with only low levels of IgG2b and IgG2a produced and no IgG3 anti-HBe production. In contrast, immunization with HBcAg in saline resulted in production of high levels of IgG2b and IgG2a anti-HBc antibodies, intermediate levels IgG3 anti-HBc antibodies, and no IgG1 anti-HBc. These unique HBcAg- and PC-HBeAg-specific IgG isotype profiles persisted even when an oil adjuvant was used. Interestingly, immunization of athymic mice with HBcAg elicited both IgG2b and IgG3 anti-HBc production, suggesting T-cell-independent mechanisms for IgG2b and IgG3 isotype switching. Previous studies have documented T-cell-independent IgG3 induction mediated by non-T-cell-derived IFN-γ (42) and transforming growth factor β (TGF-β)-mediated induction of IgG2b secretion by lipopolysaccharide-activated B cells (21).

It is notable that the dominant IgG isotype elicited by the HBcAg in athymic as well as in euthymic mice and in the context of all immunization schemes was IgG2b. In mice, the only cytokine known to regulate B-cell IgG2b switching is TGF-β (21). In human B cells, TGF-β is thought to influence IgA production (7, 43). Surprisingly high levels of IgA anti-HBc antibodies are produced during HBV infection (34, 36). TGF-β is generally believed to be a potent anti-inflammatory cytokine (2, 6, 38). If significant amounts of TGF-β are produced in response to HBcAg during a chronic HBV infection, its anti-inflammatory effects may down-regulate viral clearance mechanisms and favor persistence. However, recent evidence suggests that TGF-β may act as an inflammatory cytokine in the context of greater amounts of IL-2 and may actually enhance Th_1-like activity (13, 32, 44). In any event, the mechanism underlying high levels of IgG2b and IgA anti-HBc antibody production in mice and humans, respectively, deserves further study. Setting aside the non-T-cell influences apparent in IgG isotype production to HBcAg, the main distinction between HBcAg and PC-HBeAg is that HBcAg elicits primarily IgG2a anti-HBc antibody production and PC-HBeAg elicits primarily IgG1 anti-HBe antibody production. In the mouse, IgG1 isotype switching is influenced by the Th_2 cell cytokine IL-4, and IgG2a isotype switching is regulated by the Th_1 cell cytokine IFN-γ (10).

Because the IgG isotype patterns of anti-HBc and anti-HBe antibodies suggested a preferential induction of Th_1-like cells by HBcAg and a preferential induction of Th_2-like cells by PC-HBeAg, HBc/HBeAg-specific in vitro cytokine production was examined. When HBcAg was used as the immunogen, the highest levels of IFN-γ were produced, especially by spleen cells cultured with HBcAg in vitro, although both PC-HBeAg and P16 were also able to recall IFN-γ production by HBcAg-primed spleen cells. Note that peptide antigen recalled little or no IFN-γ production. Priming with PC-HBeAg also elicited IFN-γ production, which was recalled to the greatest degree by HBcAg in vitro; however, spleen cell production of IFN-γ by PC-HBeAg-primed mice was always inferior to HBcAg priming. These results suggest that at both the level of Th cell priming in vivo and the level of antigen recall in vitro, HBcAg elicits IFN-γ production to a greater extent than PC-HBeAg. In contrast, immunization with HBcAg elicits relatively weak IL-4 production compared to PC-HBeAg immunization. This difference in IL-4 production became more obvious when anti-IFN-γ antibody was added to the in vitro antigen cultures in order to neutralize endogenous IFN-γ. The IL-4 levels in culture supernatants of PC-HBeAg-primed spleen cells were significantly increased in the presence of anti-IFN-γ, whereas IL-4 production in HBcAg-primed spleen cell cultures was not or was only minimally increased by anti-IFN-γ. This finding indicates that both IFN-γ and IL-4 were being produced in the PC-HBeAg-primed spleen cultures, whereas IFN-γ was clearly predominant in the HBcAg-primed spleen cultures, with little IL-4 production. The results obtained from measuring HBc/HBeAg-specific in vitro cytokine production are consistent with the IgG isotype profile data. These results indicate that HBcAg and PC-HBeAg preferentially, but not exclusively, induce different Th cell subsets. Immunization with HBcAg preferentially elicits Th_1-like cells, and PC-HBeAg preferentially elicits Th_0 or Th_2-like cells. However, the most dramatic difference is the relatively inefficient IL-4 production elicited by HBcAg immunization.

In the mouse strain combinations [i.e., $(B10.S \times B10)F_1$] used for these studies, HBcAg and the PC-HBeAg are totally cross-reactive at the level of Th cell recognition since the dominant Th cell sites (i.e., 120-131 in B10.S mice and 129-140 in B10 mice) are present in both antigens. Therefore, this study demonstrates that Th cells with the same specificity can belong to different Th cell subsets and can produce different cytokine responses. This finding is not unique in that similar results were obtained in studies using monoclonal Th cells, derived from T-cell receptor Tg mice, which were induced to differentiate along a Th_1 or Th_2 cell pathway dependent on the cytokine milieu during activation (15). What is novel is the finding that the structure of the protein housing the Th cell site can influence Th cell subset selection. The particulate structure (HBcAg) preferentially induced a Th_1-like response, and the nonparticulate forms of the antigen (PC-HBeAg, P16, and peptide) appeared to preferentially induce Th_2-like cells. In experiments designed to separate the Th cell priming event from Th-B cell interactions, the structure of the antigen available at the time of Th-B cell interaction appeared to have more influence on the IgG isotype profile than the method used to prime the Th cells. For example, peptide-primed Th cells transferred into HBeAg Tg mice elicited anti-HBe antibodies

composed almost exclusively of the IgG1 isotype, whereas the same Th cell population transferred into HBcAg Tg recipients resulted in an IgG2a- and IgG2b-dominated anti-HBc response (Fig. 7). This result suggests the possibility that HBcAg-specific B cells preferentially interact with Th_1-like cells and PC-HBeAg-specific B cells preferentially interact with Th_2-like cells. An alternative interpretation is that peptide-primed Th cells may initiate B-cell expansion, and thereafter, HBcAg-specific B cells may act as a primary source of antigen-presenting cells (APCs) for naive HBcAg-specific Th_1-like cells. Conversely, PC-HBeAg-specific B cells acting as a primary source of APCs may preferentially prime PC-HBeAg-specific Th_2-like cells or may not be able to act as APCs for PC-HBeAg at all. While the ability of antigen-specific B cells to act as primary APCs for naive Th cells remains controversial (1, 8, 11, 17), we have preliminary evidence that HBcAg can be presented to HBcAg/HBeAg-specific Th cell hybridomas significantly more efficiently by B-cell APCs than can PC-HBeAg. It may well be that the enhanced immunogenicity of HBcAg as well as the unique isotype profile induced by HBcAg immunization is due to efficient B-cell APC function for this particulate antigen (21a). Indeed, it may appear contradictory that HBcAg elicits very high levels of anti-HBc antibody production yet primarily induces Th_1-like cells. This may be explained by a unique antigen presentation pathway for HBcAg or by the non-Th cell influences on anti-HBc antibody production discussed previously.

Whatever the mechanism, the observation that the two forms of the HBV nucleoprotein can preferentially elicit different Th cell subsets with the same antigen specificity may have profound implications in terms of the mechanisms of viral persistence and immunopathogenesis. Although preferential Th cell subset selection was shown after primary immunization with HBcAg and PC-HBeAg, the adoptive transfer experiments into HBcAg and HBeAg Tg mice extend this observation to a situation in which these antigens are constitutively produced, similar to the case in chronic HBV infection. A significant number of recent studies have elucidated the importance of the T-cell response to the HBV nucleoproteins in terms of viral clearance. Clearance of this noncytopathic virus is believed to be immune system mediated as is the accompanying liver injury. Previous serologic studies suggested that the balance between HBcAg/HBeAg-specific Th_1 and Th_2-like cells may influence either the induction or maintenance of chronicity. For example, dominance of HBcAg/HBeAg-specific Th_1-like cells may favor an acute or resolving infection, whereas dominance of Th_2-like cells may promote HBV persistence (19, 20). Furthermore, a recent study of cytokine production in liver-derived Th cells suggested that Th_1-like cytokines were associated with increasing hepatitis activity and Th_2-like cytokines were associated with decreasing liver disease during chronic HBV infection (12). These findings raise the question of how a Th_1-Th_2 cell imbalance may occur in the context of an HBV infection. In a murine Tg system, it was shown that the HBeAg can act as a tolerogen in utero by crossing the placenta (23). Subsequent experiments demonstrated that HBeAg-specific Th_2-like cells can preferentially survive tolerance induction to a greater extent than HBeAg-specific Th_1-like cells (29). Therefore, chronicity resulting from vertical transmission of HBV may be characterized by a predominance of HBeAg-specific Th_2-like cells. The results of the current study suggest another pathway by which the HBcAg/HBeAg-specific Th_1-Th_2 cell balance may be modulated. If HBcAg preferentially induces Th_1-like cell activity and the secretion of IL-2, IFN-γ, and tumor necrosis factor during an HBV infection, then it follows that inflammation, cytotoxic T-lymphocyte induction, liver injury, and inhibition of viral replication would characterize the HBcAg-specific T-cell response. Conversely, secretion of the anti-inflammatory cytokines IL-4, IL-5, and IL-10, enhanced antibody production, and viral persistence would characterize the HBeAg-specific T-cell response if HBeAg preferentially elicits Th_2-like cells. Because both HBcAg and HBeAg are produced during a wild-type HBV infection and because the Th_1 and Th_2 subsets are cross-regulatory (15, 41), a dynamic balance between the HBcAg/HBeAg-specific Th_1 and Th_2 subsets may exist. If so, alterations in the Th_1-Th_2 cell balance would be expected to influence the course of the infection in terms of both liver injury and persistence. Interestingly, emergence of an HBeAg-negative HBV mutant during the chronic active phase often results in an exacerbation of liver injury and a worse prognosis (3). Loss of the circulating HBeAg would, predictably, decrease the induction of HBeAg-specific Th_2 cell activity and could result in a predominance of inflammatory Th_1-like cells. Similarly, adult infection with the HBeAg-negative mutant HBV can be associated with fulminant hepatitis (4, 18, 35). Again, the absence of an anti-inflammatory HBeAg-specific Th_2-like response may increase the risk of an unchecked and overly aggressive HBcAg/HBeAg-specific Th_1 cell-dominated response. In fact, recent experiments in HBeAg, HBcAg, and dual HBeAg/HBcAg Tg mice indicate that circulating HBeAg can actually modulate the immune response to the HBcAg by altering the HBcAg/HBeAg-specific Th_1-Th_2 cell balance (21a). Therefore, the evolutionary conservation of HBeAg secretion may represent a viral strategy to maintain a long-term infection without eliciting an overly destructive immune response which would eliminate the virus and/or kill the host.

It is interesting that IFN-α, which is the most effective treatment for chronic HBV infection, appears capable of altering the HBeAg-specific Th_1-Th_2 cell balance toward the Th_1 subset at least in a Tg model of HBeAg/anti-HBe seroconversion. Indeed, the ability of IFN-α to alter the Th cell phenotype (9) may be at least one reason why IFN-α is beneficial in the treatment of chronic HBV infection. In a previous study, we demonstrated that IL-12 treatment in vivo was also capable of skewing HBeAg-specific Th_2 cells toward the Th_1 subset, and we suggested that this cytokine may be useful for therapeutic purposes (31). A previous study also indicated that the major histocompatibility complex genotype of the host and the specificity of the Th cell site recognized can effect HBcAg/HBeAg-specific Th cell subset selection (28). These findings indicate that differentiation of HBcAg/HBeAg-specific Th cell subsets even in a noninfectious system is a complex process. Therefore, the influence of HBcAg or PC-HBeAg structure on Th cell subset development should not be viewed as absolute and can be altered by factors such as the cytokine milieu, which may provide new opportunities for treatment of chronic HBV infection.

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