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DNA-mediated immunization in a transgenic mouse model of the hepatitis B surface antigen chronic carrier state

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ABSTRACT **Transgenic mice expressing the sequences coding for the envelope proteins of the hepatitis B virus (HBV) in the liver have been used as a model of the HBV chronic carrier state. We evaluated the possibility of inducing a specific immune response to the viral envelope antigens and thus potentially controlling chronic HBV infection. Using HBV-specific DNA-mediated immunization in this transgenic model, we show that the immune response induced after a single intramuscular injection of DNA resulted in the complete clearance of circulating hepatitis B surface antigen and in the long-term control of transgene expression in hepatocytes. This response does not involve a detectable cytopathic effect in the liver. Adoptive transfer of fractionated primed spleen cells from DNA-immunized mice shows that T cells are responsible for the down-regulation of HBV mRNA in the liver of transgenic mice. To our knowledge, this is the first demonstration of a potential immunotherapeutic application of DNA-mediated immunization against an infectious disease and raises the possibility of designing more effective ways of treating HBV chronic carriers.**

Hepatitis B virus (HBV) is the most common etiologic agent for infectious liver disease. Following initial infection, some individuals fail to resolve their infection and thereby become chronic carriers. The proportion of HBV-infected persons proceeding to the chronic carrier state is 5–20% of those infected as adults and as high as 95% with perinatal transmission such as occurs in areas where HBV is endemic. There are estimated to be more than 250 million chronic HBV carriers in the world today and there is a significant association between persistent infection and liver cirrhosis or hepatocellular carcinoma (1). The control of HBV infection is thought to be mediated by both humoral and cellular immune responses involving neutralizing antibodies as well as class I and class II major histocompatibility complex (MHC)-restricted T cells (2–4). Patients who successfully clear the virus following acute infection mount a multispecific polyclonal immune response to several HBV antigens, whereas those who remain persistently infected develop only a weak and more restricted antiviral response (5–9). In infected newborns, a diminished antiviral immune response that may be due to neonatal tolerance mechanisms (see ref. 10) probably plays an important role in viral persistence. In chronically infected patients empty viral particles carrying the HBV surface antigen (HBsAg) are produced and secreted in large amounts by the hepatocytes, whether or not the virus replicates in the liver. These particles persist in the serum and the corresponding HBsAg-specific neutralizing antibodies (anti-HBs) are either not induced or remain undetectable by conventional techniques due to the formation of immune complexes (11).

We have used transgenic (Tg) mice that constitutively express the HBsAg (12) as a model of chronic HBV carriers to study the possibility of inducing an immune response to HBsAg and thus control the infection. The transgene in these mice consists of a copy of the HBV genome with the core gene deleted. The sequences encoding the small, middle, and large HBV envelope proteins under the control of an endogenous HBV promoter are expressed principally in the liver from before birth (13). As a consequence, the serum contains 200-9000 ng/ml of HBsAg, but there is no accumulation of HBsAg within hepatocytes or liver pathology. We have previously shown that B-cell nonresponsiveness can be overcome in these mice by immunization with recombinant HBsAg particles in Freund's adjuvant (14). These findings provided the rationale for a pilot clinical study, in which we have demonstrated that specific vaccine therapy by standard anti-HBV vaccination can reduce HBV replication and circumvent nonresponsiveness to circulating HBsAg in half of the chronic carrier subjects (15).

DNA-mediated immunization has been shown to be an effective way to induce both humoral and cell-mediated immune responses against many different antigens (for reviews, see refs. 16 and 17) including HBsAg (18–20). In the experiments described here, we have investigated the potential of this approach for immunotherapy of chronic HBV carriers using the HBsAg transgenic mouse as a model. We show that this novel method of immunization is able to overcome functional tolerance to HBsAg and to control transgene expression by a mechanism that does not involve a detectable cytopathic effect on the liver.

MATERIALS AND METHODS

Mice. The generation and characterization of the HBV envelope transgenic mouse lineage E36 have been previously reported (12, 13). These mice were produced on a C57BL/6 × SJL/J background and were back crossed against C57BL/6 (H-2^b) at least 15 times before use. Only female mice, 5–8 weeks old, heterozygous for the HBV envelope transgene and their nontransgenic littermates were used. All experiments involving mice were carried out in accordance with institutional guidelines.

DNA Immunization. Each mouse was injected on a single occasion with 100 μg recombinant plasmid DNA expressing the S and pre-S2 domains of the gene encoding the HBV

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Abbreviations: HBV, hepatitis B virus; Tg, transgenic; MHC, major histocompatibility complex; HBsAg, HBV surface antigen; IL, interleukin; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; APC, antigen presenting cell; CTL, cytotoxic T lymphocyte.

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envelope protein (pCMV–S2.S) (20) or the *Escherichia coli LacZ* gene encoding β-galactosidase (β-gal) (pCMV–LacZ) (21). The DNA was purified by anion-exchange chromatography (Qiagen, Hilden, Germany) and was injected directly into regenerating tibialis anterior muscles as described (22).

Serology. Blood was collected from anesthetized mice by retrobulbar puncture using heparinized glass pipettes and HBsAg was measured in the plasma using a commercial ELISA kit (Monolisa AgHBs; Diagnostics Pasteur, Marnes la Coquette, France). Quantitation of mouse anti-HBs antibody was performed by an ELISA using purified recombinant particles of the same subtype (*ayw*) with or without the middle HBV envelope protein (14). For isotype determination, serial dilutions of plasma from immunized mice were added to HBsAg-coated wells (1 μg/ml) and bound antibodies were detected by anti-mouse subclass-specific biotinylated antibodies followed by streptavidin-horseradish peroxidase staining (Amersham). Antibody titers were determined by the serial end-point dilution method. The end-point was defined as the highest serum dilution that resulted in an absorbance value two times greater than that of nonimmune or control serum, with a cutoff value of 0.05. Serum alanine aminotransferase activity in the plasma was measured using a commercial kit (Enzyline; BioMérieux, Marcy P'etoile, France).

Histological Procedures. Tissues were fixed in alcoholic Bouins' fixative. Sections of paraffin-embedded tissues were cut at 5 μm thickness and stained with hematoxylin/eosin and trichrome.

Northern Blot Analysis. Total RNA of liver was extracted from mechanically pulverized frozen tissue by the hot-phenol procedure (13). The RNA (50 μg) was fractionated on a 1.2% formaldehyde/agarose gels and blotted onto nylon membranes, which were then hybridized with [^{32}P]DNA probes synthesized from HBV or β-actin DNA fragments using the Megaprime DNA labeling system (Amersham).

Adoptive Transfer of Spleen Cells. Splenocytes obtained from individual naive or primed mice were prepared for adoptive transfer by lysis of red blood cells in single cell suspension (incubation with Tris-buffered ammonium chloride for 5 min at 4°C). After four washes with RPMI 1640 medium, the remaining white blood cells were counted and resuspended in 200 μl of PBS. Approximately 5–10 × 10^7 cells were injected into the retroorbital cavity of recipient mice that had been sublethally irradiated (500 rads) immediately before transfer. For transfer of subpopulations of B or T cells, each recipient was injected with cells obtained from a single spleen.

Lymphocyte Subset Fractionation. T and B cells were separated from the total spleen cell population by nylon wool adherence (23). Adherent B cells were incubated with a rat anti-mouse Thy-1 (Valbiotech, Paris) and Low-Tox guinea-pig complement (Cedarlane Laboratories) to eliminate T cells. B cells were removed from the T-cell population by incubating the cells with sheep anti-mouse IgG-coated magnetic beads (Dynal, Great Neck, NY). The purity of the T- and B-cell populations was monitored by immunolabeling with an anti-Thy1.2-R-PE and an anti-B220-FITC (Tebu, Le Perray en Yvelines, France). These procedures resulted in <0.3% contaminating cells, as assessed by flow cytometry.

Cytokines Production. Spleen cell suspensions from pCMV-S2.S-immunized Tg or non-Tg mice were obtained as described above. About 7 × 10^6 cells/ml were cultured for 72 hr with different concentrations of HBsAg particles (3 and 0.3 μg/ml), preS2 synthetic peptide (AA 121–133 *ayw* subtype, 10 and 1 μg/ml), or medium alone. Culture supernatants were collected each day, and concentrations of interleukin 2 (IL-2), IL-4, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) were determined by ELISA using commercial kits (Genzyme).

RESULTS

DNA Immunization of Tg Mice. We have previously described a mouse model of DNA-mediated immunization to HBV that involves intramuscular injection of plasmid DNA expression vectors encoding different HBV envelope proteins (18, 20). Injection of a vector encoding the small and the middle forms of HBV envelope protein (pCMV-S2.S) into normal mice induced a strong and long-lasting humoral response to the preS2 domain of the middle protein and to subtype- and group-specific HBsAg determinants (20).

A single injection of this pCMV–S2.S DNA into HBsAg-transgenic mice provoked a decrease in the levels of circulating HBsAg (Fig. 1) and the concomitant appearance of anti-HBs antibodies which increased over time (Fig. 2). In some of the mice, antigen was eliminated from the serum as early as 4 weeks after injection of the DNA and in all the mice remained undetectable for at least 12 weeks without further injections of DNA. These effects were not due to nonspecific immune stimulation induced by the injection procedure or the presence of DNA *per se*, since HBsAg levels were unaffected (Fig. 1) and no anti-HBs was detected (Fig. 2) in control Tg mice injected with PBS alone or a DNA vector expressing β-gal (pCMV–LacZ) (21), even though the latter procedure induced high levels of anti-β-gal antibodies (ELISA titers >10^5 by 12 weeks postimmunization).

Free antibodies, which were first detectable in the plasma of Tg mice 2–4 weeks following injection of pCMV–S2.S DNA, were initially preS2-specific since they reacted only with particles carrying this epitope but not with particles devoid of it (Fig. 2). Antibodies against S-specific epitopes were not observed until 8 weeks, at which time there was a complete clearance of circulating HBsAg (see Fig. 1). It is clear that, even though Tg mice demonstrate high levels of circulating HBsAg from before birth (13), DNA-based immunization was able to induce titers of anti-HBs comparable to those induced in non-Tg controls and

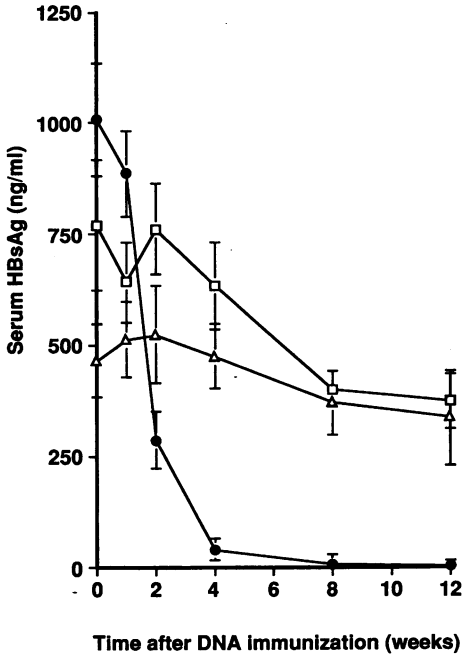

FIG. 1. DNA-based immunization of Tg mice. Groups of six female Tg mice were immunized once by intramuscular injection of 100 μg of the DNA plasmid pCMV–S2.S (●) or pCMV–LacZ (□) 5 days after cardiotoxin treatment. A group of eight Tg mice were injected with PBS instead of DNA (nonimmunized controls, △). Mice were bled at weekly intervals and the sera were analyzed for HBsAg (expressed as ng/ml). Each point represents the mean titer for the group, and error bars represent the SEM.

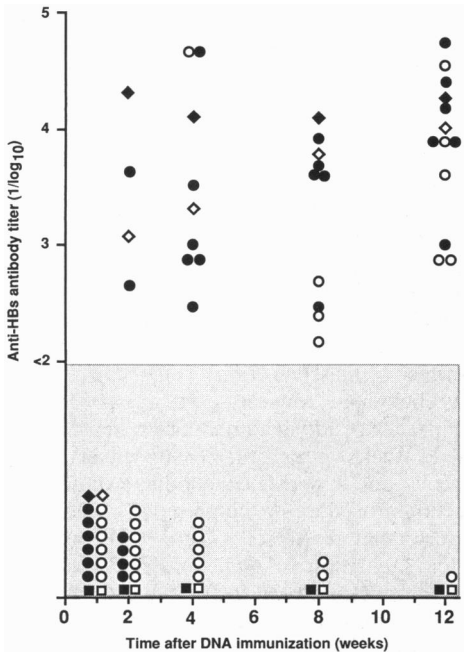

FIG. 2. Kinetics of appearance of anti-HBs antibodies in mice following injection of pCMV–S2.S DNA. Sera were obtained as in Fig. 1, and the fine specificity of the antibodies was determined by using HBsAg particles containing either the S (open symbols) or the S plus preS2 domains (filled symbols). Anti-HBs antibodies (Ig) were expressed as $1/\log_{10}$ of the antibody titer (determined by serial end-point dilution analysis). Circles represent the immunized Tg mice shown in Fig. 1, diamonds are non-Tg immunized mice, and squares are Tg mice injected with pCMV–LacZ. The symbols in the grey area correspond to mice which gave no detectable seroconversion (titer $<10^2$).

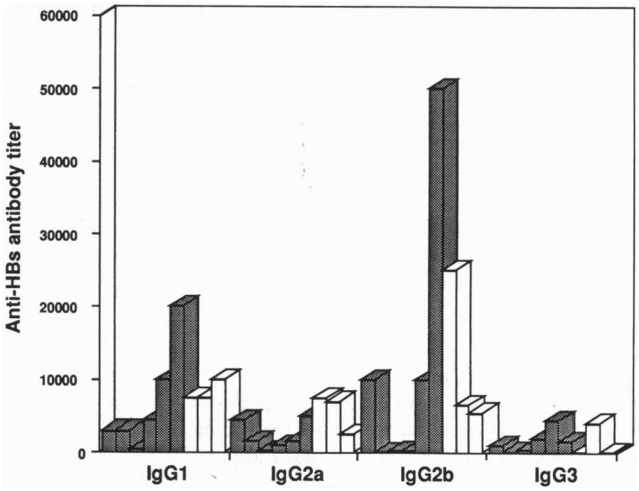

FIG. 3. Anti-HBs IgG isotype profile in the sera of six individual Tg mice (filled columns) and three non-Tg mice (open columns) at 12 weeks after immunization with pCMV–S2.S DNA. HBsAg-specific IgG1, IgG2a, IgG2b, and IgG3 antibodies were detected by ELISA with specific secondary antibodies. Antibody titers are expressed as a serial end-point dilutions.

that these antibodies were able to completely neutralize the circulating HBsAg. The isotype profile of the anti-HBs antibodies determined at the time of Tg mice seroconversion (12 weeks) was identical in Tg and in non-Tg mice and included IgG2a and IgG2b as well as IgG1 with some IgG3 (Fig. 3). The presence of these isotypes indicates that DNA-mediated immunization triggered $CD4^+$ T-helper (Th) cells.

To further characterize the T-helper subset, we analyzed cytokine production from spleen cells in culture at the time of initiation of the immune response. Spleens were removed from mice at 2 weeks after DNA injection and cell suspensions were specifically stimulated *in vitro* with HBsAg particles or preS2 peptide. The cytokine profile was identical for immunized Tg and non-Tg mice. These cultures produced IFN-γ and TNF-α, but no detectable IL-4 (Table 1). The presence of IgG2a antibodies and of IFN-γ-secreting spleen cells is consistent with a ongoing Th1 response; however, detection of IgG1 at the later times (12 weeks) suggests that a Th2 response has also been induced.

Assessment of Liver Cell Injury. To assess the effect of DNA immunization on the liver, the serum level of transaminases were measured after injection of DNA at the times indicated on Fig. 1, when the sera where taken for detection of anti-HBs. The rapid clearance of circulating antigen from immunized mice did not appear to result from a significant or persistent HBsAg-specific cytopathic effect on the liver since levels of transaminase activity in the plasma remained normal subsequent to injection of DNA. Furthermore, histological analysis of the liver was carried out at 12 and 20 weeks after DNA injection when HBsAg was cleared in all mice. Microscopic examination of liver sections showed no evidence of necrosis or inflammation.

Regulation of Transgene Expression. Since the persistent clearance of the transgene product was not associated with any apparent destruction of the transgene-expressing liver cells, we evaluated the HBV mRNA content in the livers of Tg mice. At 12 weeks after immunization with pCMV–S2.S, the mRNA was decreased in the livers of those mice which had partially cleared the antigen, and was undetectable in those which had completely eliminated HBsAg from their sera (Fig. 4*A*, lanes 5 and 6–7, respectively). This effect is persistent since HBV mRNA remained undetectable in the livers of mice analyzed 20 weeks after DNA injection (Fig. 4*A*, lanes 8 and 9). In contrast, HBV mRNA was not diminished in livers taken from

Table 1. Secretion of cytokines by spleen cells in culture

Mice	Cytokines	Medium	ConA	preS2 peptide	HBsAg
Non-Tg	IFN-γ	2 ± 1	1611 ± 377	92 ± 42	60 ± 28
	TNF-α	28 ± 13	846 ± 87	267 ± 23	58 ± 17
	IL-2	2 ± 1	2584 ± 233	3 ± 2	4 ± 2
	IL-4	6 ± 4	62 ± 15	4 ± 4	8 ± 6
Tg	IFN-γ	2 ± 2	1709 ± 12	329 ± 227	39 ± 25
	TNF-α	28 ± 12	871 ± 13	405 ± 137	83 ± 46
	IL-2	1 ± 1	4136 ± 578	1 ± 1	1 ± 1
	IL-4	11 ± 8	49 ± 3	12 ± 6	11 ± 9

Splenocytes of pCMV–S2.S-immunized Tg and non-Tg mice were incubated with medium or stimulated with concanavalin A (ConA; 2.5 μg/ml), preS2 peptide (10 μg/ml), or HBsAg particles (3 μg/ml) for 72 hr. Antigen-specific culture supernatants were harvested for determination of cytokine levels (pg/ml) at 24 hr for TNF-α and IL-2 determinations and at 48 hr for IFN-γ and IL-4. Data are as the arithmetic mean ± SEM of 5–6 spleens independently tested in two experiments.

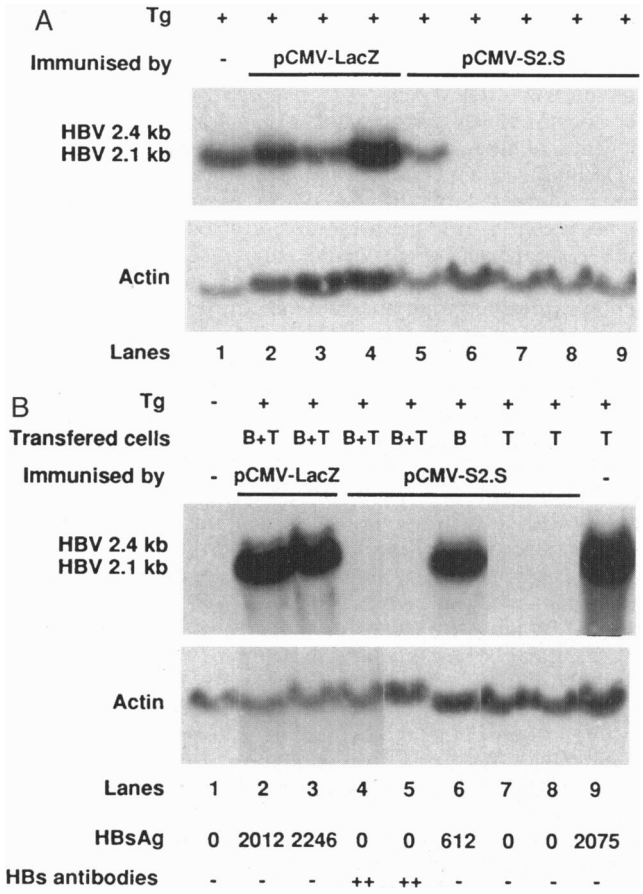

FIG. 4. Expression of HBV mRNA in the livers of Tg and non-Tg mice. Northern blot analysis was carried out on 50 μg of total RNA isolated from the livers of Tg mice (+) or their non-Tg littermates (−) after direct injection of DNA (*A*) or at 26 days after adoptive transfer of primed spleen cells (*B*). ^{32}P-labeled DNA probes specific for HBV and β-actin were used. The molecular weights (in kb) of the two mRNAs encoded by the transgene are indicated. (*A*) Lanes: 1, nonimmunized Tg mouse; 2–4: pCMV–LacZ immunized Tg mice; 5–9, Tg mice immunized with pCMV–S2.S DNA. (*B*) Tg mice receiving primed spleen cells harvested from non-Tg mice 3–6 weeks after immunization with pCMV–LacZ (lanes 2–3) or with pCMV–S2.S (lanes 4–8). Lane 9, Tg mouse receiving unprimed spleen T cells. Lane 1, RNA from the liver of non-Tg mouse is shown as a negative control. The transferred spleen cell population is indicated on the top. HBsAg titers (ng/ml) and the presence (++) or the absence (−) of anti-HBs antibody at the time of sacrifice are indicated.

untreated Tg mice or Tg mice which had been injected with pCMV–LacZ DNA (Fig. 4*A*, lane 1 and lanes 2–4, respectively). This indicates that the inhibition of viral gene expression in Tg mice injected with pCMV–S2.S was not due to a nonspecific effect such as the release of cytokines with injection-induced inflammation and/or with an immune response against transfected muscle cells expressing a foreign antigen (i.e., β-gal). Thus, the HBsAg-specific immune response induced by immunization with plasmid DNA appears to be responsible for controlling hepatic transgene expression by some noncytopathic mechanism.

To determine which component of the immune response is implicated in the down-regulation of HBV-specific mRNA and in the observed decrease or elimination of the circulating antigen, we performed adoptive transfer experiments. Fully immunocompetent non-Tg mice were immunized once with the pCMV–S2.S DNA vector and when ELISA titers of serum antibodies to HBsAg had reached at least 10^4, both the serum and the primed spleen cells were harvested from the mice for transfer into their Tg littermates.

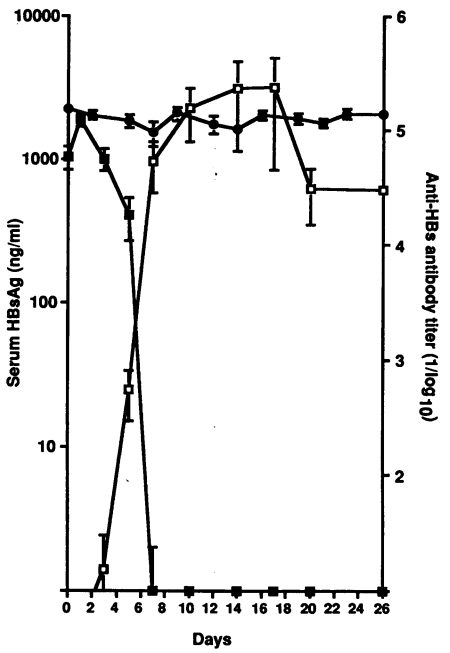

FIG. 5. Adoptive transfer of primed spleen cells into Tg mice. Non-Tg mice were immunized by intramuscular injection of pCMV–S2.S or pCMV–LacZ DNA to produce primed spleen cells for adoptive transfer into their Tg littermates. The mean titer of antibodies in the serum of donor mice at the time of spleen harvest was 1×10^5. Eleven recipient Tg mice were bled at 2 or 3 days intervals and their sera were analyzed for HBsAg (ng/ml) (■) and antibodies to HBsAg (□), (ELISA, end-point dilution titers). Results are shown as mean titers ± SEM. ●, Mean titers of serum HBsAg in five control Tg recipient mice receiving either unprimed or pCMV–LacZ-primed spleen cells.

Passive transfer of serum-derived antibodies on a single occasion into Tg mice induced a rapid but transient decrease in circulating HBsAg levels (mice 2-21 and 4-26, Table 2). In other Tg mice, circulating antigen was maintained at undetectable levels for a longer period by intraperitoneal injection of hyperimmune sera every 2–3 days over a period of 17 days (mice 1-3-5 and 1-3-6, Table 2). Neither single nor chronic administration of antibodies resulted in decreased HBV-specific mRNA in the liver (not shown), indicating that the

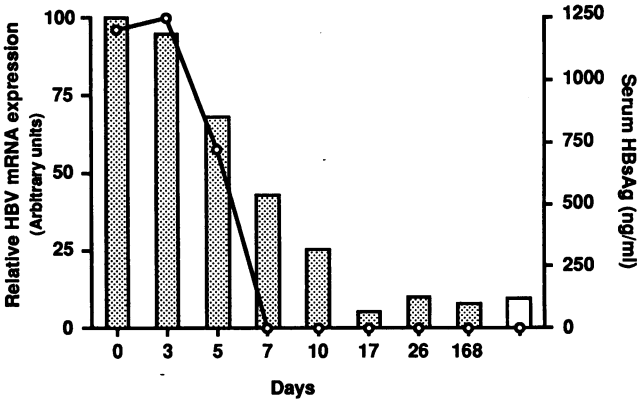

FIG. 6. HBV mRNA content in the livers of Tg mice taken at various times after adoptive transfer of HBsAg-primed spleen cells. Northern blots were performed as in Fig. 4, and quantitative determination of the HBV mRNA levels was done by PhosphorImager analysis after correction for mRNA loading and variations in transfer efficiency as assessed by β-actin expression. The results are expressed as arbitrary units and represented by grey columns. The background level of hybridization is shown for RNA extracted from a non-Tg mouse liver (open column on right). Serum HBsAg concentrations (ng/ml) at the time of liver harvest are shown (○).

humoral response after DNA-based immunization was not responsible for the down-regulation of transgene expression or the long-term elimination of the transgene product.

Injection of primed spleen cell suspensions obtained from pCMV–S2.S immunized non-Tg donor mice into Tg littermate recipients resulted in a rapid clearance of circulating HBsAg (by 7 days) along with a concomitant and sustained appearance of anti-HBs antibodies (Fig. 5). This indicates that the transferred B cells were activated in the Tg mice, probably by the circulating antigen. In contrast, injection of β-gal-primed spleen cells into Tg recipients had no effect on the level of serum HBsAg (Fig. 5). Adoptive transfer of HBsAg- but not β-gal-primed spleen cells was also able to induce a complete disappearance of HBV mRNA in the liver by 17 days (Fig. 6 and Fig. 4*B*, lanes 4 and 5, and 2 and 3, respectively). Serum HBsAg became undetectable 10 days prior to the disappearance of HBV mRNA (Fig. 6), suggesting that two separate mechanisms may be responsible for the observed clearance of the antigen: an initial transient elimination due to formation of immune complexes and a subsequent more permanent control of transgene transcription.

Serum transaminase activity and histological examination of liver sections were monitored every 2–3 days after adoptive transfer and found to be normal for up to 17 days, after which some histological sections of liver exhibited a few small necrotic foci. These were sometimes accompanied by the presence of inflammatory cells, but in no case did the necrotic regions involve more than 5% of the hepatic cells. In addition, a few apoptotic hepatocytes were detected in centrilobular areas within some randomly distributed lobules (not shown).

T Cells Are Able to Control Transgene Expression in the Absence of Antibody Production. To determine which spleen cell population was involved in the decrease or disappearance of circulating HBsAg and liver HBV mRNA, we carried out adoptive transfer with fractionated B- or T-spleen cells obtained from non-Tg donor mice immunized with pCMV–S2.S. After depletion of T cells, the transfer of HBsAg-primed spleen cells into Tg mice did not induce anti-HBs and had no effect on levels of circulating HBsAg or liver HBV mRNA (Fig. 4*B*, lane 6). This indicates that production of antibody detected in DNA-immunized Tg mice is dependent on an exogenous T-cell help (see Fig. 2). In contrast, transfer of B-cell-depleted spleen cells resulted in clearance of circulating HBsAg within 14–17 days although antibody to HBsAg was not detected in these recipient mice at these times (not shown). Thus, antigen-antibody complex formation is not required for, but has a synergistic effect on the elimination of circulating antigen (Fig. 5). It appears nonetheless that HBsAg-specific T cells are responsible for down-regulation of transgene expression since transfer of only primed T cells but not B cells or unprimed T-cells was able to reduce HBV mRNA in the liver to undetectable levels (Fig. 4*B*, lanes 6–9).

DISCUSSION

In the present study, a single intramuscular injection of a plasmid DNA encoding the HBV envelope proteins was shown to be sufficient to break B- and T-cell unresponsiveness to these antigenic proteins in transgenic mice which expressed the same envelope sequences in their liver. This may be due to the presentation of different antigenic peptides processed from the envelope proteins, possibly because different antigen presenting cells (APCs) are involved or perhaps the antigen is presented in a different fashion when synthesized in another type of cell. In addition, relatively more of the preS2 sequences may be expressed by the plasmid vector relative to expression from the transgene, as suggested by the fact that antibodies to preS2 epitopes appear first in Tg mice as in non-Tg C57BL/6 mice (20). The identity of the APCs involved in DNA-based immunization has not yet been determined; however, professional APCs such as bone marrow-derived infiltrating cells present at the injection site or interstitial dendritic cells normally present in muscle tissue could efficiently capture and present the antigen produced by the transfected muscle cells. In contrast, presentation of HBV peptides by nonprofessional APCs such as hepatocytes, which lack costimulatory molecules, may favor the induction of anergy rather than T-cell expansion (24).

Despite the high concentration of the transgene product in the circulation, antibodies to HBsAg are induced soon after DNA injection. Initially these are directed only against the preS2 epitope and it is possible that the Tg mice are less tolerant to this determinant than they are to the more abundantly expressed group and subtype determinants of HBsAg. The formation of antigen–antibody immune complexes after immunization could enhance the capture of HBsAg particles by APCs bearing the Fc receptor and potentiate the proliferation of T-cell clones as has been demonstrated *in vitro* (25). Moreover, the Th1 response induced after DNA-based immunization (refs. 26 and 27, and C. Leclerc, E. Dériaux, and R.G.W., unpublished results) could potentiate the presentation of HBsAg epitopes and thus facilitate the auto immune response since the Th1 cytokines IL-2 and IFN-γ can stimulate MHC class I and II as well as proteasome subunit expression, and can activate other proteases involved in epitopes processing (28). Whatever the explanation, B- and T-cell nonresponsiveness can be overcome and the immune response resulting from plasmid-based expression of the HBsAg in muscle cells leads to both clearance of the circulating transgene-encoded HBsAg as well as the induction of T-cell responses capable of suppressing HBV mRNA accumulation in the liver.

Interestingly this HBsAg-specific immune response did not cause an increase in transaminase activity, as would be expected in the case of a cytolytic response in the liver. We have previously found that DNA-based immunization induces a strong HBsAg-specific $CD8^+$ cytotoxic T lymphocyte (CTL) response in non-Tg mice of the $H\text{-}2^d$ haplotype (29), and Schirmbeck *et al.* (30) have recently reported that nonresponsiveness to HBsAg at the CTL level can be broken through DNA immunization in the $H\text{-}2^b$ haplotype used in this study. The absence of a detectable cytotoxic attack on the HBsAg-expressing liver cells, even after transfer of fully competent T cells, may be due to poor expression of MHC class I molecules on hepatocytes. Inefficient presentation of antigenic peptides could protect these cells from becoming targets of HBsAg-specific cytotoxic T cells. Using a Tg model similar to that used in this study, Guidotti *et al.* (31) have reported that passive transfer of HBsAg-specific $CD8^+$ CTL clones, which secreted IFN-γ and TNF-α, caused only a limited cytopathic response in the liver, but that this in turn activated a complex regulatory cascade which transiently inhibited transgene expression in the remaining liver cells without killing them. In the present study, using plasmid DNA for immunization, clonal expansion of HBsAg-specific T cells, either $CD4^+$ Th1 or $CD8^+$ CTLs or both might be the effector cells responsible for the down-regulation of HBV mRNA in the Tg liver cells via secretion of IFN-γ or other cytokines. In this Tg model the immune response induced by a single injection of DNA was remarkably well balanced between antibody production, which probably facilitated antigen clearance, and induction of HBsAg-specific T cells, which regulated transgene expression. These responses, which persisted for at least 5 months without further DNA injections, could be ascribed to the long-lasting immunity induced by DNA-based immunization (20, 32).

During HBV infection in humans, a T-helper-dependent B-cell response directed toward envelope determinants is required for clearance of the circulating particles (2). In some chronic carriers of HBV, the absence of anti-HBs production and of a CTL response could be due to a lack of helper function from HBV-specific $CD4^+$ T cells, which are found in all patients who successfully clear the virus but not in those who remain chronically infected (5). Thus, in some individuals with chronic hepatitis, there

Table 2. Serum titers of HBsAg in Tg mice after passive transfer of antibodies to HBsAg

Mouse no.	Injected serum	Bleeding (days)								
		0	0.25	1	2	3	6	10	15	17
4-23	NMS	429	404	477	420	440	452	ND	252	502*
2-21	Anti-HBs Ab	1321	0	13	61	373	725	ND	1028	1356*
6-11	NMS	696	548	442*						
4-26	Anti-HBs Ab	1080	0	22*						
1-3-16	NMS	565	ND	542	ND	326	562	328	647	693*
1-3-5	Anti-HBs Ab	721	ND	0	ND	0	0	0	3	0*
1-3-6	Anti-HBs Ab	548	ND	3	ND	0	0	3	6	0*

Results are shown for seven Tg mice injected intraperitoneally once (mice 4-23, 2-21, 6-11, and 4-26) or every 2-3 days (mice 1-3-16, 1-3-5, and 1-3-6) with either anti-HBs immune sera (anti-HBs Ab) or normal mouse sera (NMS). HBsAg titers (ng/ml) were determined in the sera collected at the indicated time. Mice were killed (*) at 26 hours or 17 days after transfer and their livers were harvested for extraction of mRNA and Northern blot analysis. ND, not done.

may be a deficit in the T-cell repertoire which results in nonresponsiveness to the HBV envelope protein. This may induce functional immunological tolerance, particularly if infection occurs early in life, as evidenced by the high level of chronicity associated with neonatal HBV infection.

In our murine model of HBV chronic carriers, the transgene is expressed prior to birth in the liver (13) and although expression is undetectable in the thymus, it is likely that the high amount of circulating HBsAg maintains peripheral tolerance to this antigen. Nevertheless, we have shown here that B- and T-cell nonresponsiveness can be overcome by using DNA-mediated immunization and that the response induced mimics in some aspects that required to clear a viral infection, namely an adequate cellular immune response able to regulate viral gene expression without killing the infected cells and an adequate humoral response to prevent the spread of free virus to uninfected cells.

This suggests that DNA-based immunization may be an effective tool in the treatment of HBV chronic carriers. To our knowledge, this is the first report of a potential immunotherapeutic application for DNA-based immunization against a chronic viral infection.

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