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Immunology

Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance *in utero*?

(hepatitis B virus/T cell/transgenic mice/persistent infection)

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ABSTRACT **Infants born to hepatitis B virus carrier mothers, who express a secreted form of the nucleocapsid antigen designated HBeAg, invariably become persistently infected. To investigate the role of immunologic tolerance mechanisms in chronic infection of the newborn, we have generated HBeAg-expressing transgenic mice. HBeAg-expressing transgenic mice were tolerant to both HBeAg and the nonsecreted nucleocapsid (hepatitis B cor antigen/HBcAg) at the T-cell level. Transgenic mice did not produce antibody to HBeAg but did produce anti-HBc antibody *in vivo* and *in vitro*. The coexistence of tolerance to HBc/HBe T-cell determinants and anti-HBc antibody production *in vivo* parallels the immunologic status of neonates born to carrier mothers. It was also demonstrated that the maintenance of T-cell tolerance to HBcAg/HBeAg required the continued presence of the tolerogen and in its absence persisted for <16 weeks. The reversibility of T-cell tolerance to HBcAg/HBeAg may explain the inverse correlation between age of infection and rates of viral persistence. These observations suggest that a function of the HBeAg may be to induce immunologic tolerance *in utero*. Expression of HBeAg may represent a viral strategy to guarantee persistence after perinatal infection.**

The nucleocapsid of the hepatitis B virus (HBV) is a 27-nm particle composed of multiple copies of a single polypeptide (p21), and the intact structure exhibits hepatitis B core antigen (HBcAg) antigenicity. A nonparticulate form of HBcAg designated HBeAg is secreted into the serum during HBV infection. HBeAg synthesis results from initiation of translation from the first initiation codon of the open reading frame, which yields a polypeptide containing a signal sequence (1–3), whereas translation from the second initiation codon yields unprocessed p21, which is assembled into HBcAg particles (3). Unlike HBcAg, the function of HBeAg is unknown; however, it is not required for viral replication or infection in an avian HBV system (4, 5).

Although HBcAg and HBeAg are serologically distinct, the primary amino acid sequences show significant identity [serum HBeAg lacks the C-terminal 34 residues of HBcAg (6) and possesses an additional 10 N-terminal residues (7)]. Because of this sequence identity, the HBcAg and HBeAg are highly crossreactive in terms of T helper (Th)-cell recognition (8, 9). For example, the dominant Th-cell recognition site in B10.S mice is p120-131, which is shared between HBcAg and HBeAg (10). Antibody production to HBcAg can occur via T-cell-independent as well as T-cell-dependent pathways, whereas HBeAg is a T-cell-dependent antigen (8, 9).

The HBV is not directly cytopathic, and the immune response of the host appears to mediate hepatocellular tissue injury and subsequent viral clearance (11). Women who are chronic carriers of HBV often infect infants in the perinatal or postnatal periods, whereas intrauterine infection is much less common (12–15). The vast majority of untreated infants born to HBeAg-positive mothers become infected, and >90% of them become chronic carriers (16). In contrast, ≈90% of HBV infections occurring in adults are resolved as acute infections, and only 5–10% result in chronic infections (17). This dramatic difference in chronicity rates is believed to reflect the immunologic status of the host at the time of infection. For example, neonates born to HBV carrier mothers may be immunologically tolerant to viral proteins to which they were exposed *in utero* (18). The tolerogenic potential of the HBcAg/HBeAg is of particular interest because there is evidence that these antigens represent important "targets" for immune-mediated viral clearance mechanisms (19, 20). This is an attractive hypothesis; however, the tolerogenic potential of HBV antigens has not been previously investigated. For this purpose, we have produced HBeAg-expressing transgenic mice, and we used a neonatal tolerance system to investigate the tolerogenic characteristics of the HBcAg/HBeAg.

MATERIALS AND METHODS

Recombinant (r) HBcAg/HBeAg and Synthetic Peptides. rHBcAg of the *ayw* subtype produced in *Escherichia coli*, and a rHBcAg deletion mutant lacking the C-terminal 39 amino acids produced in *E. coli* (21), designated HBeAg, were provided by Stephen Stahl (Biogen). This HBeAg preparation has previously been shown to express dual HBc/HBe antigenicity dependent on pH (9). At pH 9.6 the HBeAg is nonparticulate and expresses HBe antigenicity; however, at pH 7.2 it is particulate and expresses both HBc and HBe antigenicity. Synthetic peptides derived from the HBcAg sequence p89-100 and p120-131 were synthesized by the Merrifield solid-phase method in the peptide laboratory of the R. W. Johnson Pharmaceutical Research Institute (Sorrento Valley, CA) and were provided by G. B. Thornton.

Serology. HBeAg was measured in diluted transgenic mouse serum by a commercial ELISA (HBe EIA diagnostic kit; Abbott), and rHBeAg was used as a standard. This ELISA was converted into a HBcAg-specific assay by the use of a peroxidase-labeled anti-HBc antibody (Corzyme; Abbott) as the final antibody. Anti-HBc and anti-HBe antibodies were measured in murine serum or cell culture supernatant (SN) by indirect solid-phase ELISA using rHBcAg or rHBeAg (100 ng per well)-coated wells. The data are reported as antibody titer expressed as the reciprocal of the dilution

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Abbreviations: HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; Th cell, T-helper cell; r, recombinant; SN, supernatant; PLN, popliteal lymph node; PPD, purified protein derivative; ORF, open reading frame; MTp, metallothionein I promoter.

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required to yield 3 times the OD reading of preimmunization sera or control cell culture SN.

***In Vitro* Anti-HBc Production.** To examine anti-HBc antibody production *in vitro*, groups of at least five mice were primed with 5.0 μg of HBcAg emulsified in complete Freund's adjuvant (CFA) intradermally (i.d.). Ten days later, the primed spleen cells (3.5×10^6 cells per ml) were cultured in RPMI 1640 medium containing 5% fetal calf serum with or without HBcAg (0.05 μg/ml). Cell culture SN was harvested on days 3 and 7 and was analyzed for IgM, total IgG, IgG1, IgG2a, IgG2b, or IgG3 anti-HBc antibody by indirect solid-phase ELISA using IgM and IgG subclass-specific secondary antibodies. The data are reported as a reciprocal of the dilution of cell culture SN to yield 3 times the OD reading of culture SN without HBcAg.

T-Cell Proliferation Assay. Groups of at least five mice each were primed with either 5.0 μg of HBcAg or HBeAg emulsified in CFA at pH 7.2 or 9.6 in CFA by hind footpad injection. Ten days after immunization, draining popliteal lymph node (PLN) cells were harvested from individual mice, and T-cell proliferative responses were determined as described (10). Purified protein derivative (PPD), which is contained within CFA, served as the positive control antigen. The data are expressed as cpm corrected for background proliferation in the absence of antigen (Δcpm). Each data point represents the mean (±SD) cpm from five individual mice, or in selected experiments PLN cells were pooled from each group.

Neonatal Tolerance Induction. Neonatal tolerance was induced by subcutaneous (s.c.) injection of newborn mice (<24 hr old) with 40 μg of HBcAg suspended in saline, and control mice were injected with saline alone. Mice were rested after neonatal injection for at least 8 weeks prior to immunization.

Production of Transgenic Mice. The transgenic mouse lineage designated B10.S Tg-31e expressing HBeAg was produced by standard procedures (22) at the Research Institute of Scripps Clinic Transgenic Research Facility. Briefly, the HBV DNA fragment (subtype *ayw*, coordinates 1804–2804) containing the complete precore plus core open reading frame (ORF) was cloned between the mouse metallothionein I promoter (MTp) (coordinates −700 to +64) and polyadenylylation recognition sequences (coordinates +930 to +1241) such that expression of HBeAg was controlled by the MTp (Fig. 1*A*) (3). This DNA fragment was microinjected into the male pronucleus of fertilized one-cell ova of B10.S × B10.S mice. The progeny were screened for the presence of the microinjected DNA by polymerase chain reaction analysis of tail DNA by using HBV-specific oligonucleotides. DNA and RNA analysis of transgenic mice was performed by standard procedures (23).

RESULTS

Characterization of Gene Expression in Transgenic Mice. The inbred transgenic mouse lineage, B10.S Tg-31e, was derived by breeding the founder mouse with nontransgenic B10.S mice. The structure of the transgene in these mice was analyzed by DNA filter hybridization analysis (Southern blotting) (Fig. 1*B*). The HBV probe hybridized to an *Acc* I fragment of 1.01 kilobase pairs (kbp), a *Sty* I fragment of 0.58 kbp, and *Ava* II fragments of 0.82 and 0.51 kbp. Hybridization of the HBV probe to fragments of these sizes is consistent with the integration of at least one complete copy of the injected DNA (Fig. 1*A*). From the intensity of the 1.01-kbp hybridization signal observed in *Acc* I-digested DNA, it was estimated that the B10.S Tg-31e mice contain two to five copies of integrated DNA. Expression of the transgene was examined by RNA filter hybridization analysis (Northern blotting) of various B10.S Tg-31e tissue RNAs (Fig. 1*C*). A 1.6-kb transcript was observed in liver and to a much lesser extent in kidney. This transcript is consistent with the predicted size [1.24 kb plus poly(A) tail] and tissue distribution expected for the injected transgene (24). Expression of the transgene is expected to result in the synthesis of HBeAg, which should be secreted into the serum of this mice. Analysis of transgenic mouse serum by ELISA demonstrated that HBeAg was present at 8–10 ng/ml and that this level could be increased ≈9-fold by Zn^{Zn} administration (Fig. 1*D*). These results are consistent with the expression of HBeAg being controlled by the MTp. The HBeAg-positive sera of B10.S Tg-31e mice were unreactive in a HBcAg-specific ELISA (data not shown).

HBeAg-Expressing Transgenic Mice Are Immunologically Tolerant to HBeAg and HBcAg at the T-Cell Level. To determine T-cell responsiveness, groups of five B10.S control or B10.S Tg-31e mice were immunized with HBeAg, and PLN T-cell proliferative responses specific for HBeAg, HBcAg, and p120–131 were determined (Fig. 2). HBeAg-primed T cells of B10.S control mice responded equivalently to HBeAg and HBcAg, which confirms the crossreactivity of

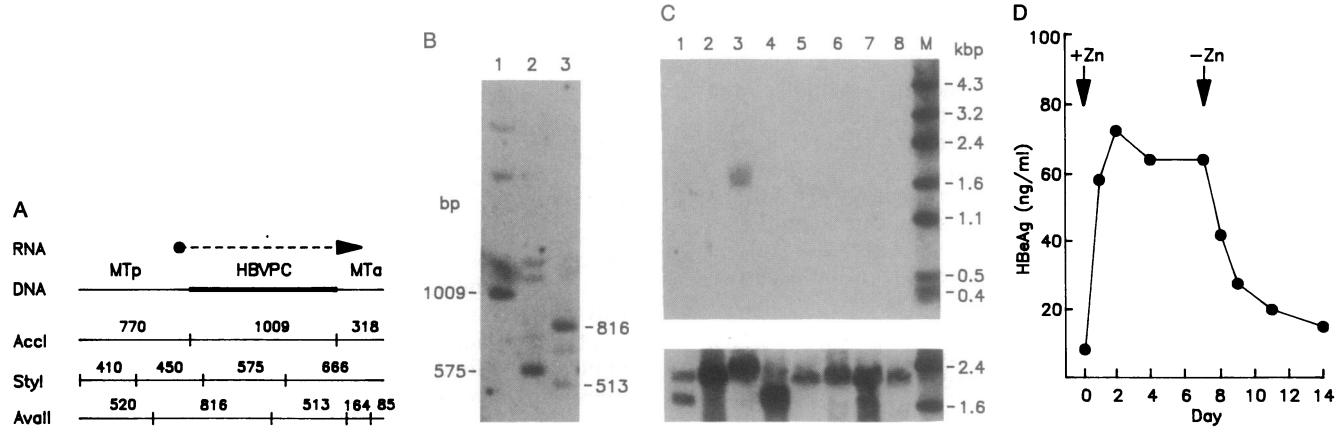

FIG. 1. (*A*) Restriction enzyme map of the DNA fragment microinjected into B10.S mice. Mta, mouse metallothionein I poly(A) recognition sequence; HBVPC, HBV precore plus core ORF. Thin lines, mouse DNA sequences; thick line, HBV DNA sequences. (*B*) DNA filter hybridization analysis of B10.S Tg-31e genomic DNA. Mouse genomic DNA (15 μg) was digested with *Acc* I (lane 1), *Sty* I (lane 2), and *Ava* II (lane 3) and was then probed with the precore plus core ORF HBV DNA fragment (coordinates 1804 to 2804). (*C*) RNA filter hybridization analysis of B10.S Tg-31e tissue RNA (10 μg) after induction with Zn^{Zn} for 2 days. Mouse RNA from heart (lane 1), lung (lane 2), liver (lane 3), skeletal muscle (lane 4), kidney (lane 5), brain (lane 6), spleen (lane 7), and stomach (lane 8) was probed with the precore plus core ORF HBV DNA fragment (*Upper*) or a human β-actin cDNA (pHFβA-1 DNA) (*Lower*). M, molecular size markers. (*D*) Induction of serum HBeAg in B10.S Tg-31e mice. Induction (+Zn) was performed by administration of 25 mM zinc sulfate in the drinking water. Subsequently, the mice were given normal drinking water (−Zn).

these antigens at the T-cell level in the B10.S strain, and responded efficiently to the dominant T-cell site represented by p120-131 (Fig. 2*A*). In contrast, HBeAg-primed T cells of B10.S Tg-31e mice were totally nonresponsive to HBeAg, HBcAg, and p120-131 (Fig. 2*B*). Similarly, immunization with HBcAg yielded the same results (data not shown). Therefore, HBeAg-expressing transgenic mice are functionally tolerant to both HBeAg and HBcAg as well as p120-131 at the level of T-cell proliferation. This T-cell tolerance did not appear to involve T suppressor cells inasmuch as HBeAg-primed T cells from transgenic mice did not inhibit the proliferation of HBeAg-primed T cells from control mice in mixing experiments (data not shown).

***In Vivo* Antibody Production in Transgenic and Control Mice.** HBeAg-expressing transgenic mice did not produce anti-HBe antibody spontaneously. It was of interest to determine the ability of B10.S Tg-31e mice to produce IgG anti-HBe and anti-HBc antibodies *in vivo* after immunization. For this purpose, B10.S control and B10.S Tg-31e mice were immunized with the particulate form of HBeAg, which possesses both HBe and HBc antigenicity. As shown in Fig. 3, B10.S control mice produce both anti-HBe and anti-HBc 10 and 24 days after primary immunization, and these responses were boosted upon secondary immunization. In contrast to control mice, B10.S Tg-31e mice produce anti-HBc but no anti-HBe antibody after primary immunization and only minimal anti-HBe after secondary immunization (i.e., 1:64 compared with 1:65,536 in B10.S control mice). The IgG anti-HBc titers of B10.S Tg-31e mice were 4-fold and 16-fold less than those of control mice 10 and 24 days after primary immunization, and 64-fold less after secondary immunization.

HBcAg-specific B cells from B10.S Tg-31e mice appear normal, as expected in the absence of *in utero* exposure to HBcAg B-cell epitopes. The status of HBeAg-specific B cells in B10.S Tg-31e mice is not as clear. In theory, the absence of detectable anti-HBe could be due to complexing with circulating HBeAg; however, the level of HBeAg in the serum of B10.S Tg-31e mice is not sufficient to prevent the detection of anti-HBe produced after adoptive transfer of nontransgenic spleen cells (data not shown). The limited anti-HBe antibody that is produced in B10.S Tg-31e mice suggests that HBeAg-specific B cells are not tolerant in these transgenic mice. Therefore, the dramatically reduced anti-

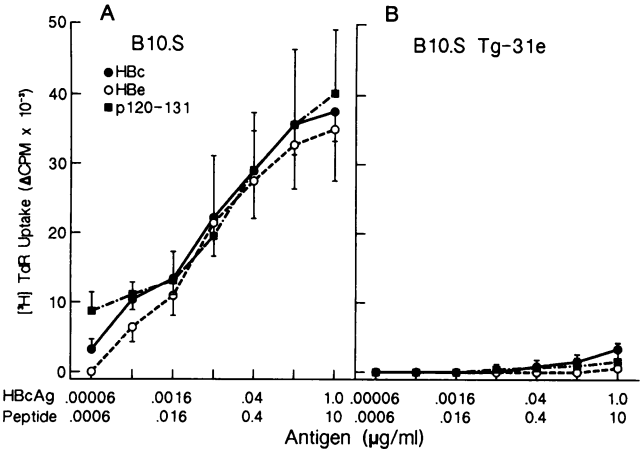

FIG. 2. T-cell responsiveness of B10.S and B10.S Tg-31e mice immunized with HBeAg. Groups of five B10.S control (*A*) or B10.S Tg-31e (*B*) mice were immunized with HBeAg, and draining PLN cells were harvested from individual mice 10 days later. The T-cell proliferative responses induced by various concentrations of the indicated antigens were determined. Each data point represents the mean (±SD) cpm from five mice. The mean T-cell proliferation specific for the PPD positive control antigen was 48,670 ± 6,850 in *A* and 44,333 ± 4,365 in *B*.

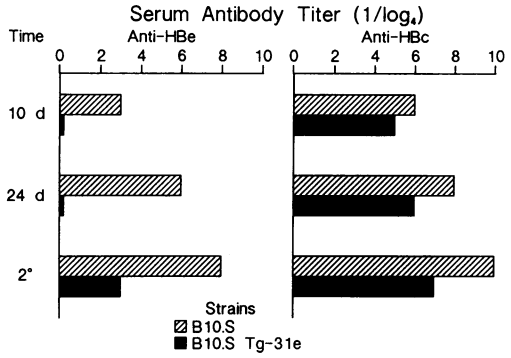

FIG. 3. *In vivo* antibody production in B10.S and B10.S Tg-31e mice. Groups of five B10.S control (hatched bars) or B10.S Tg-31e (solid bars) mice were immunized with 5.0 μg of the particulate form of HBeAg, which expresses both HBe and HBc antigenicity. Sera were collected 10 and 24 days after primary immunization and 2 weeks after secondary immunization (2°), and they were analyzed for IgG anti-HBe and anti-HBc antibodies by solid-phase ELISA. Antibody titer is expressed as the reciprocal of the dilution ($1/\log_4$) of serum, which yielded an OD reading 3 times that of preimmunization sera.

HBe antibody produced *in vivo* in B10.S Tg-31e mice reflects diminished Th-cell function as illustrated in the next section.

***In Vitro* Anti-HBc Antibody Production in Transgenic and Control Mice.** To test HBc/HBe-specific Th-cell function directly in B10.S Tg-31e mice, and to examine this function in the absence of circulating HBeAg, *in vitro* anti-HBc production was determined. As shown in Fig. 4, HBcAg-primed spleen cells of B10.S control mice produced IgM and IgG anti-HBc after 3 days in culture. In contrast, HBcAg-primed B10.S Tg-31e spleen cells produced only IgM (T-cell independent) anti-HBc after 3 days in culture. After 7 days in culture, B10.S control spleen cells produced significantly elevated levels of both IgM and IgG anti-HBc antibodies, and the IgG2b subclass predominated. In contrast, B10.S Tg-31e spleen cells produced elevated levels of IgM anti-HBc, but IgG anti-HBc production was minimal (i.e., 1:10) after 7 days in culture. The B10.S Tg-31e pattern of *in vitro* anti-HBc production and the reduced IgG anti-HBc produced *in vivo* indicates a HBcAg-specific Th-cell deficit compared with B10.S control mice. The extremely low levels of IgG anti-HBc that are produced by B10.S Tg-31e spleen cells *in vitro*

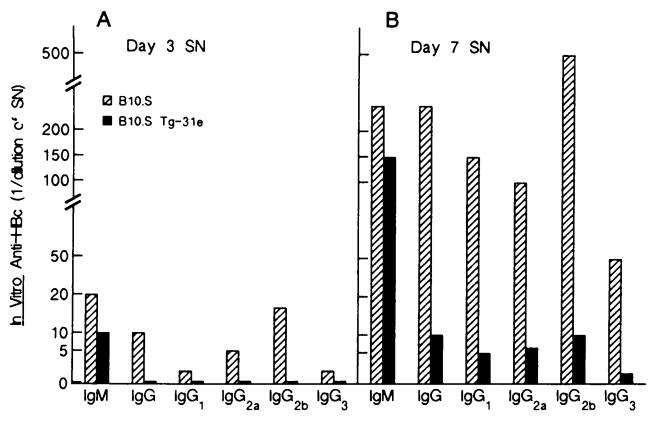

FIG. 4. *In vitro* anti-HBc production in B10.S and B10.S Tg-31e mice. Groups of five B10.S control (hatched bars) or B10.S Tg-31e (solid bars) mice were immunized with 5.0 μg of HBcAg. Ten days later pooled spleen cells (3.5×10^6 cells per ml) were cultured with or without HBcAg (0.05 μg/ml), and cell SNs were collected at days 3 (*A*) and 7 (*B*) and were analyzed for IgM, total IgG, IgG1, IgG2a, IgG2b, and IgG3 anti-HBc antibody by direct solid-phase ELISA. Data are reported as a reciprocal of the dilution of cell culture SNs to yield 3 times the OD reading of culture SN without antigen.

suggests that reduced Th-cell function is more limiting *in vitro* than *in vivo*. In any event, T-cell tolerance in HBeAg-expressing transgenic mice extends to Th cells as well as to proliferating T cells.

T-Cell Tolerance to HBcAg Is Reversible. Although B10.S Tg-31e mice are tolerant to HBcAg and HBeAg at the T-cell level indefinitely, the extent to which T-cell tolerance would persist in the absence of continued exposure to the tolerogen was examined. For this purpose, a neonatal tolerance model was used. Normal B10.S neonatal mice were injected subcutaneously with 40 μg of HBcAg in saline or with saline alone and were rested for at least 8 weeks. The HBcAg rather than HBeAg was used for this experiment to observe anti-HBc antibody production. Starting at 8 weeks of age and at 4-week intervals, groups of three neonatally HBcAg injected and control mice were immunized with HBcAg and the HBcAg- and p120-131-specific T-cell proliferative responses were determined. As shown in Fig. 5 *A* and *B*, T cells of B10.S mice injected as neonates with HBcAg and immunized at 8 and 12 weeks of age were highly tolerant to HBcAg, as demonstrated by minimal HBcAg- and p120-131-specific T-cell proliferative responses compared with control mice. It was also notable that mice tolerant to HBcAg at the T-cell level, nevertheless, produced anti-HBc antibody (data not shown). Therefore, even in mice neonatally exposed to a dose of HBcAg sufficient to induce T-cell tolerance, HBcAg-specific B cells were not tolerant. By 16 weeks of age the HBcAg-specific T-cell proliferative responses of B10.S mice injected as neonates with HBcAg were approximately the same as the control responses (Fig. 5*C*). Therefore, it appears that HBcAg-specific T-cell tolerance is an active process and requires the continued presence of the tolerogen to be maintained.

Nontransgenic Offspring of B10.S Tg-31e Mothers Demonstrate Reduced T-Cell Responsiveness. The observation that a single neonatal exposure to HBcAg was sufficient to render mice T-cell nonresponsive to HBcAg prompted us to examine the T-cell responder status of nontransgenic offspring of B10.S Tg-31e females, which may have been exposed *in utero* to HBeAg transplacentally. For this purpose, pregnant B10.S Tg-31e or control females were given zinc to increase the HBeAg serum concentration in B10.S Tg-31e females to ≈72 ng/ml. When the offspring were 6 weeks old, T-cell proliferative responses specific for HBeAg, p120-131, and the minor T-cell site p89-100 were determined (Fig. 6). As expected, HBeAg-expressing transgenic offspring demonstrated significant T-cell tolerance to HBeAg and to the constituent peptides (Fig. 6*A*). The nontransgenic littermates were not completely tolerant, as shown by T-cell proliferative responses to the entire antigen panel (Fig. 6*B*); however, the T-cell proliferative responses were significantly reduced compared with control mice (Fig. 6*C*) and were intermediate between control mice and transgenic mice. Interestingly, the T-cell tolerance demonstrated by mice exposed to HBeAg *in utero* is enhanced if the mother is also antibody (anti-HBc/HBe) positive (data not shown). This suggests a role for anti-HBc/HBe antibodies either directly or as a mechanism to transport HBeAg across the placenta.

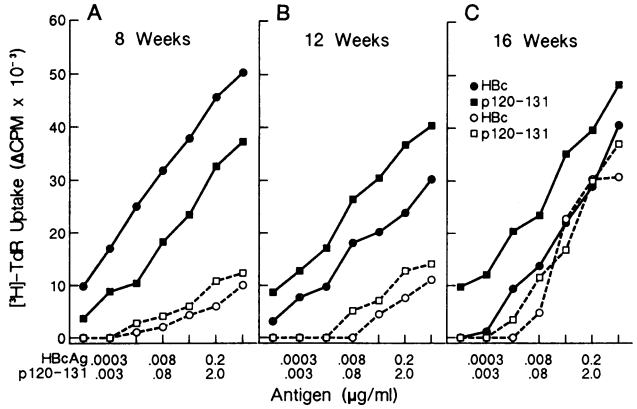

FIG. 5. The persistence of HBcAg-specific T-cell tolerance after a single neonatal dose of HBcAg. Neonatal tolerance was induced by the injection of newborn B10.S mice (<24 hr) with 40 μg of HBcAg in saline (open symbols) or with saline alone (solid symbols). At intervals of 8 (*A*), 12 (*B*), and 16 (*C*) weeks, groups of three experimental or saline control mice were immunized with 5.0 μg of HBcAg. Ten days later, T-cell proliferation induced by various concentrations of HBcAg and p120-131 was determined as described in Fig. 2.

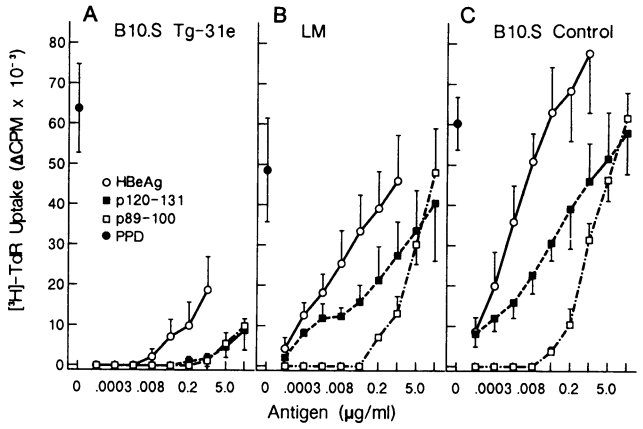

FIG. 6. T-cell responsiveness of nontransgenic offspring of B10.S Tg-31e females. B10.S Tg-31e or control B10.S females were treated with zinc during the last week of pregnancy. Groups of five of the transgenic (*A*), nontransgenic littermates (LM; *B*), and control (*C*) offspring were immunized at 6 weeks of age with HBeAg (5.0 μg), and T-cell proliferative responses were determined. Compared at antigen concentrations required for half-maximal stimulation of B10.S control T cells, the HBeAg- and peptide-specific T-cell responses of B10.S Tg-31 mice were statistically different from the LM ($P < 0.001$) and the control ($P < 0.001$) mice. Similarly, the HBeAg- and peptide-specific T-cell responses of the nontransgenic LM were statistically different from B10.S Tg-31e ($P < 0.001$) and control mice ($P < 0.01$) by Student's *t* test. The PPD-specific T-cell proliferative responses were not significantly different.

DISCUSSION

HBeAg-expressing transgenic mice represent a model system to examine the consequences of *in utero* exposure to HBeAg on HBcAg/HBeAg-specific immune responses. Characterization of HBeAg/HBcAg-specific tolerance in B10.S Tg-31e and neonatally injected mice indicated (*i*) T cells but not B cells are made tolerant by HBeAg present in the serum at a concentration of 10 ng/ml; (*ii*) T-cell tolerance elicited by HBeAg also extends to HBcAg-specific T cells; (*iii*) B10.S Tg-31e mice produce anti-HBc but not anti-HBe antibodies upon immunization; (*iv*) the IgG but not the IgM anti-HBc response is diminished in B10.S Tg-31e mice; and (*v*) the T-cell tolerance induced by a single neonatal exposure to HBcAg is reversible and persists for 12-16 weeks. It is of interest that many characteristics of immune tolerance demonstrated by B10.S Tg-31e mice parallel the long-term immunologic status of neonates born to HBeAg-positive HBV carrier mothers. For example, infants infected perinatally often remain HBeAg positive and produce anti-HBc but not anti-HBe antibodies. The correlation of the human serological observations with the murine experimental data suggest the hypothesis that the aberrant immunological responses of neonates born to carrier mothers may also result from *in utero* exposure to HBeAg as occurs in the transgenic model.

The finding that *in utero* exposure to HBeAg renders T cells of B10.S Tg-31e mice nonresponsive to HBcAg as well as to HBeAg may be relevant to mechanisms of T-cell

tolerance in human HBV infection because HBeAg is more likely than HBcAg to traverse the placenta. The HBeAg is a nonparticulate small molecular weight protein, which is secreted into the serum, whereas HBcAg is a particulate, intracellular antigen, which is present in serum at low levels. In support of the possibility that maternal HBeAg may traverse the placenta are the reports that HBeAg has been detected in neonate cord serum of 88% (25, 29), 63% (26), and 54% (27) of infants born to HBeAg-positive HBV carrier mothers. The particulate envelope antigen (HBsAg) was detected in cord serum with half or less than half the frequency of HBeAg (26, 27, 29). The presence of HBsAg in cord serum may indicate contamination with maternal blood, whereas HBeAg positivity in the absence of HBsAg suggests that HBeAg can cross the placenta. Placental transit of the intact HBeAg may not be necessary since a peptide fragment of HBeAg (p120-131) injected into neonatal B10.S mice has been shown to elicit T-cell tolerance to the entire HBeAg (28). It seems unlikely that HBsAg functions as a tolerogen *in utero* because a high percentage of infants born to HBeAg-positive mothers vaccinated with HBsAg at birth produce anti-HBs antibodies very efficiently (30). Consistent with the possibility that HBeAg may cross the placenta and induce tolerance *in utero*, the T-cell proliferative responses of nontransgenic offspring of B10.S Tg-31e mothers were reduced compared with control B10.S mice and were intermediate between transgenic and control mice. The lack of complete T-cell tolerance may reflect exposure to a suboptimal concentration of the tolerogen *in utero*. For example, B10.S Tg-31e mice treated with zinc express quantities of HBeAg detectable in serum at a dilution of 1:100, whereas HBeAg has been detected in maternal serum of HBV carriers at dilutions of 1:2000 to 1:8000 using the same ELISA (31).

Because HBeAg is a secreted protein, it most likely gains access to the thymus through the circulation. It can be predicted that this mode of antigen presentation within the thymus would lead to the functional deletion of major histocompatibility complex class II-restricted Th cells. We suggest that the basic immunologic "defect" in neonates born to HBeAg-positive carrier mothers resides in Th-cell tolerance specific for the HBcAg/HBeAg. Such tolerance would preclude Th-cell function necessary for anti-HBe and maximal IgG anti-HBc antibody production, and the ability of HBc/HBe-specific Th cells to elicit anti-envelope antibodies (32) would also be impaired. In addition, HBc/HBe-specific Th-cell tolerance may diminish development of a cytotoxic T-lymphocyte response, which may be required for the elimination of virus-infected hepatocytes.

Although infants born to HBeAg-positive HBV carrier mothers are at high risk for infection and subsequent viral persistence, the infants' age at the time of HBV infection has been shown to be inversely correlated with the rate of chronicity (13). In the context of an HBc/HBe-specific Th-cell tolerance model, this phenomenon can be explained by the reversibility of T-cell tolerance. In the murine system, it was shown that a single neonatal dose of HBcAg resulted in T-cell tolerance apparent at 8 and 12 weeks of age, but by 16 weeks tolerance had waned. Therefore, for T-cell tolerance to the HBcAg/HBeAg to be maintained, the tolerogen must be continually present. This suggests that in the absence of the tolerogen, HBc/HBe-specific thymocytes can emerge from the thymus, and this "repertoire renewal process" requires ≈16 weeks in this murine model. Similarly, in the human system the fetus may be exposed *in utero* to tolerogenic HBeAg but not infected at birth. The longer the elapsed time before HBV infection, the greater the probability of renewing the HBc/HBe-specific T-cell repertoire because the neonate would no longer be exposed to the tolerogen (HBeAg).

Cumulatively, the human epidemiological and serological observations and the murine experimental data suggest that a function of the HBeAg may be to induce T-cell tolerance *in utero*. Although infections in infants born to anti-HBe-positive mothers are rare, if infection occurs these infants usually demonstrate transient or fulminant rather than chronic infections (33). Indeed, expression of HBeAg may represent a viral strategy to persist in the host after perinatal infection, which would confer a selective advantage on the conservation of the precore domain. Further studies in this transgenic system and in animal models of hepadnavirus infection are now possible and will be necessary to confirm this hypothesis.

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