The Secreted Hepatitis B Precore Antigen Can Modulate the Immune Response to the Nucleocapsid: A Mechanism for Persistence

David R. Milich, Margaret K. Chen, Janice L. Hughes and Joyce E. Jones

http://www.jimmunol.org/content/160/4/2013

Why The JI?

• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Speedy Publication! 4 weeks from acceptance to publication

References
This article cites 47 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/160/4/2013.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Secreted Hepatitis B Precore Antigen Can Modulate the Immune Response to the Nucleocapsid: A Mechanism for Persistence

David R. Milich, Margaret K. Chen, Janice L. Hughes, and Joyce E. Jones

The hepatitis B precore Ag (HBeAg) is a secreted nonparticulate version of the viral nucleocapsid hepatitis B core Ag (HBcAg), and its function is unknown. A proportion of HBeAg-specific Th cells evade deletion/anoergy in HBeAg-transgenic (Tg) mice and mediate anti-HBe “autoantibody” (autoAb) production after in vivo activation with the appropriate Th cell peptide. This model system was used to determine how secretory HBeAg may effect deletion of Th cells in the periphery. For this purpose, HBeAg-Tg mice were bred with Fas and Fas ligand (FasL)-defective lpr/lpr and gld/gld mutant mice. Fas-FasL interactions mediate activation-induced apoptosis in the periphery. In HBeAg-Tg/+ mice, high-titered anti-HBe autoAb was produced that was exclusively composed of the IgG1 isotype (i.e., Th2-like profile). In contrast, HBeAg-Tg/lpr and HBeAg-Tg/gld mice produced significantly less anti-HBe autoAb, and the IgG isotype patterns were broadened to include IgG2a, IgG2b and IgG3 as well as IgG1 (i.e., mixed Th1/Th2-like profile). These results suggest that HBeAg-specific Th cells are preferentially depleted by Fas-FasL-mediated interactions. The effect of circulating HBeAg on HBeAg-specific Th1 cells was also examined by transferring HBeAg/HBeAg-specific Th cells into dual HBeAg- and HBcAg-expressing Tg recipient mice. The presence of serum HBeAg ablated the expected Th1-mediated anti-HBe Ab response and shifted it toward a Th2 phenotype. These results suggest that in the context of a hepatitis B viral infection, circulating HBeAg has the potential to preferentially deplete inflammatory HBeAg- and HBcAg-specific Th1 cells that are necessary for viral clearance, thereby promoting hepatitis B virus persistence.


A unique feature of the hepatitis B virus (HBV) is the production of a secreted, nonparticulate form of the nucleoprotein designated hepatitis B precore Ag (HBeAg). The nucleocapsid, known as hepatitis B core Ag (HBcAg) is expressed as an intracellular protein that self-assembles to form particles that encapsulate the viral genome and polymerase and are essential to the maturation of the virion. In contrast, the function of secretory HBcAg in the viral life cycle is unknown inasmuch as it is not required either for infection or replication (1–3). We and others have proposed that the HBcAg may have an immunoregulatory function in promoting viral persistence (4–6). For example, in HBcAg-expressing transgenic (Tg) mice it was demonstrated that the HBcAg can cross the placenta in non-Tg littermates and establish Th cell tolerance specific for HBcAg and HBeAg, which are largely cross-reactive at the level of Th cell recognition (5). It was proposed that such a mechanism may be responsible for the high chronicity rates (~90%) (7, 8) observed in babies infected perinatally by their HBeAg-positive mothers (4). In fact, perinatal infection with an HBeAg-negative variant of the HBV results in acute rather than chronic infection (9). A similar finding has been reported in the woodchuck model of chronic infection (10). Therefore, conservation of secretion of the HBeAg may represent a viral strategy to guarantee persistence during vertical transmission of HBV, which is the major source of chronic infection in endemic areas.

However, HBeAg secretion is also conserved in the avian hepatitis viruses, in which in utero tolerance mechanisms are not relevant. Furthermore, adult infection with the HBeAg-negative mutant is often associated with a fulminant course of infection rather than the relatively benign acute course that characterizes most adult-onset infections with wild-type HBV (11, 12). In addition, emergence of the HBeAg-negative mutant during chronic active HBV infection can correlate with an exacerbation of liver injury and a worse prognosis (13). These observations suggest that the HBeAg may function to modulate the immune response during chronic HBV infection in the adult in addition to its effects on neonatal tolerance.

One means of examining the effects of continuous exposure of the immune system to the secreted HBeAg has been to develop HBeAg-expressing Tg mice. Studies in HBeAg-Tg mice revealed that the level of Th cell tolerance was dependent upon the MHC background and Th cell site recognized by the Tg murine strain. A proportion of Th cells of HBeAg-Tg mice on an H-2b background (resides 129–140-specific) evade tolerance induction and can be activated in vivo (14). This HBeAg-Tg model has provided the opportunity to examine the immunoregulatory properties of circulating HBeAg. For example, a single injection of the HBeAg-derived Th cell site (peptide 129–140) into HBeAg-Tg (H-2b) mice...
leads to sufficient anti-HBe “autoantibody” (autoAb) production to complex with and mask the detection of serum HBeAg (14), and Fig. 1). Subsequent studies revealed that the HBeAg-specific (i.e., self-reactive) Th cells that evade tolerance and mediate anti-HBe autoAb production in HBeAg-Tg mice are significantly “altered” by their coexistence with the circulating HBeAg. The HBeAg/self-reactive Th cells surviving in HBeAg-Tg mice exhibit a unique fine specificity that can be distinguished from the HBeAg-specific Th cell repertoire of non-Tg mice and are comprised predominantly of Th2-like cells (15). The preferential survival of HBeAg-specific Th cells of the Th2-type in HBeAg-Tg mice is of particular interest because of the serologic evidence that an imbalance in HBe/HBeAg-specific Th1/Th2 cell function may contribute to the induction and/or maintenance of persistent HBV infection (16).

Because the HBeAg is a secreted protein, its tolerogenic effects on the HBe/HBeAg-specific Th cell repertoire can be mediated within the thymus and/or within the peripheral lymphoid compartment. We are particularly interested in the effects of circulating HBeAg on Th cells in the periphery because of the possible implications for HBV infection in the adult. This current study addresses this issue as well as possible cross-regulatory effects of HBeAg-specific Th cells on the HBeAg-specific immune response. For this purpose, HBeAg-Tg mice were bred with Fas-defective lpr/lpr mice and Fas ligand (Fasl)-defective gld/gld mice. Stimulation of previously activated T cells or repeated antigen stimulation induces activation-induced apoptosis (AIA) mediated by Fas-FasL interactions (17–19). Importantly for this study, thymic T cell deletion (i.e., negative selection) is apparently not Fas-mediated (20–22), whereas peripheral clonal deletion appears to be Fas-mediated and impaired in lpr/lpr and gld/gld mice leading to the accumulation of self-reactive T and B cells and a predisposition for autoimmune disease (22, 23). Therefore, the availability of HBeAg-Tg mice on Fas-expressing or Fas-deficient backgrounds enabled us to examine the ability of secretory HBeAg to deplete HBeAg-specific Th cells in the periphery and determine whether Th1 or Th2 cells were preferentially affected by this mechanism. Second, armed with this information, adoptive transfer experiments were designed to examine possible cross-regulation between HBeAg-specific and HBeAg-specific Th cells in the periphery.

### Materials and Methods

**Mice**

C57BL/10 (B10) (H-2b) and B10 × B10.SIF, mice were obtained from the breeding colony of The Scripps Research Institute. The designated HBeAg-Tg and HBeAg-Tg mice, which express either HBeAg (9–13 ng/ml) in the serum or HBeAg (0.25 ng/ml) intracellularly, respectively, were produced as described previously (5, 24). Briefly, for the HBeAg-Tg line, the HBV-DNA fragment (subtype ayw, coordinates 1804–2804) containing the complete precore plus core open reading frame was cloned between the mouse metallothionein I promoter (MtP) (coordinates −700 to +64) and polyadenylation recognition sequences (coordinates 930–1241), such that expression of HBeAg was controlled by the Mtp. The microinjected HBV-DNA fragment used to produce the HBeAg-Tg lineage consisted of the HBV (ayw) core sequences (coordinates 1890–2804) under the control of the mouse MtP and polyadenylation sequences. B10 MRL-Fas−/− (H-2b) mice were obtained from The Scripps Research Institute breeding colony. B6Smn.C3H-Fas−/− (H-2b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice homozygous for the gld or lpr mutation and either homozygous or hemizygous for the HBeAg transgene were produced by first crossing homozygous lpr or gld mice with HBeAg-Tg mice that were homozygous for the HBeAg transgene. The F1 mice produced were then intercrossed to produce F2 mice of the appropriate genotypes. Subsequently, mice of the desired genotype were produced by intercrossing F1 mice. Nontransgenic and Fas-FasL wild-type or heterozygous littermates were used as control mice. All mice designated as lpr or gld were homozygous for the lpr or gld mutations. The mice designated as HBeAg-Tg or HBeAg-Tg mice were hemizygous for the HBe/HBeAg transgenes with the exception of the HBeAg-Tg mice used for cytokine analysis, which were homozygous. (HBeAg-Tg × lpr/lpr) or (HBeAg-Tg × gld/gld) mice were designated elpr or elgd.

**Genotyping by PCR**

Fas−/− and Fas+ were typed by conventional PCR methodology (D. C. Roopenian, personal communication, The Jackson Laboratory, Bar Harbor, ME). The following oligonucleotides were purchased from Life Technologies and had the following sequences: FAS2, 5′-TTT CAT AAG TGG TGG CGC AA-3′; and FAS3, 5′-CAC GGA GAA GTA GCA AGA TGA-3′. Amplification was conducted in 50 µl DNA, 1.5 mM MgCl2, 20 µM each of three oligonucleotides, 100 µM each of four deoxynucleoside triphosphates, 1.25 U Taq polymerase, and cycling conditions were as follows: (94°C × 1); (94°C × 1, 55°C × 1, 72°C × 1) × 20; (72°C × 1) × 20, and (72°C × 1); FAS1 + FAS3 yields a 135-bp product for the wild-type allele and FAS2 + FAS3 yields a 271-bp product for the lpr allele. The products were visualized on a 1.5% agarose gel. Fas−/− was distinguished from Fas+ using mutagenically separated PCR technology as described by Rust et al. (25). Primers were purchased from Life Technologies and had the following sequences: GLD1, (forward) 5′-TGC ACT GGT CAA ATG ACC CAC A-3′; GLD2, (normal reverse) 5′-AGA TCA TTT TAA ATT CTT TCT AAG GCT TGA A-3′, and GLD3 (mutant reverse) 5′-TCT TTT AAA GCT TAT ACA AGC CAA GAT-3′. Amplification was conducted in a PTC 150 MJ Research thermal cycler (Cambridge, MA) using 0.5 ml thin-walled tubes. The 100 µl reaction volume contained 10 µM DNA, 1.5 mM MgCl2, 20 µM each of four deoxynucleoside triphosphates, 0.1 µM each GLD1 and GLD2, 0.2 µM GLD3, 2.5 U Taq polymerase, and cycling conditions were as follows: (94°C × 1); (94°C × 1, 60°C × 1, 72°C × 1) × 20; and (72°C × 1); GLD1 and GLD2 yields a 176-bp product for the normal gene and GLD1 + GLD3 yields a 155-bp product for the mutant form of the gene. The products were visualized on a 3.5% NuSieve GTG Agarose/1% agarose (catalogue no. 50072, FMC Bioproducts, Rockland, ME) composite gel.

**Recombinant proteins and synthetic peptides**

The HBV core 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 particles that comprise the virion nucleocapsid (HBcAg). Although HBcAg and HBeAg are serologically distinct, these Ags are cross-reactive at the level of T cell recognition because they are colinear throughout most of their primary sequence. HBcAg of the ayw subtype was produced in Escherichia coli and purified as described previously (20). An rHBeAg 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 (27). The presence of the 10 precore amino acids prevents particle assembly, and HBeAg was recognized efficiently by HBeAg-specific mAbs but displays little HBe antigenicity (27). An aliquot of truncated HBeAg was reduced and denatured by boiling in SDS/2-ME (1.0%) and then alkylated. The SDS was removed by dialysis, and the material was resublimated in 3 M urea. This preparation consisted predominantly of monomers (16 kDa) with some dimer formation upon nonreducing PAGE and was designated P16. P16 does not bind HBeAg- or HBeAg-specific mAbs.

Peptides were synthesized by the simultaneous multiple peptide synthesis method (28) and were kindly provided by Richard Houghten (Torrey Pines Institute for Molecular Studies, La Jolla, CA.). The following Hbc/HBcAg-derived synthetic peptides representing Th cell recognition sites were used and designated by amino acid position from the N terminus of HBcAg: 129–140, PPAYPFPNAPIL; and 120–140, YSPGWWRPTPPAYPFPNAPIL.

**Serology**

HBeAg was measured in diluted Tg mouse sera by a commercial ELISA (HBc enzyme immunoassay; Abbott Laboratories, Chicago, IL), and rHBeAg was used as a standard. Anti-HBe and anti-HBe IgG Ab was measured in murine sera by an indirect solid-phase ELISA using HBcAg or rHBeAg as the solid-phase ligands as described previously (29). The data are expressed as Ab titers representing the reciprocal of the highest dilution of sera required to yield an OD492 reading three times that of preimmunization sera. IgG isotype-specific ELISAs were performed using IgG1-, IgG2a-, IgG2b-, and IgG3-specific second Abs (Southern Biotechnology, Birmingham, AL).

**Adoptive transfer of autoAb production in HBeAg- and HBcAg-Tg mice**

Non-Tg donor mice were immunized in the hind footpads either with 100 μg of the peptide T cell site 129–140 emulsified in CFA or with CFA alone, and draining popliteal lymph node (PLN) cells were harvested after 10 days and cultured with peptide 120–140 (0.2 μg/ml) or purified protein derivatives for an additional 3 days. In vitro-activated PLN cells were then transferred into sublethally irradiated (500 R) HBcAg- or HBeAg-expressing Tg recipient sera. Sera were collected before and at various times after adoptive transfer and analyzed for anti-HBe and anti-HBe Abs by ELISA. In selected experiments, T cells were enriched from the PLN preparation before adoptive transfer, producing 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 (30), unselected PLN cells were routinely used to transfer HBc/HBe-specific T cell help in adoptive transfer experiments.

**Determination of Ag-specific cytokine production**

Groups of four HBeAg-Tg/Fas-FasL-expressing (e/lpr) or HBeAg-Tg/Fas-deficient (el/lpr) mice were immunized i.p. with rHBeAg (10 μg) emulsified in IFA, and 10 to 14 days later spleen cells were harvested, pooled, and cultured in 10 × 10^6/ml with various concentrations of Ags. Culture supernatants (SN) were harvested at 24 h for IL-2 and IL-4 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 (31). IL-4 was measured by the ability of SN to stimulate proliferation of the IL-4-sensitive CT-3 cell line generously provided by William Paul, National Institutes of Health, Bethesda, MD (32). IFN-γ was measured by two-site ELISA using mAb HB170 and a polyclonal goat anti-mouse IFN-γ Abs (Southern Biotechnology, Birmingham, AL.). The following HBc/HBeAg-specific peptides were used: 129–140, e/lpr, or HBeAg-Tg/Fas-deficient (el/lpr) mice were injected with the peptide T cell site 129–140 (50 μg in IFA). The HBeAg-Tg mice were bled before injection and at 2 wk intervals for the determination of total IgG anti-HBe as well as isotype-specific anti-HBe Ab levels by ELISA. Because mouse to mouse variation was minimal (=2-fold) in this system, sera were pooled before analysis.

**Results**

**Induced anti-HBe autoAb production is actually reduced in HBeAg transgenic mice carrying the lpr and gld mutations**

Although the MRL-lpr/lpr and MRL-gld/gld strains are most severely affected, other strains carrying the lpr and gld mutations also develop lymphadenopathy and are prone to developing autoimmunity with differing kinetics (33). Therefore, the simplistic prediction was that HBeAg-Tg/lpr or gld mice would be more susceptible to induced anti-HBe autoAb production as compared with HBeAg-Tg/+ mice. In fact, quite the opposite occurred. As shown in Figure 1, el/lpr mice produced significantly less anti-HBe autoAb (i.e., ~16- to 32-fold less) as compared with el/+ mice during the first 12 wk after the HBeAg Th cell peptide 129–140 was injected. Thereafter, the anti-HBe autoAb titers of the el/+ and el/lpr mice began to merge. It should be noted that by wk 32, lymphadenopathy was beginning to become evident in el/lpr mice. The diminished early anti-HBe autoAb production demonstrated by el/lpr mice cannot be attributed to a generalized hyporesponsiveness conferred by the lpr mutation, since in a control experiment littermate non-Tg wild-type (+/+ ) and lpr (+/lpr) mice differed by only 2- to 4-fold in their anti-HBe Ab responses to immunization with recombinant HBeAg (Fig. 1, inset). It was also notable that the IgG isotype distribution of anti-HBe Abs produced by +/+ vs +/lpr mice was not significantly different (data not shown). This result indicates that the lpr mutation has no general effect on IgG isotype (see next section).

Similar experiments were performed comparing induced anti-HBe autoAb production in HBeAg-Tg/+ mice with HBeAg-Tg/gld mice. Groups of five el/+ or el/gld mice were injected with the HBeAg-specific Th cell site 129–140 and anti-HBe autoAb titers were measured by ELISA thereafter. The el/gld mice produced from 8- to 64-fold less anti-HBe autoAb during the first 20 wk of observation as compared with el/+ mice (Fig. 2). In view of the

![FIGURE 2. Comparative anti-HBe autoAb production in HBeAg-Tg/+ and HBeAg-Tg/gld mice. Groups of five el/+ or el/gld mice were injected with the synthetic Th cell site 129–140 (50 μg, IFA). Mice were bled at 2- to 4-wk intervals, sera were pooled from five mice per group, and anti-HBe IgG autoAb end-point titers were measured by ELISA. Titers are expressed as the reciprocal of the highest dilution of sera required to yield an OD492 value three times that of pre-129–140 injection sera. This experiment was performed on two separate occasions and is representative.](http://www.jimmunol.org/content//185/10/6393/F2.large.jpg)
injected with the Th cell peptide 129–140 (50 μg, IFA). Mice were bled 6 wk after 129–140 injection, sera were pooled from five mice per group, and anti-HBe autoAb end-point titers of the IgG1, IgG2a, IgG2b, and IgG3 isotypes were measured by ELISA. Titers are expressed as the reciprocal of the highest dilution of sera required to yield an OD492 value three times that of pre-129–140 injection sera. NR, no response. These data are representative of experiments performed on two separate occasions.

The IgG isotype profile of anti-HBe autoAb differs between HBeAg-Tg/+ and HBeAg-Tg/lpr or gld mice

One explanation for why the absence of Th cell clonal deletion in the periphery in Fas-Fasl-defective HBeAg-Tg mice results in diminished as opposed to increased anti-HBe autoAb production would be a preferential depletion of the Th1 subset of HBeAg-specific Th cells by Fas-mediated apoptosis. To examine this possibility, the IgG isotype patterns of anti-HBe autoAb produced by HBeAg-Tg/+ and HBeAg-Tg/lpr or gld mice were determined. As illustrated in Figure 3, the anti-HBe autoAb response in e/+ mice induced by injection of the Th cell site 129–140 is exclusively comprised of the IgG1 isotype. Only the peak response at 6 wk is shown; however, the predominance of the IgG1 isotype is evident from wk 2 throughout the observation period. In marked contrast, the induced anti-HBe autoAb response in e/lpr mice is characterized by a 16-fold reduction in the IgG1 isotype as compared with e/+ mice and also by the production of significant quantities of IgG2a and IgG2b anti-HBe Abs (Fig. 3). Injection of peptide 129–140 into +/+ or +/lpr control mice did not elicit anti-HBe autoAb production due to the absence of endogenous HBeAg.

An analysis comparing the anti-HBe IgG isotypes produced in e/+ and e/gld mice injected with the Th cell site 129–140 revealed IgG isotype profiles similar to those observed in e/+ and e/lpr mice (Fig. 4). The overall anti-HBe autoAb response in e/gld mice is reduced and the IgG isotype pattern is broadened to include IgG2a, IgG2b, and IgG3 as compared with the IgG1 predominance observed in e/+ mice. In the mouse, IgG1 switching is influenced by IL-4, a cytokine predominantly produced by Th2 cells, and IgG2a and IgG3 switching are mediated by IFN-γ, a cytokine produced by Th1 cells and NK cells (34, 35). Therefore, the anti-HBe IgG isotype data indicate that a mixed population of HBeAg-specific Th1 and Th2 cells coexist in the periphery of e/lpr and e/gld mice. In e/+ mice, the induced anti-HBe autoAb response is almost exclusively dominated by IgG1 production, suggesting that HBeAg-specific Th1 cells are depleted by Fas-mediated mechanisms, and HBeAg-specific Th2 cells are spared.

Cytokine production in HBeAg-Tg/+ and HBeAg-Tg/lpr mice

To determine whether Fas-mediated mechanisms affected HBeAg-specific cytokine production in HBeAg-Tg mice, e/+ and e/lpr mice were immunized with 10 μg of rHBeAg and Ag-specific in vitro cytokine production by splenic Th cells was measured. A panel of HBe/HBe-derived Ags was used (i.e., HBeAg; HBeAg: P16, the structural subunit polypeptide; and peptide 120–140) to recall Th cell cytokine production in vitro. Splenic Th cells of HBeAg-primed e/+ mice produced IL-4 significantly more efficiently than e/lpr HBeAg-primed Th cells recalled in vitro with

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Anti-HBe autoAb IgG isotype analysis in HBeAg-Tg/+ and HBeAg-Tg/lpr mice. Groups of five e/+ or e/lpr mice were injected with the Th cell peptide 129–140 (50 μg, IFA). Mice were bled 4 wk after 129–140 injection, sera were pooled from five mice per group, and anti-HBe autoAb end-point titers of the IgG1, IgG2a, IgG2b, and IgG3 isotypes were measured by ELISA. Titers are expressed as the reciprocal of the highest dilution of sera required to yield an OD492 value three times that of pre-129–140 injection sera. NR, no response. These data are representative of experiments performed on two separate occasions.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Anti-HBe autoAb IgG isotype analysis in HBeAg-Tg/+ and HBeAg-Tg/gld mice. Groups of five e/+ or e/gld mice were injected with the Th cell peptide 129–140 (50 μg, IFA). Mice were bled 4 wk after 129–140 injection, sera were pooled from five mice per group, and anti-HBe autoAb end-point titers of the IgG1, IgG2a, IgG2b, and IgG3 isotypes were measured by ELISA. Titers are expressed as the reciprocal of the highest dilution of sera required to yield an OD492 value three times that of pre-129–140 injection sera. NR, no response. These data are representative of experiments performed on two separate occasions.
HBcAg-specific e/Th cells. When comparing e/1 HBcAg-specific e/Th cells, the in vivo Ab data suggesting a relative Th1-like bias in HBe/lpr mice, these cytokine profiles clearly indicate the presence of mixed Th1/Th2 cell populations in the in vitro cytokine production. No differences were observed in Ag-specific IL-2 production vs in vitro cytokine production may also reflect the fact that for the cytokine studies exogenous HBeAg was used for immunization. In the other studies (i.e., anti-HBe autoAb production and adoptive transfers) endogenous/transgenic HBcAg or HBeAg served as the source of Ag in vivo. Endogenous Ags may better mimic Ag exposure during an HBV infection and reduce possible artifacts due to immunization and in vitro culture.

**The presence of HBeAg in the serum can regulate the HBcAg-specific immune response**

Because the experiments performed in el/lpr and el/gld mice suggested that HBeAg in the serum preferentially deleted HBcAg-specific Th1 cells, and because previous studies indicated that HBcAg preferentially elicits Th1-like cells (36), it was of interest to determine whether the Th1-mediated HBcAg-specific immune response would be influenced by the presence of HBeAg in the serum. For this purpose, peptide 129–140–primed Th cells were adoptively transferred into HBeAg-Tg, HBcAg-Tg, or dual HBeAg/HBcAg-Tg recipients, and the IgG isotype profile of the anti-HBe and anti-HBc Abs produced were determined. As depicted in Figure 6, adoptive transfer of 129–140–primed Th cells into e+/c mice resulted in anti-HBe Ab production dominated by IgG1 isotype (i.e., Th2-like pattern) and no anti-HBc Abs were produced (not shown). In contrast, adoptive transfer of the same peptide 129–140–primed Th cells into e/lpr or e/c recipient mice did not elicit Ab production (not shown). This experiment was performed on two separate occasions and is representative.

**FIGURE 5.** Comparative Ag-specific cytokine production in HBeAg-Tg/+ and HBeAg-Tg/lpr mice. Groups of four e/+ or el/lpr mice were immunized with rHBeAg (10 µg, IFA). Spleen cells were harvested on day 10, and pooled splenic cells were cultured (8 × 10^6/ml) with rHBcAg (1.0 µg/ml) and rHBeAg (50 µg/ml). Culture SN were collected for IL-2 (24 h), IL-4 (48 h), and IFN-γ (48 h) measurements. The data represent mean cytokine levels ± SD from five separate experiments. The levels of IL-4 produced by e/+ and el/lpr T cells cultured in vitro with HBcAg and peptide 120–140 were statistically different (p < 0.01, Student’s t test).

**FIGURE 6.** Circulating HBeAg effects the Th cells adoptively transferred into HBeAg-Tg, HBcAg-Tg, or dual HBeAg/HBcAg-Tg recipient mice. Non-Tg (B10 × B10.S)F1 donor mice (F1+/-) were primed in the hind footpads either with 100 µg of the synthetic Th cell site 129–140 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 HBe/HBeAg-derived peptide 120–140 (0.2 µg/ml) for 3 days. In vitro activated Th cells (1.2 × 10^7) were then transferred into five sublethally irradiated (500 R) F1 e/+ and F1 e/lpr recipient mice. Sera were collected before transfer and at 8 wk after adoptive transfer and analyzed for IgG isotype-specific anti-HBe or anti-HBc Abs by ELISA. Ab titers were 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 e/+ or e/lpr or e/c recipient mice did not elicit Ab production (not shown). This experiment was performed on two separate occasions and is representative.
dramatic effect circulating HBeAg had on the anti-HBe response in dual Tg e/c mice. Anti-HBe production in e/c Tg mice was almost totally ablated, and the low titer anti-HBe Ab that was produced was composed entirely of the IgG1 isotype, which was not produced in +/c Tg recipients. These results indicate that HBeAg circulating in the serum of e/c Tg mice inhibited the production of the Th1 cell-dependent anti-HBe IgG isotypes and promoted the production of Th2 cell-dependent IgG1 anti-HBe Abs. At least two mechanisms may explain the effect of serum HBeAg on the anti-HBe response. First, serum HBeAg preferentially depletes HBeAg-specific Th1 cells in the periphery of HBeAg-Tg mice (Figs. 3 and 4), and it is these same HBe/HBeAg-specific Th1 cells that mediate IgG2a anti-HBe Ab production in HBeAg-Tg mice. Second, the preponderance of HBe/HBeAg-specific Th2-like cells surviving in the periphery of e/c or e/c Tg mice may down-regulate HBe/HBeAg-specific Th1 cells by virtue of the production of cross-regulatory Th2-type cytokines (i.e., IL-4, IL-10). It is important to note that the effects of serum HBeAg on the Th cell repertoire are not limited to Th cells “shaped” in an HBeAg-Tg setting. Adoptively transferred Th cells from a non-Tg donor were efficiently skewed toward the Th2 phenotype by circulating HBeAg in e/c Tg recipients.

Discussion

Previous studies using this HBeAg-Tg model demonstrated that HBeAg-specific Th cell tolerance was incomplete on an H-2b background, and that Th2 cells preferentially survived tolerance induction (14, 15). Because the HBeAg is a secreted protein, it could be assumed that depletion of immature HBeAg-specific Th cells occurs primarily in the thymus. However, this current study suggests that mature HBeAg-specific Th1 cells are preferentially depleted in the periphery after contact with the secreted HBeAg. It appears that the Fas system is not involved in positive and negative selection in the thymus (20, 21). However, Fas-FasL interactions appear that the Fas system is not involved in positive and negative selection in the periphery (22, 23). Yet the lpr and gld mutations in the Fas and FasL genes, which abolish Fas activity, actually resulted in decreased rather than increased anti-HBe autoAb production in this transgenic model of induced autoAb production. One explanation for this apparent paradox would be the preferential depletion of regulatory HBeAg-specific Th1-like cells in the periphery via Fas-mediated AIA. In fact, FasL is expressed predominantly on Th1 cells (37) and is only expressed on a subset of Th2 cells if at all (38). Alternatively, a recent study suggests that differential sensitivity of Th cell subsets to AIA resides in the ability of Th2 cells to express high levels of Fas-associated phosphatase 1, which may inhibit Fas signaling (39). Correspondingly, Th1 clones are highly sensitive to AIA while Th2 clones are relatively resistant (37, 39–41). These observations suggest that Th1 cells are more susceptible to Fas-mediated apoptosis than are Th2 cells. In addition to reduced anti-HBe autoAb production in e/lpr and e/gld mice as compared with e/+ mice, the IgG isotype profiles of the anti-HBe Abs were also quite different. In e/+ mice the anti-HBe Ab response is composed almost exclusively of the IgG1 isotype (i.e., Th2-like). In contrast, e/lpr and e/gld mice efficiently produce IgG2a, IgG2b, and IgG3 as well as IgG1 anti-HBe autoAbs (i.e., mixed Th1- and Th2-like). This same shift in the anti-HBe autoAb IgG isotype pattern from a Th2-like to a Th1-like or mixed phenotype can be elicited in e/+ mice by in vivo treatment with IL-12 (42) or IFN-α (36), both of which enhance Th1 cell differentiation.

We suggest that circulating HBeAg in HBeAg-Tg mice preferentially depletes HBeAg-specific Th1-like cells in the periphery via Fas-mediated apoptosis, and that HBeAg-specific Th2-like cells survive this process to a greater degree. Reciprocally, HBeAg-specific Th1-like cells can survive in e/lpr and e/gld mice, and the resulting Th2/Th1-mixed population of HBeAg-specific Th cells mediates lower Ab production with a broader IgG isotype profile. It appears that HBeAg-specific Th1 cells can down-regulate HBeAg-specific Th2 cells in e/lpr and e/gld mice, in which HBeAg-specific Th1 cells have not been fully depleted. Therefore, much like self-reactive Th2 cells that have been proposed to be regulatory for pathogenic self-reactive Th1 cells (43, 44), it appears that autoreactive Th1 cells can regulate autoAb-inducing Th2 cells specific for a secreted self Ag.

Although the HBeAg and the HBeAg are cross-reactive at the level of Th cell recognition, these two structural forms have quite different properties. The HBeAg is a nonparticulate secreted protein and the HBeAg is a particulate intracellular protein. The HBeAg can function as a Th cell-independent Ag, whereas the HBeAg is strictly a Th cell-dependent Ag (45). Recent studies indicate that HBeAg and HBeAg use different Ag presentation pathways as well (3). These different characteristics may explain why the same Th cell population (129–140-specific) adoptively transferred into HBeAg-Tg recipients results in IgG1 anti-HBe Ab production and, to the contrary, IgG2a/IgG2b anti-HBe Ab production in HBeAg-Tg recipients. Because the immune response to HBeAg is biased toward Th1-like cells (36) and the secreted HBeAg appears to preferentially deplete Th1-like cells in the periphery, it was of interest to determine whether the presence of HBeAg in the serum would alter the immune response to the intracellular HBeAg. The results of transferring 129–140-specific Th cells into dual HBeAg/HBeAg-expressing Tg recipients were quite revealing. The presence of HBeAg in the serum ablated the characteristic Th1-like anti-HBe Ab response and promoted a Th2-like anti-HBe response (i.e., IgG1 anti-HBe), which did not occur in Tg mice expressing only HBeAg. In addition to independently confirming the ability of HBeAg to deplete Th1-like cells in the periphery, this result has important implications for understanding mechanisms of viral persistence and immunopathogenesis in chronic HBV infection.

Earlier studies suggested that exposure to the HBeAg in utero may establish Th cell tolerance to the HBe/HBeAg and promote chronicity upon perinatal infection by an HBeAg-positive mother (4, 5). This current study suggests an additional mechanism by which the HBeAg may maintain or induce chronicity even during an adult infection. The fact that the HBeAg is secreted and widely disseminated coupled with its ability to deplete HBe/HBeAg-specific Th1-like cells and spare Th2-like cells make it a likely candidate to promote viral persistence. HBeAg-specific Th2-like cells produce anti-inflammatory cytokines (i.e., IL-4, IL-10) that would be expected to inhibit the expansion of HBe/HBeAg-specific CTL and Th1 effector cells necessary for the clearance of this noncytolytic virus. Recent serologic evidence suggests that a Th1/Th2 subset imbalance in favor of HBe/HBeAg-specific Th2 cells may play a role in promoting chronic HBV infection (16). Furthermore, recent studies of cytokine production in liver-derived Th cells indicated that Th0-like cells were predominant in chronic HBV patients, Th1-like cytokines were associated with increasing hepatitis activity, and Th2-like cytokines were associated with decreasing liver disease during chronic HBV infection (46, 47). Because both HBeAg and HBeAg are produced during a wild-type HBV infection and because HBe/HBeAg-specific Th1 and Th2 cells can be cross-regulatory (Fig. 6), a dynamic balance between these Th cell subsets may exist during an HBV infection. Alterations in the HBe/HBeAg-specific Th1/Th2 cell balance would be expected to influence the course of the infection both in terms of liver injury...
and viral persistence. Interestingly, emergence of an HBV mutant that does not produce the HBeAg during chronic active HBV infection often results in an exacerbation of liver injury and a worse prognosis (13). Similarly, adult infection with the HBeAg-negative HBV mutant can be associated with severe fulminant hepatitis (11, 12). Therefore, the loss or absence of serum HBeAg can be associated with increased inflammation and liver injury. Both of these clinical observations are consistent with the hypothesis that the secreted HBeAg may preferentially deplete inflammatory HBc/HBeAg-specific Th1 cells and bias the Th1/Th2 balance toward the anti-inflammatory Th2 phenotype as suggested by this Tg model. Interestingly, human HBeAg-specific CD4+ Th1 clones, but not Th0 clones, can be anergized in vitro by stimulation with specific peptides (48). Secretion of the HBeAg may represent a viral strategy to promote or maintain a long term infection without eliciting an overly destructive immune response that would eliminate the virus and/or kill the infected host.

Several forms of Th1/Th2 cell cross-regulation were observed in this Tg model system. 1) In the absence of Fas activity (i.e., in el/Pr and e/gld mice), HBeAg-specific Th1 cells down-regulate HBeAg-specific Th2 cells resulting in lower anti-HBe Ab production and induction of the IgG2a, IgG2b, and IgG3 isotypes of anti-HBe. 2) In the presence of Fas activity (i.e., in e/+ mice), HBeAg-specific Th2 cells preferentially survive Fas-mediated depletion in the periphery and predominate over HBeAg-specific Th1 cells. 3) As demonstrated by the adoptive transfer of 129–140-specific Th cells into dual HBeAg/HBcAg-Tg recipients, the circulating HBeAg preferentially depletes or alters the function of the Th1 cells that would otherwise mediate IgG2a and IgG2b anti-HBe Ab production. Therefore, at the HBeAg concentration in the serum and the level of HBeAg expression in the liver of the dual HBeAg/ HBcAg-Tg mice in this study, HBeAg-specific Th2 cells predominated over HBcAg-specific Th1 cells. Of course, this balance may shift depending on the relative concentrations of the two Ags. These results indicate how dynamic the balance between HBc/HBeAg-specific Th1 and Th2 cells can be even in this noninfectious Tg system. Similar variables and perhaps many more that are unique to an infectious system may influence the HBc/HBeAg-specific Th1/Th2 cell balance during an HBV infection.

Although most of the conditions examined in this study favored the predominance of HBeAg-specific Th2-like cells, the HBeAg also has the potential to elicit Th1-like cells (36, 42) and serve as a CTL target (49, 50). The immune function of HBeAg cannot be viewed only in the context of in utero tolerance and perinatal infection. For example, during an adult acute HBV infection, in which in utero T cell tolerance is not relevant, production of IL-12 and the family of IFNs may be sufficient to bias the HBc/HBeAg-specific immune response toward the Th1 phenotype. However, even in the case of adult infection, it appears that secretory HBeAg may still modulate the vigor of the inflammatory response to some degree in view of the observation that adult onset infection with the HBeAg-negative mutant often results in fulminant hepatitis (11, 12). During a chronic HBV infection, HBcAg-specific Th cell tolerance may wane with age (5), allowing the emergence of Th1-like cells. Even in the setting of HBeAg-Tg mice, in vivo treatment with either IL-12 or IFN-α can shift the HBeAg-specific Ab response from Th2 to Th1 cell predominance (36, 42). Therefore, the context of the exposure to HBeAg may be important in determining the effect secretory HBeAg will have on the outcome of an acute or chronic HBV infection (see Fig. 7).

During the so-called “tolerance phase” of chronic HBV infection, secretory HBeAg would be expected to bias the HBc/HBeAg immune response toward the Th2 phenotype and help to maintain viral persistence. However, in the injury phase of chronic HBV infection, when by definition HBeAg-specific tolerance has been broken, the HBeAg-specific Th1/Th2 cell balance may shift toward the Th1 phenotype and the HBeAg may serve as an additional target for CTL recognition as well. A number of factors may contribute to a time-related shift in HBeAg-specific Th cells from a Th2-like to a Th1-like phenotype during chronic HBV infection including: the waning of HBeAg-specific Th1 cell tolerance with age; neutralization of circulating HBeAg by anti-HBe resulting in less efficient Fas-mediated Th1 cell depletion in the periphery; release of HBeAg from hepatocytes, thereby increasing the role of HBcAg-specific Th cells that tend to be of the Th1 phenotype; and increased production of IL-12 and the family of IFNs that promote Th1 cell differentiation.

In the tolerance phase of chronic HBV infection, production of secretory HBeAg would enhance the persistence of the wild-type virus and, therefore, an HBeAg-negative mutant would have no selective advantage. In contrast, in the injury phase of chronic HBV infection production of secretory HBeAg may become disadvantageous for the virus (i.e., as a target for the now active CTL response) and an HBeAg-negative variant may be selected as an escape mutant by Th1-biased HBeAg-specific immune pressure (Fig. 7). This view is consistent with the accumulation of multiple mutations within the precore and core gene regions in chronic HBV patients with liver injury and the relative absence of
precore and core region mutations in chronic HBV patients without liver injury (13, 51–54). This hypothesis also explains the apparent paradox of how an HBeAg-negative mutant can become predominant during a chronic HBV infection if circulating HBeAg promotes viral persistence. Serum HBeAg only promotes viral persistence in the context of a predominant Th2-like HBeAg-specific Th cell repertoire.

In summary, this HBeAg-Tg model illustrates that the secretion of a viral protein (i.e., HBeAg), which may preferentially deplete inflammatory Th1 cells, may represent a viral strategy to promote persistence. Furthermore, skewing of the Th1/Th2 cell balance specific for one viral Ag (i.e., HBeAg) can affect the immune response to another viral Ag (i.e., HBeAg) via cross-regulatory mechanisms. Infections with HBV as well as HIV are characterized by the secretion of excess envelope proteins (i.e., hepatitis B surface Ag and gp120, respectively). It is anticipated that secretory HBsAg may also exert important immunomodulatory effects on Th1/Th2 cell subset distribution during chronic HBV infection as has been proposed for gp120 during chronic HIV infection (37).

Acknowledgments

The authors thank Drs. F. Schödel and D. Peterson for providing recombinant Ags and Ms. Rene Lang for preparation of the manuscript.

References


