HBsAg/HLA-A2 transgenic mice: a model for T cell tolerance to hepatitis B surface antigen in chronic hepatitis B virus infection

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Abstract

A humanized murine model was developed to study T cell tolerance to the hepatitis B surface antigen (HBsAg) that is present in sera of hepatitis B virus chronic carriers. The HBsAg/HLA-A2 double-transgenic mice express a chimeric HLA-A2 MHC class I molecule and a high amount of the HBsAg in the liver that is secreted and present in sera during the animal’s lifetime. In these mice, injection of plasmid DNA encoding HBsAg induced a high frequency of CD8+ T cells secreting IFN-γ in the periphery, with in vitro cytolytic activity and specificity for two dominant HBs-specific HLA-A2-restricted epitopes. Nevertheless, the DNA-based immunization elicited neither Th1 nor Th2 CD4+ T cell responses. Despite a high concentration of HBsAg in sera, these mice developed an immunocompetent CD8+ T cell repertoire towards the viral self-antigen, whereas the CD4+ T cell repertoire was tolerized. In the absence of a CD4+ T cell response, the IFN-γ-secreting CD8+ T cells primed by DNA-based immunization were unable to exert their antiviral functions in vivo on liver cells expressing the transgene product. However, when pro-inflammatory stimuli were given before or after DNA-based immunization, the HBsAg was cleared from the serum. This effect was antibody dependent and associated with the detection of an HBs-specific Th1 CD4+ T cell response in the periphery. This model provides evidence that HBsAg displayed a strong tolerogenic effect on the CD4+ T cell compartment that is associated with a defect in CD8+ T cell effector functions in vivo.

Introduction

Transgenic models have been developed to study the mechanisms of tolerance and their implications for immune-mediated diseases. Expression of neo-self-antigens in the liver presents a particular interest because of the putative tolerogenic role of the liver in the immune response (1–3) and because of the direct access of hepatocytes to circulating T cells (4). In addition to central thymic selection, peripheral tolerance mechanisms that include deletion, anergy or ignorance have been defined. In situations where peripheral anergy or ignorance are involved, tolerance at the T cell level can be broken by using either viral infection, activated dendritic cell injection or DNA-based immunization (5, 6).

The mechanisms responsible for T cell hypo-responsiveness or tolerance to hepatitis B virus (HBV) proteins in chronic HBV infection are not completely understood. In chronically infected patients, the CD8+ T cell response is undetectable or weak and narrow in the periphery (7), and the CD4+ T cell response is much less vigorous than in patients with acute hepatitis (8, 9). In children born to infected mothers, HBV antigens are considered as self-antigens and this may explain the high rate of chronic infection in this population. Thus, in people who are chronically infected, the immune response should be switched from tolerance or peripheral anergy to priming of effector T cells. To achieve this goal, antigen-specific immunotherapy is a promising approach in inducing efficient cellular immune responses. Here we have developed a novel animal model for HBV chronic carriers. The hepatitis B surface antigen (HBsAg)/HLA-A2 mouse is a humanized lineage that has as unique MHC class I molecule, the human HLA-A2 molecule (10), and expresses HBV envelope proteins in hepatocytes. This lineage was derived from an HBsAg transgenic mouse (6), in which we have previously reported...
that immunological tolerance to HBsAg can be broken by injection of a DNA vector encoding HBsAg. The HBsAg/HLA-A2 mouse allows us to investigate the induction of CD8+ T cells with specificities comparable to those observed in humans in the presence of a high level of circulating antigen.

In this study, the role of HBsAg in T cell tolerance was further clarified. We showed that peripheral specific cytotoxic CD8+ T cells could be primed by DNA-based immunization in a tolerant environment, whereas HBsAg-specific CD4+ T cells were not detectable under the same experimental conditions. In the absence of specific Tc cells, these CD8+ T cells were unable to modulate HBsAg mRNA expression or to mediate immune injury in the liver. Clearance of circulating HBsAg by inducing anti-HBs antibodies (anti-HBs) was also not achieved. However, a pro-inflammatory signal, such as irradiation or CpG-containing oligonucleotides, led to a partial restoration of antiviral effector functions in vivo by enabling clearance of the antigen produced by the liver. Finally, we provided some evidence that, in this model, CD4+ T cell tolerance was maintained by the presence of circulating HBsAg.

Methods

Mice

The HBsAg/HLA-A2 double-transgenic mice were obtained from an intermediate HBsAg transgenic and MHC class I knockout lineage. HBsAg transgenic mice were previously described [E36 lineage (11)]. To obtain HBsAg transgenic and MHC class I knockout mice, males from the E36 lineage (6,11) were bred with MHC class I-deficient females (12) until a founder male knockout for the b2m gene (b2m±/±) and the Dd (Db±/±) MHC class I encoding genes and expressing HBsAg was produced. The inactivation of b2m gene and the Dd genes was confirmed by Southern blot. Then founder males were mated with mice transgenic for a chimeric HLA-A2 molecule (HHD) and deficient for mouse MHC class I molecules [HHD+/+ b2m±/± Dd±/± (10)]. This breeding resulted in the B8HHD lineage characterized by deficiency in mouse MHC class I molecules, low-level expression of human HLA-A2 MHC class I molecule on the surface of all cells including hepatocytes ([10] and data not shown) and expression of HBsAg in the liver. In this lineage, 50% of progeny expresses the HBV transgene due to the heterozygous status of the transgene. HBsAg+ HLA-A2+ mice were used as controls for immunization. All experiments involving animals were performed according to European guidelines.

DNA vectors and immunization

pCMV-S2.S (13) encodes the small (S) and middle (preS2+S) proteins of the HBV envelope (ayw subtype) under the control of the cytomegalovirus (CMV) immediate early gene promoter. The pMAS-core plasmid (a kind gift of H. L. Davis) used as control DNA encodes the HBV capsid. The plasmid DNA used for gene transfer was purified using anion-exchange chromatography columns (Qiagen, Hilden, Germany), dissolved in endotoxin-free PBS (Sigma, St Louis, MO) at 1 mg/ml and stored at or below -20°C.

HBsAg+ and HBsAg− littermate HLA-A2 female mice, 6–8 weeks old, were immunized 1 or 2 times at 2–3 weeks interval by intramuscular injection bilaterally into cardiotoxin-treated tibialis anterior muscles as previously described (14,15) with 100 μg of recombinant plasmid DNA. All intramuscular injections were carried out under anesthesia (sodium pentobarbital, 75 mg/kg, i.p.).

CpG oligodeoxynucleotide (ODN) (sequence 1826: 5′-TCCATGACGTCCTCAGGT-3′) or non-CpG control ODN (sequence 1982: 5′-TCCAGGACTCTCAGGT-3′) (16) were provided by Coley (Wellesley, MA) and were synthesized with a nuclease-resistant phosphorothioate backbone. ODN were injected i.p. (50 μg in 200 μl of PBS per mouse) 1 week after the DNA booster injection. Sublethal irradiation (500 rad) prior to immunization was performed 3 days before DNA injection, when required.

Serology

Blood was collected from mice by retrobulbar puncture using heparinized glass pipettes at various times before and after DNA injection or adoptive transfer, and sera recovered by centrifugation were assayed for anti-HBs antibodies by specific ELISA as previously described (17). The sera from DNA-immunized or non-immunized HBsAg/HLA-A2 transgenic mice were also used for detection of HBsAg by a commercial ELISA kit (Monolisa AgHBs Plus; Bio-Rad, Marnes la Coquette, France).

Hepatocellular injury was monitored biochemically as serum alanine transferase (ALT) activity using a commercial kit (Enzyline; BioMérieux, Lyon, France).

ELISPOT assay

IFN-γ-releasing cells were quantified ex vivo after a short-term peptide or protein stimulation as previously described (18). Freshly isolated splenocytes (1 × 10^6 cells/well) were incubated in anti-IFN-γ coated wells for 40 h using different antigenic stimulations in αMEM medium (Life Technologies, Cergy-Pontoise, France). Cells were incubated either with HBV-derived HLA-A*0201-restricted epitopic peptides (HBs183–191, FLLTRILTI; Aventis Pasteur, France; A183–191, FLLTTLRILTI; HBs348–357, GLSPTVWLSV and HBc18–27, FLPSDFPPSV, 2 μg/ml), with I-Aβ-restricted preS2-decapeptide (HBs126–138, RGLYFPAGGSSGG, 3 μg/ml), with HBc-derived peptide (HBc128–140, TPPATRPNNAPIL, 3 μg/ml) or with recombinant HBsAg particles produced in mammalian cells [3 μg/ml; Aventis Pasteur, France (19)]. Alternatively cells were infected with recombinant vaccinia virus encoding the large HBV envelope protein [rVVS1.S2.S (18)] at the multiplicity of infection of 1/1. Wells containing cells in culture medium or infected with wild type vaccinia virus were used as negative controls to evaluate background level. Spot-forming cells (SFC) were revealed with secondary biotin-conjugated antibody and with BCIP + NBT substrates (Promega, Madison, WI). Spots were counted in a double-blind fashion under a stereo binocular. The number of specific IFN-γ-secreting T cells was obtained by subtracting the number of spots in control wells from spots obtained after specific stimulation. The response measured by ELISPOT was considered significant when the number of specific spots was >10 spots and at least 2 times higher than background. Quantification of CD8+ T cells in splenocytes was performed.
by cytofluorometry analysis using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) with CellQuest software, following staining with FITC- or phycoerythrin-conjugated anti-mAb (PharMingen, San Diego, CA).

**Measurement of cytotoxic T lymphocyte (CTL) activity**

CTL activity was measured 2 weeks after DNA immunization on splenocytes stimulated in vitro with peptide for 1 week as previously described (18). Specific cytolytic activity of effector cells was tested in short-term $^5$Cr-release assay against either previously described (18). Specific cytolytic activity of effector on splenocytes stimulated in vitro. CTL activity was measured 2 weeks after DNA immunization. Measurement of cytotoxic T lymphocyte (CTL) activity was calculated in triplicate as

$\frac{\text{experimental} - \text{spontaneous release}}{\text{maximum} - \text{spontaneous release}} \times 100$

**CD4+ T lymphocyte subset fractionation and adoptive transfer**

Splenocytes obtained from individual naive or DNA-primed mouse were prepared for adoptive transfer by lysis of red blood cells in single-cell suspension. After 4 washes with RPMI medium, the remaining white blood cells were counted and resuspended in 200 µl of PBS. The CD4+ T cell subpopulation was isolated by positive selection using magnetic cell sorting (MACS; Miltenyi Biotec, Paris, France) with CD4+ microbeads. The purity of the CD4+ T cell was confirmed by cytofluorometry analysis. The percentage of undesirable CD8+ T cells population was <0.05%. The purified CD4+ T cells were pooled according to the type of immunization and resuspended in 200 µl of endotoxin-free PBS. CD4+ T cells (6–7 $\times$ 10^6 cells) were injected into the retro-orbital cavity of recipient mice that had been sublethally irradiated (500 rad) before transfer. Quantification of HBsAg-specific CD4+ T cells contained in the splenocyte preparation was performed by ELISPOT assay in parallel with cell transfer.

**Results**

**HBsAg/HLA-A2 double-transgenic mice**

In the HBsAg/HLA-A2 transgenic lineage, the transgene consists of a complete HBV genome deleted of the gene encoding nucleocapsid. This construct allows liver-restricted expression of the three envelope proteins under the control of internal viral promoter and enhancer. These proteins synthesized by the hepatocytes are assembled into 22-nm HBsAg-containing empty viral particles, which are highly secreted into the serum. The level of circulating HBsAg ranges from 1000 to 6000 ng/ml depending on the mouse for 6- to 8-week-old females. Expression of HBsAg lasts from birth and slowly decreases over time due to hormonal regulation of the transgene (20), and methylation of the transgene does not occur in this lineage over time (21). In this novel lineage, mice have a reduced CD8+ T cell population (1–6% of splenocytes) due to the expression of the HHD transgene as the only MHC class I molecule. However, this reduced number of CD8+ T cells does not constrain the width of the repertoire (18,22).

The non-immunized HBsAg/HLA-A2 mice are tolerant to the viral antigen encoded by the transgene. They have neither anti-HBs antibody nor liver damage (normal ALT level, data not shown).

**Induction of HBs-specific CD8+ T cells in HBsAg/HLA-A2 mice**

In the HBsAg transgenic parental E36 lineage we have previously shown that DNA immunization with a plasmid encoding HBsAg (pCMV-S2.S) was able to overcome tolerance to this antigen (6). However, in this C57 BL/6 genetic background, only one H-2d-restricted epitope was defined in mice immunized with HBsAg (23). In contrast, HBs-specific, HLA-A2-restricted epitopes were extensively studied both in acutely HBV-infected humans and in mice transgenic for HLA-A2 (18,24,25). Therefore, the HBsAg/HLA-A2 double-transgenic mice could be useful to further define the functions and the fine specificity of CD8+ T cells involved in breaking tolerance to HBsAg after DNA-based immunization.

After two injections of pCMV-S2.S plasmid, a high frequency of HBV-specific IFN-γ-secreting CD8+ T cells was induced in HBsAg+ and HBsAg− mice (Fig. 1A). Spot-forming CD8+ T cells reacting with two HLA-A2-restricted epitopes (HBs183–191 and HBs348–357) were detected in all animals of both groups without any in vitro amplification. These two epitopes have been previously referred to as superdominant, due to the high rate of specific response induced in all pCMV-S2S-immunized HLA-A2 mice (18). Higher frequencies of CD8+ T cells specific for the peptide HBs348–357 were induced by DNA-based immunization in HBsAg+ compared to HBsAg− mice (0.8 and 0.4% of CD8+ T cells respectively, Fig. 1A). These CD8+ T cells were long lasting as they were still detected 10 weeks post-immunization (data not shown). No significant response was observed in non-immunized mice even in animals expressing HBsAg (see Fig. 1A).

To analyze the capacity of CD8+ T cells from pCMV-S2.S-immunized HBsAg+ mice to recognize epitopes derived from the processing of endogenously produced antigen, we performed IFN-γ ELISPOT with recombinant vaccinia virus expressing the large HBV envelope protein. We have previously shown that infection of splenocytes with this virus allowed presentation of epitopes recognized by CD8+ T cells only (18). Comparison of responses obtained from either HBsAg+ or HBsAg− mice provided evidence that CD8+ T cells primed in the periphery by DNA immunization recognized murine spleen cells presenting epitopes derived from the endogenous processing with the same efficiency (Fig. 1B). In addition to the secretory function of CD8+ T cells induced by DNA immunization in HBsAg+ and HBsAg− HLA-A2 mice, their ability to mediate lysis was also studied. Following 1 week of in vitro peptide stimulation, splenocytes from either HBsAg+ or HBsAg− HLA-A2 immunized mice were able to lyse autologous targets presenting the two superdominant epitopes (i.e. HBs183–191 and HBs348–357) with comparable efficiency (Fig. 1C). Because a high frequency of CD8+ T cells recognizing epitopes derived from HBsAg was found in the periphery, we asked if these cells could have mediated the lysis of liver cells. No significant elevation of transaminase activities was found in sera taken sequentially after DNA immunization or after booster injection (data not shown).
In this new animal model DNA immunization with pCMV-S2.S vector induced peripheral HBV-specific cells with the full characteristics of autoreactive T cells: (i) high frequency, (ii) secretion of IFN-\(\gamma\), (iii) recognition of endogenously processed antigen and (iv) \textit{in vitro} lytic activity. However, these cells seemed unable to lyse transgene-expressing liver cells. These results suggest that spleen-derived CD8\(^+\) T cells primed by DNA-based immunization, although fully competent \textit{in vitro}, were functionally tolerant \textit{in vivo} to the viral antigen expressed by liver cells.

### Ability of HBsAg/HLA-A2 mice to develop T\(_{h1}\) response

To measure CD4\(^+\) T cells primed by DNA immunization and hence determine whether the inefficient function of CD8\(^+\) T cells could be associated with a defect in T\(_n\) response, we performed IFN-\(\gamma\) or IL-4 ELISPOT using either HBsAg recombinant particles or I-A\(^b\)-restricted epitope present in the preS2 domain of the middle HBV envelope protein as stimulating antigens. These antigens were previously shown to stimulate CD4\(^+\) T cells in particular (18). In HBsAg\(^+\) mice, IFN-\(\gamma\)-secreting CD4\(^+\) T cells recognizing native antigen or preS2-specific I-A\(^b\)-restricted peptide were induced by pCMV-S2.S immunization, whereas in HBsAg\(^+\) mice, neither HBs-specific IFN-\(\gamma\)-secreting nor IL-4-secreting CD4\(^+\) T cells were detected (Fig. 2A and data not shown). ELISPOT assay performed with T cells from non-immunized mice did not detect specific CD4\(^+\) T cells.

To determine if the failure in induction of the HBs-specific CD4\(^+\) T cell response in HBsAg/HLA-A2 mice immunized with HBsAg-encoding DNA was due to a global defect in the CD4\(^+\) T cell response, we immunized mice with a plasmid encoding an irrelevant antigen. HBsAg\(^+\) and HBsAg\(^-\) HLA-A2 transgenic mice were injected twice with pMAS-core plasmid encoding the HBV core protein. The HBsAg\(^+\) mice do not express this...
antigen because the viral genome used as a transgene has been deleted of the core gene (11). IFN-γ-secreting CD4+ and CD8+ T cells were tested by ELISPOT assay using HBc128±140- and HBc18±27-derived peptides as stimuli, corresponding to the well-described I-Aβ- and HLA-A2-restricted epitopes respectively (26,27). Two weeks after the second injection, all mice mounted core-specific CD4+ and CD8+ T cell responses. The frequencies of core-specific CD4+ and CD8+ T cells were not significantly different between HBsAg+ and HBsAg- mice (Fig. 2B). These results suggested that in HBsAg/HLA-A2 transgenic mice there is a specific defect in priming HBs-specific CD4+ T cells after pCMV-S2.S immunization. This defect could be due to a specific tolerance in the CD4+ T cell compartment.

To further analyze if the CD4+ T cell tolerance was spread over all preS2-specific epitopes, we studied the Th1 response induced by pCMV-S2.S immunization towards the complete preS2 region of the HBV middle protein. For this purpose we used overlapping peptides covering the preS2 region as stimulator peptides in ELISPOT assay (Fig. 3A). We analyzed the T1 response after two pCMV-S2.S immunizations in HBsAg+ and HBsAg- HLA-A2 transgenic mice. As described above, HBsAg+ mice mounted a response against native HBsAg and the dominant I-Aβ preS2 peptide (preS2126-138) (Fig. 3B), whereas HBsAg+ mice did not. In addition, SFC were detected using the preS2109-134 peptide. Regarding the HBsAg+ HLA-A2 transgenic mice, a T1 response specific for preS2139-163 peptide could be detected in four of seven HBsAg+ HLA-A2 transgenic tested mice following DNA-based immunization. In contrast, in HBsAg- HLA-A2 transgenic mice, a specific response against preS2139-163 peptide was detected in only two of nine mice and at a lower frequency. These results suggest that CD4+ T cells specific for the subdominant epitope could be stimulated by DNA immunization in HBsAg+ HLA-A2 transgenic mice, whereas CD4+ T cells specific for dominant epitopes were tolerized.

HBsAg clearance did not occur in HBsAg/HLA-A2 mice following DNA-based immunization

In the E36 parental lineage of HBsAg transgenic mice we have previously shown that HBs-specific CD4+ and CD8+ T cells induced by DNA immunization were able to control HBs mRNA expression in hepatocytes via an IFN-γ-dependent mechanism (28). In addition, CD4+ T cells also contributed to the clearance of circulating HBsAg in the sera by providing help to HBs-specific B cells. To determine if pCMV-S2.S-primed CD8+ and CD4+ T cells have the same antiviral effect in the context of another MHC class I molecule, HBsAg/HLA-A2 mice were bled and the HBsAg concentration in the sera was measured by specific ELISA. Non-immunized HBsAg/HLA-A2 mice were followed as a control. A natural decrease of HBsAg in the sera related to age of mice was observed in the control group (20) (Fig. 4). A significant 2-fold decrease in circulating HBsAg was observed in DNA-immunized mice during the first 2 weeks following DNA immunization and then the rate of decrease of the antigen did not differ from controls (Fig. 4). However, the HBsAg in the sera of DNA-immunized mice remained consistently lower than in non-immunized transgenic mice. Even immunization followed by a booster injection did not induce the complete clearance of HBsAg, but only led to a drop in the HBsAg level after each injection (data not shown). Northern blot analysis performed on mRNA extracted from liver of DNA-immunized mice showed no decrease in HBV mRNA either (data not shown).

Thus, in HBsAg/HLA-A2 transgenic mice, HBs-specific IFN-γ-secreting CD8+ T cells present in the periphery are unable to control viral mRNA expression in the liver and this correlated with HBsAg persistence in the sera.

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Clearance of HBsAg following adoptive transfer of specific CD4+ T cells occurred only in irradiated mice

In HBsAg/HLA-A2 transgenic mice, DNA immunization was not efficient at inducing the clearance of viral antigen and that correlated with a partial tolerization of HBs-specific CD4+ T cells (see Figs 2 and 3). To further define the role of CD4+ T lymphocytes in this model, we performed adoptive transfer of pCMV-S2.S-primed CD4+ T cells from HBsAg- HLA-A2 transgenic mice into HBsAg+ HLA-A2 transgenic mice as recipients.

CD4+ T cells (5–6 × 10^6) purified from either pCMV-S2.S-immunized or naive HBsAg- mice were injected i.v. into their sublethally or non-irradiated HBsAg+ littermates. When HBs-specific CD4+ T cells were injected in non-irradiated HBsAg+ HLA-A2 transgenic mice as control, no significant decrease of circulating HBsAg was observed (Fig. 5). Although competent for IFN-γ secretion and for recognition of different HBs-related epitopes, these HBs-specific CD4+ T cells were unable to help for the production of anti-HBs antibodies and for the subsequent clearance of HBsAg in non-irradiated mice.

Interestingly, in irradiated mice receiving CD4+ T cells purified from pCMV-S2.S-immunized mice, a dramatic decrease of circulating antigen was observed in all mice from weeks 5 to 10 and the HBsAg level remained negative for at least 12 weeks (Fig. 5). Transfer of only 1200 HBs-specific IFN-γ-secreting CD4+ T cells per mouse, as quantified by ELISPOT performed on donor cells, was sufficient to mediate antigen clearance. One of the mice receiving CD4+ T cells from pCMV-S2.S-immunized mice had a slower decrease in HBsAg level, which could be ascribed to a partial loss of T cells at the time of injection or to the highest level of HBsAg in serum among recipient mice at the day of transfer. No significant decrease of the HBsAg level was observed in mice receiving unprimed CD4+ T cells. The immune responses of mice receiving CD4+ T cells were monitored. At week 12 post-transfer, no CTL were detected in recipient mice receiving either pCMV-S2.S primed or unprimed CD4+ T cells. In contrast, specific antibodies were detected from week 5 post-transfer in all mice receiving HBs-specific CD4+ T cells. The long time required (5 weeks) for the clearance of the antigen may correspond to the time needed for B cell restoration and their activation by circulating HBsAg together with the transferred CD4+ T cells.

These results show that HBs-specific CD4+ T cells primed in a non-transgenic environment could break peripheral tolerance in this model. The CD4+ T cells primed by DNA immunization in HBsAg- mice did not activate specific cytotoxic T cells, but acted by helping B cells to produce

Fig. 3. PreS2-specific T1 responses in HBsAg+ and HBsAg- HLA-A2 mice. (A) PreS2 amino acid sequence and peptides used to stimulate T cells. (B) Groups of seven HBsAg+ (filled bars) and nine HBsAg- (open bars) HLA-A2 mice were immunized as in Fig.1. Specific IFN-γ-secreting CD4+ T cells were detected after stimulation with preS2 overlapping peptides or recombinant HBsAg particles. Results are given as the frequency of IFN-γ SFC/10^6 splenocytes and calculated as mean ± SEM.
specific antibodies in the recipient HBsAg transgenic mice. Nevertheless, the HBs-specific CD4+ T cells require sublethal irradiation to mediate the clearance of circulating antigen.

Irradiation of HBsAg/HLA-A2 mice restored an efficient T cell response

We further investigated if irradiation could restore in vivo the antiviral functions of T cells primed by DNA-based immunization in HBsAg/HLA-A2 mice. DNA immunization was performed 2 days after sublethal γ-irradiation of mice and HBsAg levels were measured. The pMAS-core plasmid was injected in HBsAg+ irradiated mice as a positive control for immune response induction. No HBs-specific T lymphocytes were detected by ELISPOT in mice receiving pMAS-core DNA. However, IFN-γ-secreting CD4+ and CD8+ T cells specific for core antigen were detected 6 weeks after immunization (see Fig. 2B and data not shown). This indicates that restoration of the T lymphocyte population had occurred in irradiated animals, allowing initiation of CD4+ and CD8+ T cells responses following immunization with an irrelevant antigen. This also shows that production of antigen by cells transfected by plasmid DNA lasted long enough to activate new thymic emigrant cells.

No significant decrease of HBsAg was observed during the first 2 weeks in mice that were irradiated prior to pCMV-S2.S immunization (Fig. 6). Then, HBsAg strongly decreased in the sera of most mice and complete clearance of HBsAg was even observed in half of them (Table 1). The delay in the response may correspond to the restoration and priming of a new lymphocyte population after irradiation. In contrast, in irradiated mice immunized with the pMAS-core irrelevant plasmid and in non-irradiated mice immunized with pCMV-S2.S, the HBsAg level remained constant in the sera. In irradiated mice immunized with pCMV-S2.S DNA, HBs-specific IFN-γ-secreting CD8+ T cells were present from week 6 to 12 post-immunization in all tested mice (Table 1). Regarding the induction of an HBs-specific T,1 CD4+ response, mice could be classified in two groups. HBs-specific CD4+ T cells were detected by ELISPOT assay at the time of sacrifice (i.e. 6 or 12 weeks) in two mice, while no HBs-specific CD4+ T cells were detected in the three other mice. Interestingly, the serum concentration of HBsAg in the first group was <10 ng/ml or undetectable and anti-HBs antibodies were detected (Table 1). These results show that irradiation of mice prior to immunization leads to the emergence of an immune response with antiviral properties. Clearance of HBsAg in the sera and concomitant detection of a preS2-specific CD4+ T cell response in individual mice suggests that these cells provided help to B cell for anti-HBs antibody production.

Injection of immunostimulatory sequences interfered with T cell tolerance in DNA-immunized HBsAg/HLA-A2 mice

Because total-body irradiation was described to act as a pro-inflammatory signal marked by production of tumor necrosis factor (TNF)-α, IL-6 and IL-1, we next investigate if injection of
a strong stimulator of Th1 responses could promote the rupture of CD4+ T cell tolerance to HBsAg and the subsequent clearance of HBsAg in DNA-immunized HBsAg/HLA-A2 mice. We used immunostimulatory sequences containing CpG motifs that we had previously shown to strongly activate B cells to produce antibodies, and to induce Th1 cytokines secretion such as TNF-α, IL6, IFN-γ and IL-12 (17). Non-CpG ODN were used as negative controls. HBsAg+ mice were injected twice with pCMV-S2.S DNA to induce a high frequency of CD8+ T cells. One week after the booster injection CpG ODN were injected i.p. In mice receiving CpG ODN, a decrease in the HBsAg level was observed 1 week after the injection (Fig. 7A). In two out of five mice, HBsAg became undetectable 2–3 weeks after CpG ODN injection. In the three other mice HBsAg decreased significantly, but remained at an intermediate level for at least 4 weeks. In control mice receiving either DNA vaccine and non-CpG ODN or CpG ODN alone, a slow decrease in circulating HBsAg was observed (Fig. 7B and C). Immunization with DNA followed by CpG or non-CpG ODN injections induced production of anti-HBs antibodies (Fig. 7D and E), but higher titers were observed in mice receiving CpG ODN (Fig. 7D). The adjuvant effect of non-CpG ODN on B cells was previously observed in other studies and could be due to some adjuvant properties of the ODN phosphorothioate backbone (17,29). In addition, antibodies were higher in mice with a strong decrease in HBsAg.

Fig. 6. Irradiation of HBsAg+ HLA-A2 mice prior DNA-based immunization. HBsAg (ng/ml) in sera of mice that were sublethally or non-irradiated prior to a single injection of pCMV-S2.S or of pMAS-core DNA. HBsAg concentration is given for individual irradiated pCMV-S2.S-immunized mice (each type of closed symbol stands for one mouse). HBsAg concentration given as mean value for four non-irradiated pCMV-S2.S-immunized mice (open circles) and for three irradiated pMAS-core-immunized mice (open squares).

No significant HBs-specific CD4+ T cells were induced in mice receiving control ODN, as the number of spots remained under the threshold level. In contrast, in mice receiving the CpG ODN, significant frequencies of IFN-γ-secreting CD4+ T cells were detected in three of five immunized mice (Fig. 8B). This helper response was detected using whole HBsAg particles and preS2-derived peptide in individual mice. Detection of specific CD4+ T cells correlated with the strongest decrease of circulating antigen.

Thus, in HBsAg/HLA-A2 transgenic mice, DNA immunization combined with CpG ODN injection induced a decrease of circulating HBsAg and a concomitant production of specific antibodies, which correlated with specific CD4+ T cell detection. In this model, a pro-inflammatory signal is required for the emergence of a detectable CD4+ T cell response that was inhibited in the periphery by the presence of a high level of HBsAg.

Discussion

We have developed a novel murine model for HBV chronic infection, HBsAg/HLA-A2 double-transgenic mice, in order to

Table 1. HBsAg titers and HBs-specific immune responses in HBsAg+ HLA-A2 mice irradiated or not prior immunized with pCMV-S2.S DNA

<table>
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<tr>
<th>Irradiation</th>
<th>HBsAg (ng/ml)</th>
<th>HBs-specific CD4+ T cells</th>
<th>HBs-specific CD8+ T cells</th>
<th>Anti-HBs antibody (liter)</th>
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<td>&lt;10d</td>
<td>11d</td>
<td>360</td>
</tr>
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*HBsAg concentration measured in serum at the time of sacrifice.

*IFN-γ-secreting CD4+ T cells/10^6 spleen cells measured by ELISPOT performed on fresh splenocytes using the preS2-derived I-A^b-restricted peptide (HBs126-138) as stimulator antigen. Ten spots were considered as the cut-off value.

*IFN-γ-secreting CD8+ T cells/10^6 spleen cells measured by ELISPOT performed on fresh splenocytes with the Hbs-derived HLA-A2-restricted peptide (HBs348-357) as stimulator antigen. Ten spots were considered as the cut-off value.

*Mice killed at 12 weeks post-immunization (all other mice killed 6 weeks post-immunization).
study the tolerance to HBsAg expressed in the liver from before birth and highly secreted into blood, and (ii) have an animal model closely related to neonatally acquired chronic infection in which therapeutic approaches could be assessed. In this model, tolerance of both T cell subsets and their synergy to clear the circulating viral antigen were studied. Contrary to most of the studies performed using mice expressing a unique TCR directed to a self-epitope encoded by a transgene, the HBsAg/HLA-A2 transgenic mice displayed a wide HLA-A2 MHC class I-restricted CD8⁺ T cell repertoire, as previously shown in single HLA-A2 transgenic mice (18,22,30). They also display the natural H-2b MHC

**Fig. 7.** CpG or non-CpG ODN injection in pCMV-S2.S-immunized HBsAg⁺ HLA-A2 mice. Groups of mice were immunized twice intramuscularly with pCMV-S2.S plasmid at days 0 and 21 (closed arrows) and received one i.p. injection of 60 μg CpG (A and D) or non-CpG (B and E) ODN at day 27 (open arrows). A group of non-immunized HBsAg⁺ mice received the CpG ODN only (C). HBsAg (A–C) and anti-HBs antibody (D and E) were detected in sera by specific ELISA, and titers are given for individual immunized animals.

**Fig. 8.** IFN-γ-secreting T cell responses following CpG or non-CpG ODN injection in HBsAg⁺ HLA-A2 mice. Splenocytes were harvested from mice described in Fig. 7 at 4 weeks after ODN injection. (A) Frequencies of IFN-γ-secreting CD8⁺ T cells were detected using HLA-A-restricted peptides HBs₃₄₈₋₃₅₇ and HBs₁₂₆₋₁₃₈ as stimulator peptides in ELISPOT assay. (B) Frequencies of IFN-γ-secreting CD4⁺ T cells were detected using the preS2-derived I-Aβ-restricted peptide (HBs₁₂₆₋₁₃₈) or HBsAg particles in ELISPOT. Results given as mean values of IFN-γ SFC/10⁶ splenocytes. Level of HBsAg (ng/ml) at the time of sacrifice given for each individual mouse. HBsAg levels of mice displaying specific CD4⁺ T cell responses are in bold. Status of mice immunization is indicated on each panel.
Overcoming T cell tolerance in HBsAg/HLA-A2 transgenic mice

class II-restricted CD4+ T cell repertoire. Because they express the viral transgene from birth and because the amounts of HBsAg in the serum are similar to those found in humans, the HBsAg/HLA-A2 animal model will be useful to understand how the high concentration of HBsAg in the sera of HBV chronic carriers regulates the immune response, in turn impairing the viral clearance.

HBV-specific T cell responses have been related to clearance of the virus in acutely infected patients (31). It was shown in different models that replication of HBV or other hepatavirus replication is regulated by Th1 cytokines such as IFN-α/β, IFN-γ and TNF-α (32–34). In HBsAg transgenic mice, in duck infected at birth with DHBV and in WHBV-infected woodchuck, DNA-based immunization was shown to induce strong T1-type responses that resulted in viral elimination (6,35,36).

In the novel humanized model for chronic hepatitis B infection described here, DNA immunization with a plasmid encoding HBsAg induced a high frequency of HBV-specific CD8+ T cells in the periphery even in the presence of a high level of antigen in the sera. These T cells shared the same characteristics with those detected in patients resolving HBV infection (37,38). However, these CD8+ T cells were unable to exert their antiviral effector functions in vivo against hepatocytes expressing this antigen and thus failed to control viral expression in the liver. Inducing such CD8+ T cells could be considered as a mechanism of silencing antiviral immune responses during chronic infection.

In our animal model, it is clear that thymic clonal deletion of autoreactive CD8+ T cells does not occur since HBs-specific CD8+ T lymphocytes were primed by DNA-based immunization despite the presence of a high level of circulating HBsAg. The CD8+ T cell functions were very similar to those induced by the same immunization protocol in an HBsAg+ transgenic littermate. The rupture of CD8+ T cell tolerance by DNA-based immunization could result from antigen presentation by APC in the muscle after cross-priming (39), direct production of antigen in APC by plasmid transfection (40) or from conditioning of APC by CpG immunostimulatory sequences present in the plasmid backbone (41). The only difference seen in the DNA-immunized HBsAg+ mice compared to their HBsAg− counterparts was an enhancement of their frequency for the two dominant epitopes (Fig. 1A). This increased frequency could be explained by stimulation of DNA-primed T cells by HBsAg-derived peptides presented on transgenic hepatocytes, by the MHC class I alternative pathway for processing and presentation of exogenous HBsAg in peptides (42) or by activation of pre-existing, but anergized HBs-specific CD8+ T cells in HBsAg+ mice. However, this difference was not observed when recombinant vaccinia virus expressing HBV envelope proteins was used in the ELISPOT assay (Fig. 1B). This suggests that additional envelope-derived epitopes presented by cells infected with the vaccinia virus were recognized in HBsAg− mice. In the HBsAg/HLA-A2 mice, the induction of cytotoxic CD8+ T cells in the periphery does not result in autoimmune disease nor in an efficient control of the transgene expression as was previously observed in other mouse models for HBV chronic infection (6,43,44). Compared to the HBsAg parental transgenic lineage (6), this discrepancy could be ascribed to the low level of MHC class I molecules on the cell surface due to HHD transgene expression (10) resulting in a less efficient recognition between CD8+ T cells and hepatocytes expressing antigen. However, a defect in presentation of the two dominant epitopes described here on transgenic hepatocytes cannot be formally excluded. Another property of the HBsAg/HLA-A2 mouse is its low number of total CD8+ T cells (10) that is clearly below the threshold required for efficient control of HBV mRNA expression in the liver (44,45). Remarkably, we show here that this inefficient CD8+ T cell response was also associated with a defect in the CD4+ T cell response.

In the CD4+ T cell compartment, the tolerance appeared to be more established compared to CD8+ T cells. Although a shift from a T1 to T2 response was shown to be a mechanism of tolerization to prevent destruction of the organ in models in which T1 responses are related to autoimmune disease (46–48), neither T1 nor T2 HBs-specific CD4+ T cells were detected by ELISPOT in DNA-immunized HBsAg/HLA-A2 mice. Nevertheless, HBs-specific CD4 T cell responses could be restored by sublethal irradiation or CpG ODN injection in the DNA-immunized HBsAg/HLA-A2 mice, resulting in an efficient antiviral immune response and subsequent clearance of circulating HBsAg. This result indicates that HBs-specific CD4+ T cells were not clonally deleted in thymus, but rather tolerized in the periphery. Irradiation or CpG ODN injection have been involved in breaking tolerance of T cells to viral antigen (3,49,50). Irradiation mediates massive cell apoptosis and inflammation resulting in part in T cell migration into the liver (50). Since CpG motifs are known as pro-inflammatory mediators, they could have mimicked effects of γ-irradiation (3). One possible hypothesis is that, in immunized HBsAg/HLA-A2 mice, pro-inflammatory mediators conditioned the microenvironment of the liver leading to migration of DNA-primed specific CD8+ T cells. This migration could result in the partial control of the transgene expression by IFN-γ secreted by CD8+ T cells and that correlates with the transient sharp decrease of HBsAg observed in the serum after DNA-based immunization (see Fig. 4). After sublethal irradiation or CpG ODN injection, emergence of the CD4+ T cell response was only observed in mice displaying a strong decrease of viral antigen in serum. These results suggest that a high level of circulating HBsAg maintained CD4+ T cell tolerance. These CD4+ T cells might have a crucial role in maintaining the number or in post-licensing HBs-specific peripheral CD8+ T cells (51,52). CD4+ T cells have also been involved in helping activated CD8+ T cells to migrate into the liver and to secrete regulatory cytokines (51). In addition, we showed here using adoptive transfer experiments that CD4+ T cells activate HBsAg-specific B cells to produce anti-HBs antibodies. These antibodies could, in turn, mediate the decrease of HBsAg in serum of mice by immune complex formation.

In this novel animal model for HBV chronic carriers, we have shown that the role of circulating HBsAg in the maintenance of T cell tolerance was crucial. Although induced in the periphery by DNA immunization, HBs-specific polyclonal CD8+ T cells displayed no effector functions in the liver. In patients, a low-frequency population of HBs-specific CD8+ T cells with altered tetramer binding and surviving in the presence of a high quantity of HBsAg was recently described (53). In our animal model, restoration of CD4+ T cells via irradiation or CpG ODN
References


