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Dendritic Cell Immunization Breaks Cytotoxic T Lymphocyte Tolerance in Hepatitis B Virus Transgenic Mice

Yukihiro Shimizu, Luca G. Guidotti, Patricia Fowler, and Francis V. Chisari

Hepatitis B virus (HBV) transgenic mice that are immunologically tolerant to HBV-encoded Ags represent a model of chronic HBV infection suitable for the development of therapeutic immunization strategies before testing in humans. Five lineages of HBV transgenic mice were immunized with plasmid DNA that encodes hepatitis B surface Ag (HBsAg) or with cytokine-activated bone marrow-derived dendritic cells (DCs) in an attempt to break tolerance to HBsAg at the B and T cell levels. DNA immunization stimulated an Ab response but not a cytotoxic T lymphocyte response to HBsAg in two of the five transgenic lineages studied. In contrast, infusion of activated transgenic or nontransgenic DCs stimulated a splenic CTL response in all three transgenic lineages immunized in this manner at precursor frequencies comparable to those in nontransgenic mice, indicating that DC function is normal, and HBsAg-specific CTLs are present but functionally silent in these transgenic animals. Importantly, none of the animals developed hepatitis or displayed suppressed viral gene expression or replication following either DNA immunization or DC administration even in the presence of anti-hepatitis B surface (HBs) Abs and HBs-specific CTLs. These results indicate that Ag presentation by cytokine-activated DCs can break tolerance and trigger an anti-viral CTL response in HBV transgenic mice, and they suggest that this strategy is more efficient than DNA immunization in this setting. Nonetheless, more efficient immunization strategies are needed to stimulate an immune response of sufficient quality and magnitude to achieve an immunotherapeutic antiviral effect. The Journal of Immunology, 1998, 161: 4520–4529.

Cytotoxic T lymphocytes are thought to contribute to hepatitis B virus (HBV) clearance by killing infected hepatocytes and by secreting antiviral cytokines (1–3). Acutely infected patients characteristically produce a vigorous, polyclonal, and multispecific CTL response that is usually sufficient to clear the infection, while persistently infected patients produce a weak or undetectable HBV-specific CTL response (1, 2). Based on these observations, therapeutic enhancement of the T cell response to HBV has the potential to terminate chronic HBV infection. The immunologic basis for impaired T cell responsiveness to HBV in chronically infected patients must be defined, however, for rational immunotherapeutic approaches to be designed. While thymic deletion is unlikely to be operative in neonatal HBV infection, the precise mechanisms responsible for diminished T cell responsiveness to HBV in persistently infected neonates and adults are not understood.

The availability of transgenic mice that express HBV envelope proteins (3) and of mice that express all the viral gene products and replicate HBV in their hepatocytes at levels comparable to those in chronically infected patients (4) affords the opportunity to study the mechanisms responsible for neonatal tolerance to HBV and to develop immunotherapeutic approaches that have the potential to terminate persistent infection. We have previously shown that transgenic mice from a lineage that expresses the HBV envelope proteins under the control of the mouse albumin promoter (lineage 107-5) are immunologically tolerant to hepatitis B surface Ag (HBsAg) at the T cell level despite immunization with recombinant vaccinia viruses that routinely induce HBsAg-specific proliferative and CTL responses in nontransgenic littermates (5). The current study was undertaken to examine the ability of other modes of immunization to break tolerance at the CTL level in these and other lineages of transgenic mice.

Plasmid DNA immunization has recently been shown to induce HBsAg-specific Ab and CTL responses in normal mice and chimpanzees (6–8), and it has been reported to induce anti-hepatitis B surface (HBs) production and to inhibit HBV gene expression in a unique lineage of HBV envelope transgenic mice in the absence of histologic evidence of hepatitis (9). Dendritic cells (DCs) are professional APCs (10, 11) that are distributed throughout the body and play a central role in Ag presentation to CD4+ and CD8+ T cells. DCs can be expanded from murine bone marrow cells with granulocyte-macrophage CSF (GM-CSF) and IL-4 (12, 13) and, when pulsed with Ag protein or peptide, can induce specific Ab and CTLs in vivo (14–20). Accordingly, activated DC immunotherapy has recently been studied in patients with melanoma (21) and malignant lymphoma (22).

Since activated DCs express high levels of costimulatory molecules and secrete inflammatory cytokines, they have the potential to activate anergic T cells. This idea is supported by the observation that simultaneous expression of B7-1 and a transgene-encoded viral protein on pancreatic islet cells could break tolerance and induce a CTL response against the viral (self) proteins with islet destruction after infection with the corresponding virus (23, 24). Although the thymus can eliminate self-reactive T cells in fetal and neonatal life (25), the foregoing reports and other observations (26–29) raise the possibility that peripheral tolerance might play...
an important role in the establishment and/or maintenance of immunologic tolerance. If so, HBsAg-specific CTLs might be present in these HBV transgenic mice (and possibly in persistently infected patients) but be anergized.

In the present study we attempted to break tolerance to HBsAg in several lineages of HBV transgenic mice by plasmid DNA immunization or by the administration of in vitro cytokine-activated, bone marrow-derived DCs. The results demonstrate that DC administration can induce a quantitatively normal HBsAg-specific CTL response in these transgenic mice, but DNA immunization cannot. Apparently, HBsAg (self)-specific CTL precursors have escaped negative selection in the thymus and are present, but unresponsive, in the transgenic mice, yet they can be activated by appropriate stimulation. Nonetheless, the CTLs failed to induce hepatitis or to suppress viral gene expression or replication, suggesting that although CTL tolerance can be broken by DC immunization more efficient strategies must be developed to generate a functionally effective CTL response in these animals and, by extension, in humans.

Materials and Methods

Transgenic mice

Five HBV transgenic mouse strains were used in this study. Lineage 107-5 (official designation, Tg[Alb-1, HBV] Br66; inbred B10.D2, H-2d) contains the entire HBV envelope open reading frame under transcriptional control of the mouse albumin promoter and produces the large, middle, and small envelope polypeptides, all of which contain HBsAg (30). Lineage MUP-Env1 (Tg[MUP; HBV]Ch1; C57BL/6 X B10.D2 F1) contains the entire HBV open reading frame downstream of the mouse major urinary promoter; however, transcription is controlled by the internal HBs promoter, resulting in the production of the middle and small HBV envelope polypeptides, both of which contain HBsAg. Lineage pFC80-219 (Tg[4HBV]Ch219; C57BL/6 X B10.D2 F1) contains four complete HBV genomes (aww subtype) linked in a tandem head to tail orientation at the HBV EcoRI site of pBR322, but only the HBs promoter is active, resulting in expression of the middle and small HBV envelope polypeptides (30–32). Lineages 1.3.3.46 (Tg [HBV 1.3 genome] Ch146; inbred B10.D2) and 1.3.32 (Tg [HBV 1.3 genome] Ch132; C57BL/6 X BALB F1) express all the HBV proteins and replicate the virus in the liver at levels comparable to those found in patients chronically infected by HBV (4). Age- and sex-matched nontransgenic B10.D2, C57BL/6 X B10.D2 F1, and C57BL/6 X BALB F1 mice were included as controls.

DC cultures and immunization

Bone marrow cells were collected from the femurs and tibiae of transgenic and syngeneic nontransgenic mice and suspended in RPMI 1640 supplemented with 10% FBS, 2 mM t-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Life Technologies, Gaithersburg, MD), and 50 μM 2-ME (Sigma, St. Louis, MO). DCs were expanded according to the method of Lu et al. (13) with minor modifications. Briefly, the cells (2 × 10^6/well) were seeded in 24-well plates (Corning, Conning, NY) in the presence of 5 ng/ml of recombinant GM-CSF (PeproTech, Rocky Hill, NJ) and cultured to allow activated DC or DC progenitors to aggregate and lightly attach to the wall. After overnight incubation, nonadherent single cells were gently removed by swirling the plates and aspirating the medium. New medium, supplemented with 5 ng/ml of GM-CSF and 50 U/ml of IL-4 (PharMingen, San Diego, CA), was added to the wells, and four-fifths of the medium was changed every 2 days. After 2 to 3 days of culture, clusters of expanding DC were observed, and each time the medium was changed, nonadherent small cells were removed by the procedure described above. On day 5 of culture, 1000 U/ml of TNF-α (Genentech, South San Francisco, CA) was added in addition to GM-CSF and IL-4, and the cells were used on day 7 for in vivo or in vitro analysis.

The activated DCs were either injected without further manipulation or were pulsed with 10 μg/ml of the immunodominant, L^a-restricted HBs_28–35 peptide plus 10 μg/ml of human β2m (Sigma) for 2 h at 37°C. Alternatively, the DCs were pulsed with recombinant preS2/S particles produced in stably transfected Chinese hamster ovary cells (obtained from Pasteur Merieux Institute, Val de Reuil, France) at a concentration of 1 μg/ml on day 5 and further pulsed with the same Ag (100 μg/ml) for 2 h at 37°C immediately before injection. For both preparations, the cells were washed three times, and 1 × 10^6 DCs were i.v. injected into the tail vein of each mouse.

Phenotypic analysis of DC surface markers

Surface markers of fresh bone marrow cells and cultured DCs were analyzed by flow cytometry on a FACSscan (Becton Dickinson, Mountain View, CA). Staining with anti-CD80, anti-CD86, anti-L^1, anti-L^2, anti-CD14 (all purchased from PharMingen, San Diego, CA), and NLCDC-145 (anti-DEC-205, provided by Dr. Ralph M. Steinman, Rockefeller University, New York, NY) was followed by incubation with anti-rat IgG2a or anti-rat IgG2b (PharMingen), and incubation with anti-Ly-6-C was followed by phycoerythrin-labeled streptavidin (Caltag, San Francisco, CA). Phycoerythrin-labeled anti-CD11a, anti-CD11c, anti-CD40, anti-CD44, anti-CD49d, anti-CD49f, anti-LPAM-1, and anti-CD54 (all from PharMingen) Abs were also used and analyzed by 6-color immunofluorescence microscopy. To block the Fc receptor-mediated binding of the Abs, anti-CD16/32 Abs (1 μg/10^6 cells; PharMingen) were included in all reactions.

DC presentation of an immunodominant HBsAg epitope to CTLs

Splenoc DC-enriched fractions were isolated by the method described by Saki et al. (33). Briefly, spleen cells were cultured without cytokines for 2 h, and nonadherent cells were removed by gentle washing with RPMI medium. The adherent cells were cultured further for 18 h to allow DCs to detach from the dish, and low density cells were collected by density gradient centrifugation with Histo-Paque (sp. gr., 1.077; Sigma). To examine the ability of DCs to present an immunodominant HBsAg epitope to CTLs, cytokine-activated bone marrow-derived DCs or freshly isolated splenic DCs from nontransgenic and transgenic mice were labeled with 5 μM Calcein AM and incubated with HBs-pulsed P815/S transfectants, or peptide (HBs_28–35)-pulsed P815 cells as target cells as previously described (35). The percent cytotoxicity was calculated as ((experimental release – spontaneous release)/(total release – spontaneous release)) × 100, and total and spontaneous release from wells containing 4%/v/v Triton X-100 or medium, respectively, were determined. The percent specific cytotoxicity shown in this paper reflects the HBsAg-specific cytotoxic activity minus the Ag-nonspecific cytotoxic activity observed against the P815 control cells. Transgenic mouse spleen cells that had been primed in vivo were depleted of Thy1.2^+, CD4^+ or CD8^+ cells in vitro by magnetic bead-conjugated Ab (Dynal, Lake Success, NY) and analyzed for the ability to kill P815/S targets or HBs_28–35 peptide-pulsed P815 cells in a 4-h 51Cr release assay.

Limiting dilution analysis to determine the frequency of HBsAg-specific CTL precursor in spleen cells

Quantitative analysis of HBsAg-specific CTL precursors (CTL-p) was performed by seeding 1,000, 3,000, 10,000, and 30,000 spleen cells/well in a 96-well round-bottom plate with irradiated (20,000 rad) P815/preS1 transfectants (1 × 10^5/well), irradiated (3,000 rad) syngeneic nontransfectant spleen cells (1 × 10^5/well), and 3% EL-4 supernatant in 24 replicates in RPMI 1640 containing 10% FBS and 50 μg/ml of human IL-4 for 5 to 7 days, and CTL activity was measured as described above. Alternatively, the DCs were pulsed with 10 μg/ml of the immunodominant, L^a-restricted HBs_28–35 peptide-pulsed P815 cells, or peptide (HBs_28–35)-pulsed P815 cells as target cells as previously described (35). The percent cytotoxicity was calculated as ([experimental release – spontaneous release]/total release – spontaneous release)) × 100, and total and spontaneous release from wells containing 4%/v/v Triton X-100 or medium, respectively, were determined. The percent specific cytotoxicity shown in this paper reflects the HBsAg-specific cytotoxic activity minus the Ag-nonspecific cytotoxic activity observed against the P815 control cells. Transgenic mouse spleen cells that had been primed in vivo were depleted of Thy1.2^+, CD4^+ or CD8^+ cells in vitro by magnetic bead-conjugated Ab (Dynal, Lake Success, NY) and analyzed for the ability to kill P815/S targets or HBs_28–35 peptide-pulsed P815 cells in a 4-h 51Cr release assay.

Passive transfer of DC-immunized spleen cells

Lineage 107-5 mice were immunized with HBs_28–35 peptide-pulsed nontransgenic bone marrow-derived DCs expanded in the presence of GM-CSF, IL-4, and TNF-α, and their spleen cells were harvested 7 days later. The cells were cultured with irradiated (20,000 rad) P815/preS1 transfectants (1 × 10^5/well), irradiated (3,000 rad) syngeneic nontransfection spleen cells (1 × 10^5/well), and 3% EL-4 supernatant in 24 replicates in RPMI 1640 containing 10% heat-inactivated FBS. After 8 days of culture the cytotoxic activity of each well was examined against P815 cells and P815/S transfectants, and the wells in which HBsAg-specific cytolytic activity exceeded the mean ± 3 SD of unimmunized spleen cell cultures seeded at the corresponding cell numbers per well were scored as positive. The CTL-p frequencies were calculated as described by Taswell (36).

DNA immunization

Fifty micrograms of plasmid pCMV-S2/S, which contains the preS2/S coding region of HBV under the transcriptional control of the CMV immediate
FIGURE 1. Developmental expression of HBV RNA. HBV gene expression in the liver and thymus in fetus (18 days gestation), newborn (day 1), and adult (6 wk) animals of five lineages of HBV transgenic mice analyzed by Northern blot. L, liver; T, thymus.

Serologic and biochemical analysis

Serum was examined for HBsAg, hepatitis B e Ag (HBeAg), and anti-HBs titers using commercially available reagents (AUSRIA II-125, Abbott Laboratories, Abbott Park, IL), and EBK II-125 IRIA kit, Incstar (Stillwater, MN), respectively) and HBsAg and HBeAg were quantitated by comparison with known standards, as previously described (39). Hepatocellular injury was monitored at various time points after immunization by measuring serum alanine aminotransferase (ALT) activity (40).

Histologic and immunohistochemical analysis

Histologic and immunohistochemical analysis

Tissue samples were fixed in 10% zinc-buffered formalin (Anatek, Battle Creek, MI), embedded in paraffin, sectioned (3 μm), and stained with hematoxylin and eosin as previously described (40). The intracellular distribution of hepatitis B core Ag (HBcAg) was assessed by the labeled avidin-biotin detection procedure and counterstained with Mayer’s hematoxylin before mounting (41).

DNA isolation and Southern blot

Southern blot analysis was performed on total liver DNA from lineages 1.3.32 and 1.3.46 by agarose gel electrophoresis of 20 μg of restricted genomic DNA as previously described (4). Before electrophoresis, all DNA samples were digested with RNase A (Boehringer Mannheim, Indianapolis, IN) at 10 μg/ml for 1 h at 37°C. Nylon filters were hybridized with an HBV-specific 32P-radiolabeled DNA probe as previously described (42).

Northern blot analysis

Frozen tissues were mechanically pulverized and extracted by the acid-phenol-chloroform method (52). Total RNA (20 μg) was analyzed for HBV, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and albumin expression by Northern blot as previously described (42).

Results

Developmental expression of HBV mRNA in liver and thymus of HBV transgenic mice

Total cellular RNA isolated from the liver and thymus of fetal (18 days gestation), newborn (day of birth), and 6-wk-old adult transgenic mice from five transgenic mouse lineages was analyzed for HBV envelope and pregenomic RNA by Northern blot analysis. As shown in Figure 1, the adult liver from all lineages expressed high levels of the appropriate HBV transcripts, while the thymus of the adult animals was uniformly negative. Specifically, the adult lineages from lineages 1.3.32 and 1.3.46 displayed the 3.5-kb pregenomic RNA and the 2.1-kb HBs RNA, while lineage 107-5 expressed the 2.4-kb preS1 and 2.1-kb HBs transcripts, and lineages 219 and ENV-1 expressed only the 2.1-kb HBs RNA. Importantly, the 2.4-kb and/or the 2.1-kb envelope RNA species were expressed in the fetal and/or newborn liver and thymus in all lineages except 1.3.32. Furthermore, the 3.5-kb RNA was detectable in fetal liver and/or thymus in lineages 1.3.32 and 1.3.46, and it was also detectable in the newborn thymus of lineage 1.3.46. Assuming that these transcripts were translated during fetal life or immediately after birth, neonatal tolerance to HBV envelope Ags would be expected in all lineages except 1.3.32.

DC immunization

Activation and phenotypic analysis of bone marrow and splenic DCs. Lineages 107-5 and 1.3.46 (inbred B10.D2) and lineage 1.3.32 (C57BL/6 × BALB F1) were immunized with cytokine-activated transgenic and nontransgenic bone marrow-derived DCs. Between 8 and 10 × 10^5 DCs were routinely expanded from the bone marrow after 7 days of in vitro culture in the presence of GM-CSF, IL-4, and TNF-α, and 6 to 8 × 10^5 freshly isolated splenic DCs were recovered from lineages B10.D2, 107-5, and 1.3.46. The expression of CD11c, DEC-205, I-A^d, CD86, and CD40 was up-regulated with those cytokines (Fig. 2A), and both the bone marrow-derived (Fig. 2A) and splenic (Fig. 2B) DCs expressed similar levels of those markers as well as CD1d, CD11a, CD44, CD49d, CD49f, CD54, LPAM-1, and Ly-6C (not shown) regardless of their transgenic or nontransgenic origin. Thus, it appears that HBV gene expression in these transgenic animals does not affect the expansion or expression of activation markers by their splenic or bone marrow-derived DCs. HBsAg presentation by bone marrow and splenic DCs. The ability of cytokine-activated, bone marrow-derived DCs and freshly isolated splenic DCs from transgenic and nontransgenic mice to process HBsAg and to present an immunodominant HBs peptide was examined by measuring the susceptibility of HBsAg-pulsed
DCs to cytolysis by an HBs28–39-specific, L4-restricted CTL clone. Initially, we showed that nontransgenic B10.D2 DCs process and present HBsAg to CTLs in a time- and cytokine-dependent manner, becoming susceptible to lysis within 30 min of Ag pulsing, with lysis increasing thereafter (Fig. 3A) and displaying optimal lysis when they were expanded in the presence of GM-CSF, IL-4, and especially TNF-α (Fig. 3B). Importantly, HBsAg-pulsed bone marrow-derived and splenic DCs from lineages 107-5 and 1.3.46 were as susceptible to lysis as B10.D2 DCs (Fig. 4), indicating that the Ag processing and presenting functions of the transgenic DCs are normal. Furthermore, we showed that freshly isolated splenic DCs from nontransgenic lineage B10.D2 and transgenic lineages 107-5 and 1.3.46.

FIGURE 2. Phenotypic analysis of bone marrow and splenic DCs. Flow cytometric analysis of phenotypic markers on A) bone marrow-derived nontransgenic DCs expanded in the presence of 5 ng/ml GM-CSF alone, 5 ng/ml GM-CSF plus 50 U/ml IL-4 or 5 ng/ml GM-CSF, 50 U/ml IL-4, plus 1000 U/ml TNF-α and transgenic (lineages 107-5 and 1.3.46) DCs expanded with 5 ng/ml GM-CSF, 50 U/ml IL-4, plus 1000 U/ml TNF-α, and on B) freshly isolated splenic DCs from nontransgenic lineage B10.D2 and transgenic lineages 107-5 and 1.3.46.
bone marrow-derived DCs from the same lineage were not killed without continuous HBsAg pulsing (Fig. 4), presumably because the half-life of MHC class I molecules is relatively short (43), and the HBsAg peptide-MHC complexes formed in vivo could turn over in the absence of Ag after 7 days of culture in vitro.

HBsAg-specific CTL induction. The ability of DCs to induce HBsAg-specific CTLs was examined 1 wk after i.v. injection of one million bone marrow-derived nontransgenic DCs into transgenic (lineages 107-5 and 1.3.46) and syngeneic nontransgenic (B10.D2) mice. HBsAg-specific CTL activity of harvested spleen cells was examined after 1 wk of in vitro restimulation with P815/preS1 transfectants. As shown in Figure 5, CTL activity against P815/ preS1 transfectants and HBs28–39 peptide-pulsed P815 target cells was induced in all three groups of DC-immunized mice, while unimmunized spleen cells showed no CTL activity, indicating that HBsAg-specific CTL activity detected in the immunized animals reflects in vivo priming. Interestingly, prior Ag pulsing was not necessary for activated DCs to induce CTL responses in the transgenic mice (Fig. 5), suggesting that the DCs can internalize and present the HBsAg present in vivo to indigenous, class I-restricted T cells in these transgenic animals. In blocking experiments we demonstrated that the HBsAg-specific CTL activity of the DC-induced transgenic and nontransgenic CTLs was mediated by Thy1.2+ CD8+ T cells (not shown). Interestingly, by limiting dilution analysis we showed that the HBsAg-specific CTL-p frequencies in both lineages of transgenic mice were comparable to those in nontransgenic mice (Fig. 5), indicating that CTL precursors are not deleted in these animals despite the likely expression of HBsAg before or immediately after birth (Fig. 1). Furthermore, we showed that CTL responses can be induced in transgenic recipients by injection of as few as 100 bone marrow-derived nontransgenic DCs expanded with GM-CSF and IL-4.

FIGURE 3. Ag presentation by in vitro HBsAg-pulsed nontransgenic DCs. HBsAg presentation of bone marrow-derived DCs to an HBs28–39-specific CTL clone was assessed by measuring its ability to lyse 51Cr-labeled DCs with or without pulsing with HBsAg in vitro. A, Time dependence of Ag presentation. DCs, expanded from B10.D2 bone marrow cells with 5 ng/ml GM-CSF, 50 U/ml IL-4, and 1000 U/ml TNF-α, were pulsed with HBsAg particles (100 μg/ml) for 30, 60, or 120 min or with HBs28–39 peptide (10 μg/ml) for 120 min and used as targets for an HBs28–39-specific CTL clone in a 4-h 51Cr release assay. Untreated DCs (open circles); DCs pulsed with HBsAg for 30 min (closed triangles), 60 min (closed diamonds), or 120 min (closed squares); and DCs pulsed with HBs28–39 peptide for 120 min as positive controls of the assay (closed circles) are shown. B, Cytokine dependence of Ag presentation. DCs were expanded from B10.D2 bone marrow cells with various cytokine mixtures and used as targets for an HBs28–39-specific CTL clone in a 4-h 51Cr release assay. Untreated DCs (open circles); HBsAg-pulsed DCs expanded with 5 ng/ml GM-CSF and 50 U/ml IL-4 (closed triangles), 5 ng/ml GM-CSF plus 500 U/ml IL-4 (closed squares); and DCs expanded with 5 ng/ml GM-CSF, 50 U/ml IL-4, plus 1000 U/ml TNF-α (closed circles) are shown.

FIGURE 4. Spontaneous Ag presentation by transgenic DCs. HBsAg presentation by bone marrow-derived or freshly isolated splenic DCs from HBV transgenic lineages 107-5 and 1.3.46 and nontransgenic B10.D2 mice. DCs are expanded from the bone marrow in the presence of 5 ng/ml GM-CSF, 50 U/ml IL-4, and 1000 U/ml TNF-α or are isolated from the spleen cells and used as targets for an HBs28–39-specific CTL clone with or without pulsing (2 h) with HBsAg particles (100 μg/ml) in a 4-h 51Cr release assay. Untreated bone marrow-derived DCs (closed circles), HBsAg particle-pulsed bone marrow-derived DCs (open circles), untreated splenic DCs (closed triangles), and HBsAg particle-pulsed splenic DCs (open triangles) are shown. The experiments were repeated three times for each DC population, and a representative experiment is shown.
and that transgenic recipients respond more strongly than non-transgenic recipients to small numbers of DCs (Fig. 6A). Importantly, we showed that cytokine-activated bone marrow-derived transgenic DCs can induce CTL responses as efficiently as non-transgenic DCs in nontransgenic and transgenic recipients (Fig. 6, B and C), indicating that transgenic DCs can function normally in vivo, in keeping with their phenotypic (Fig. 2, A and B) and Ag-presenting functional integrity (Fig. 4) in vitro. As shown in Figure

![Graph](image-url)

**FIGURE 5.** Immunogenicity of DC in transgenic and nontransgenic mice. The cytotoxic activity of spleen cell cultures from DC-immunized mice and unimmunized mice was determined. One million bone marrow-derived nontransgenic DCs, expanded with 5 ng/ml GM-CSF, 50 U/ml IL-4, and 1000 U/ml TNF-α, were incubated with HBs$_{28-39}$ peptide (10 µg/ml), HBsAg particles (100 µg/ml), or medium alone for 2 h, after which they were washed three times and i.v. injected into HBV transgenic mice lineages 107-5 and 1.3.46 and nontransgenic B10.D2 mice. Spleen cells harvested 1 wk after injection of HBs$_{28-39}$ peptide, HBsAg particle-pulsed DCs, untreated DCs or unimmunized spleen cells were cultured with irradiated (20,000 rad) P815/preS1 and 3% EL-4 supernatant as a source of IL-2 for 7 days. The cytotoxic activity of those cells was examined against those of P815, P815/S transfectant, and P815 plus HBs$_{28-39}$ in a 4-h $^{51}$Cr release assay, and HBsAg-specific cytotoxic activities are shown after subtracting the Ag-nonspecific CTL activity against P815 targets at the corresponding E:T cell ratios. The results represent the mean ± 1 SEM from three mice per group. The numbers in each box represent HBsAg-specific CTL precursor frequencies determined by limiting dilution analysis using P815 and P815/S target cells.

![Graph](image-url)

**FIGURE 6.** Relative immunogenicity of DCs from transgenic and nontransgenic donors in homologous and heterologous recipients. A, Varying numbers of bone marrow-derived DCs, expanded from nontransgenic B10.D2 mice with 5 ng/ml GM-CSF and 50 U/ml IL-4 and pulsed with HBs$_{28-39}$ peptide (100 µg/ml), were i.v. injected into lineages 107-5 and B10.D2. Spleen cells harvested 1 wk after DC immunization were cultured with irradiated (20,000 rad) P815/preS1 transfectants for 5 days, and CTL activity was measured in a 4-h $^{51}$Cr release assay using P815 cells and P815/S transfectants. The data are the means for two mice per group and are shown as HBsAg-specific cytotoxic activity at an E:T cell ratio of 25 by subtracting the Ag-nonspecific activity against P815 cells at the same E:T cell ratio. B, Transgenic DCs induce HBsAg-specific CTLs in B10.D2 nontransgenic (B) and 107-5 (C) recipients. One million bone marrow-derived DCs, expanded from lineages 107-5 and nontransgenic B10.D2 with 5 ng/ml GM-CSF and 50 U/ml IL-4, and pulsed with HBs$_{28-39}$ peptide (10 µg/ml), were injected i.v. into both groups of mice, and cytotoxic activity was examined after 5 days of in vitro stimulation with irradiated P815/preS1 transfectants. The data are the mean of two mice per group and are shown as HBsAg-specific cytotoxic activity by subtracting the Ag nonspecific cytotoxic activity against P815 targets at the corresponding E:T cell ratios.
however, the DC-induced CTL response in all three groups was transient, being strongest 1 to 2 wk after DC immunization and waning rapidly thereafter.

Serum HBsAg levels were monitored in 58 DC-immunized animals from lineages 107-5 (19 mice), 1.3.46 (36 mice), and 1.3.32 (three mice), and serum HBeAg was monitored in 19 DC-immunized animals from lineages 1.3.46 (16 mice) and 1.3.32 (three mice). Serum levels of HBsAg in the lineage 107-5 mice did not change compared with those in 18 unimmunized transgenic controls (not shown). Interestingly, serum HBsAg levels fell in 10 of 39 DC-immunized mice from lineages 1.3.46 and 1.3.32, some of which also produced anti-HBs Abs (not shown). Since age- and sex-matched mice from these lineages spontaneously produce anti-HBs, however, the role of DC immunization in the induction of anti-HBs in these animals is unclear. In contrast, serum HBeAg levels did not decrease in any of the transgenic mice (not shown).

Despite the ability of DC immunization to break CTL tolerance to HBsAg in these animals, histologic analysis of the liver 1, 2, or 4 wk after DC immunization displayed only a very slight focal inflammatory infiltrate in the hepatic parenchyma in occasional mice from lineage 107-5 mice, and no histologic abnormalities or elevated serum ALT activity were detected in lineages 1.3.46 or 1.3.32. Similarly, hepatic HBsAg and HBCAg expression (by immunohistochemical analysis), HBV RNA (by Northern blot analysis), and (in lineages 1.3.46 and 1.3.32) HBV DNA replicative intermediates indicative of viral replication (by Southern blot analysis) were unchanged when examined 1, 2, and 5 wk after DC immunization. A total of 17 DC-immunized and nine unimmunized animals from lineages 107-5 (eight and three mice, respectively), 1.3.46 (six and three mice, respectively), and 1.3.32 (three and three mice, respectively) were examined for down-regulation of viral gene expression and replication. No differences were observed between the immunized and unimmunized animals with respect to any of these parameters (not shown).

Table I. Passive transfer of DC-immunized spleen cells into HBV transgenic and nontransgenic mice

<table>
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<th>Recipient</th>
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</table>

HBV transgenic mice were immunized with 1 million HBs28–39 peptide-pulsed nontransgenic DCs, and the spleen cells were harvested 7 days later. The cells were cultured with irradiated P815/PreS1 cells and 3% EL-4 supernatant for 7 days, and 5 × 10^6 spleen cells were intravenously injected into unimmunized transgenic and nontransgenic B10.D2 mice (2 mice per group). Serum ALT was monitored, and all mice were sacrificed on day 2 for histologic analysis.

FIGURE 7. Transience of the CTL response to DC immunization. Kinetics of CTL induction after immunization with HBs28–39 peptide-pulsed DCs (A) and HBsAg particle-pulsed DCs (B). One million bone marrow-derived DCs from B10.B2 mice, expanded with 5 ng/ml GM-CSF, 50 U/ml IL-4, and 1000 U/ml TNF-α and pulsed with either HBs28–39 (10 μg/ml) or HBsAg particles (100 μg/ml), were i.v. injected into lineages 107-5, 1.3.46, and nontransgenic B10.D2 mice. Spleen cells harvested 1, 2 and 4, or 5 wk after DC immunization were cultured with irradiated (20,000 rad) P815/preS1 transfectants and 3% EL-4 supernatant as a source of IL-2 for 7 days, and CTL activity was measured in a 4-h ^51Cr release assay using P815 cells and P815/S transfectants. The data are the mean ± 1 SEM from three to five mice per each group and are shown as HBsAg-specific cytotoxicity by subtracting the Ag nonspecific activity against P815 cells at the corresponding E:T cell ratios.

FIGURE 8. Failure of DNA immunization to induce HBsAg specific CTLs in HBV transgenic mice. CTL induction in HBV transgenic and nontransgenic mice after DNA immunization. Spleen cells harvested 4 wk after DNA immunization were cultured with irradiated (20,000 rad) P815/preS1 transfectants and 3% EL-4 supernatant as a source of IL-2 for 7 days, and CTL activity was measured in a 4-h ^51Cr release assay using P815 cells and P815/S transfectants as target cells. The data are the mean ± 1 SD from three mice and are shown as HBsAg-specific cytotoxicity by subtracting the Ag nonspecific activity against P815 cells at the corresponding E:T cell ratios.

Serum HBsAg and HBeAg levels, liver histology, and HBV gene expression or replication

Serum HBsAg levels were monitored in 58 DC-immunized animals from lineages 107-5 (19 mice), 1.3.46 (36 mice), and 1.3.32 (three mice), and serum HBeAg was monitored in 19 DC-immunized animals from lineages 1.3.46 (16 mice) and 1.3.32 (three mice). Serum levels of HBsAg in the lineage 107-5 mice did not change compared with those in 18 unimmunized transgenic controls (not shown). Interestingly, serum HBsAg levels fell in 10 of 39 DC-immunized mice from lineages 1.3.46 and 1.3.32, some of which also produced anti-HBs Abs (not shown). Since age- and sex-matched mice from these lineages spontaneously produce anti-HBs, however, the role of DC immunization in the induction of anti-HBs in these animals is unclear. In contrast, serum HBeAg levels did not decrease in any of the transgenic mice (not shown).
Passive transfer of DC-immunized spleen cells

Spleen cells from cytokine-activated, bone marrow-derived, DC-immunized mice from lineages 107-5, 1.3.32, and 1.3.46 were cultured in vitro with P815/preS1 and 3% EL-4 supernatant for 7 days, after which 5 x 10^6 cells were i.v. injected into the same lineages of transgenic mice and syngeneic nontransgenic mice. As shown in Table I, DC-immunized spleen cells from lineage 107-5 mice induced more than a 30-fold increase in serum ALT activity after transfer into homologous 107-5 animals but not into syngeneic nontransgenic B10.D2 recipients. Histologically, the liver demonstrated marked mononuclear cell infiltrates, apoptotic hepatocytes, and necroinflammatory foci (not shown). These results demonstrate that CTLs induced in DC-immunized transgenic mice are cytopathic in vivo, suggesting that the absence of hepatitis in these animals is probably due to insufficient numbers of CTLs rather than to a functional defect in homing, Ag recognition, or effector activity.

DNA immunization

Groups of at least six animals from each of the five HBV transgenic lineages described in Figure 1 plus groups of three to five syngeneic nontransgenic controls were immunized one or more times with 100 μg of pCMV-S2/S in their regenerating tibialis anterior muscles 5 days after cardiotoxin injection. Serum HBsAg, anti-HBs, and ALT levels were monitored before and at weekly intervals thereafter, and mice were sacrificed for analysis of splenic CTL activity and hepatic HBV gene expression and replication 4 and 12 wk later. A single injection of pCMV-S2/S induced a rapid and vigorous Ab response to HBsAg in nontransgenic B10.D2 mice (not shown). In contrast, anti-HBs Ab responses were not detected in any of the transgenic mice until 4 wk after immunization, when one or two mice from lineages 1.3.32 and 1.3.46 displayed low titer Abs, and serum HBsAg levels fell to undetectable or nearly undetectable levels in the same two lineages. As indicated earlier, unimmunized mice from these two lineages spontaneously produce anti-HBs and clear HBsAg from the serum at this age (not shown), suggesting that the Ab response in these mice may not have been induced by DNA immunization.

Importantly, a rapid, reproducible, and vigorous HBsAg-specific splenic CTL response was detectable in all the nontransgenic B10.D2 controls but in none of the transgenic animals when they were examined 4 wk after a single DNA immunization (Fig. 8) or at 12 wk, i.e., 4 wk after boosting performed 8 wk after the primary injection (not shown). In keeping with these results, we did not observe any histologic evidence of liver disease or changes in the hepatic content of HBcAg, HBV RNA, or replicative HBV DNA intermediates in a total of 87 mice (five from lineage 1.3.46, six each from lineages 107-5 and ENV-1, 27 from lineage 219, and 43 from lineage 1.3.32) analyzed at 4 and 12 wk after immunization (not shown).

Relative immunogenicity of DC and DNA immunization

The relative abilities of DC and DNA immunization to induce HBsAg-specific CTLs were simultaneously assessed 2 wk after immunization in lineage 1.3.32 animals and in syngeneic nontransgenic controls. As shown in Figure 9, DC immunization induced a stronger CTL response than DNA immunization in nontransgenic mice. Importantly, HBsAg-specific CTLs were only induced in HBV transgenic mice by DC immunization.

Discussion

The mechanisms responsible for diminished immune responsiveness to HBV in chronically infected patients must be elucidated to understand the pathogenesis of chronic hepatitis and to develop immunotherapeutic strategies to terminate the infection. Since HBV has not been shown to inhibit Ag processing or presentation, cytokine production or responsiveness, or T cell function, immunologic tolerance to HBV is probably a multifactorial, host-determined event involving negative selection, anergy, exhaustion, ignorance, or defective APC function depending on the immunologic status of the host and the dose and route of the infection.

In this study transgenic mice that express HBV in the liver and/or thymus during fetal or early life were used as a model of neonatal HBV infection, the commonest cause of HBV persistence worldwide (44). Using this system, we showed that the mice fail to mount a CTL response to HBsAg after DNA immunization but they produce normal numbers of HBsAg-specific CTLs after the infusion of ex vivo activated DCs, even transgenic DCs. This implies that CTL precursors are present in normal numbers in these animals, which is surprising because they do not produce a CTL.
response to HBsAg either spontaneously or after DNA immunization. This is especially puzzling because the bone marrow-derived and splenic DCs from the transgenic mice express normal levels of adhesion molecules, homing receptors, and activation markers; display normal Ag processing and presenting activities; and can break tolerance and induce normal numbers of HBsAg-specific CTLs when as few as 100 cytokine-activated transgenic DCs are injected into syngeneic transgenic recipients. It is important to note that the activated DCs did not require prior pulsing with HBsAg to induce HBsAg-specific CTLs in the transgenic mice, implying that the activated transgenic DCs can internalize, process, and present HBsAg to CTL precursors in vivo. This is consistent with evidence that exogenous HBsAg can enter the class I processing pathway and induce class I-restricted CTLs in vivo in nontransgenic animals (45, 46). Indeed, in vitro pulsing of DCs with HBsAg supported this idea, since cytokine-activated DCs pulsed with HBsAg in vitro for only 30 min could be killed by HBsAg-specific CTLs, suggesting that cytokine-activated DCs have the ability to internalize HBsAg present in the circulation, and process and present the HBs immunodominant peptide rapidly after injection into HBV transgenic mice.

These results suggest that the indigenous resting DCs in the transgenic mice are not only phenotypically and functionally normal but, when activated, they have the potential to process and present circulating HBsAg to class I-restricted T cell precursors that are present in normal numbers in these animals. Collectively, these results suggest that CTL tolerance to HBsAg in our HBV transgenic mice reflects anergy or ignorance rather than T cell exhaustion or deletion. The results also suggest that to become activated the transgenic CTLs must receive signals that are not required by nontransgenic CTLs, and they suggest that although these signals are provided by the ex vivo-activated DCs, they are not provided by the resting indigenous DCs in these animals even after DNA immunization remains. It is possible that the transgenic CTLs require more efficient help or more effective costimulation than the nontransgenic CTLs, as might occur if HBsAg-specific TCR expression or activation is down-regulated in the transgenic mice due to constant interaction with Ag presented by nonprofessional APCs (e.g., hepatocytes). Indeed, the up-regulation of costimulatory molecules on the DCs might be crucial, since they deliver not only costimulatory signals but also survival signals to CTLs through up-regulation of Bcl-XL (47). Furthermore, costimulation of T cells is reported to lower the activation threshold of T cells (48) and decreases the time of commitment for T cell activation (49). It is also possible that the ex vivo activated DCs deliver Ag more efficiently to the lymphoid tissues for CTL induction than occurs either spontaneously or after DNA immunization. Additional studies will be required to test these various hypotheses. It will also be interesting to determine whether pharmacologic activation of transgenic DCs in vivo will be sufficient to induce a CTL response to HBV in these transgenic mice, because if similar mechanisms are operative in chronically infected patients, the current results suggest that cytokine-activated DCs from chronically infected patients might have immunotherapeutic value. The immunogenicity of this approach must be improved, however, because despite the fact that HBsAg-specific CTLs were induced in the transgenic mice by DC immunization, they did not cause liver disease or down-regulate HBV RNA expression or HBV DNA replication (50). This could be due to the induction of insufficient numbers of CTLs, the brief duration of the CTL response, defective trafficking of the CTLs to the liver, or the activation of low avidity CTLs. We favor the first two alternatives because viral clearance during the acute phase of many viral infections is associated with a sustained CTL response with splenic CTL-p frequencies of >1 in 100 T cells (23) while the CTL response to HBsAg induced by DC immunization was transient and only reached 1 in 6,000–20,000 at its peak. Functional CTL deficiencies, including their trafficking and homing potential, are unlikely, since DC-immunized transgenic spleen cells were able to cause hepatitis when injected into transgenic recipients. Therefore, we consider it most likely that a higher number of HBsAg-specific CTLs must be present for a longer period of time to cause hepatitis and to inhibit HBV gene expression and replication in this system. Although additional experiments are required to confirm this hypothesis, our results suggest that Ag presentation by activated DCs can trigger an anti-viral CTL response in HBV transgenic mice and that this strategy is more efficient than DNA immunization in this setting.

Additional experiments are clearly required to understand why CTL tolerance in these transgenic mice can be broken by DC immunization but not by plasmid DNA or recombinant vaccinia virus (5), both of which induce HBsAg-specific CTLs in syngeneic nontransgenic animals very efficiently (35, 51). Additional studies are also needed to understand the discrepancy between our results and a report by Mancini et al. (9) that DNA immunization can suppress hepatic HBsAg-specific RNA content in an independent lineage of HBsAg transgenic mice in the absence of evidence of a CTL response or inflammatory liver disease. Since DNA immunization did not reduce the hepatic HBV RNA content in the five HBV transgenic lineages we studied, it is possible that lineage-specific effects (e.g., transgene structure or integration site) unique to the transgenic mice used in our experiments rather than the virus-specific effects (i.e., inhibition of HBV gene expression by plasmid DNA-induced cytokine production) proposed by Mancini et al. (9), which should be shared by all of the lineages, may be responsible for the conflicting results. This idea is also supported by the fact that the genetic backgrounds of two of our lineages (219 and 1.3.32) and the lineage used by Mancini et al. are similar or identical and because the same plasmid (pCMV-S2S) was used under the same immunization conditions in their studies and ours. Until the basis for these conflicting results is resolved, generalizations regarding the value of DNA immunization for the treatment of chronic HBV infection (9) may be premature.

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