Regulation of Hepatitis B Virus mRNA Expression in a Hepatitis B Surface Antigen Transgenic Mouse Model by IFN-γ-Secreting T Cells After DNA-Based Immunization

Maryline Mancini,* Michelle Hadchouel,† Pierre Tiollais,* and Marie-Louise Michel²*

The immunotherapeutic effect of DNA-mediated immunization against chronic hepatitis B virus (HBV) infection has been evaluated in transgenic mice expressing the sequences that code for the envelope proteins of HBV in the liver. In this model of HBV chronic carriers, a single i.m. injection of plasmid DNA encoding HBV envelope proteins is sufficient to generate specific immune responses leading to the clearance of the transgene expression product and the control of HBV mRNA. The relative contributions of the T cell subpopulations induced by DNA immunization were examined using adoptive transfer experiments. It was shown that either CD8⁺ or CD4⁺ T lymphocytes from immunocompetent DNA-immunized animals were sufficient to control viral gene expression in the livers of the recipient transgenic mice. This effect was mediated by a cytokine-dependent mechanism common to both T cell subpopulations; this mechanism did not require cell lysis, but did involve the production of IFN-γ by the activated T cells. The Journal of Immunology, 1998, 161: 5564–5570.

During hepatitis B virus (HBV) infection, both cellular and humoral limbs of the immune response are required for viral clearance to occur. The humoral Ab response contributes to the clearance of circulating virus particles and the prevention of viral spread within the host, whereas the cellular immune response eliminates infected cells (1). Currently available vaccines usually fail to activate both arms of the immune response.

DNA-based immunization may contribute to immunotherapeutic strategies by inducing both cellular and humoral responses. This novel method of immunization refers to the induction of an immune response to a protein expressed in vivo from a gene introduced directly in the form of pure plasmid DNA (2, 3). To date, DNA-based immunization has been found to result in a broad range of immune responses, including neutralizing Abs as well as cytotoxic and Th cell responses. It was shown recently that immunization with plasmid DNA vectors can induce a strong bias toward a Th1 response in animals (4). Th1-type cytokines such as IL-2 and IFN-γ are associated principally with cell-mediated immune responses and play a crucial role in protection from intracellular pathogens, including viruses. In HBV infection, it has been suggested that an imbalance between Th2 and Th1 responses may contribute to chronicity (5).

As a model for the HBV chronic carrier state, we have used transgenic (Tg) mice that constitutively express hepatitis B surface Ag (HBsAg) (6). 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 (7). As observed for chronic HBV carriers, large amounts of subviral particles are present in the sera. In this model, we have shown previously that the immune response induced after a single i.m. injection of DNA results in the complete clearance of circulating HBsAg and in the long-term control of transgene expression in hepatocytes (8). This response did not involve a detectable cytopathic effect in the liver. We now report, using adoptive transfer of fractionated primed spleen cells from non-Tg DNA-immunized mice, that both HBs-specific CD4⁺ and CD8⁺ T lymphocytes are involved in the regulation of the HBV mRNA expressed in the livers of Tg mice. These T cells operate without major cytopathic effects. These results suggested that both CD4⁺ and CD8⁺ T cells act by a common mechanism. Following in vitro antigenic stimulation, both subpopulations secreted type 1 cytokines, especially IFN-γ. The role of this cytokine was confirmed using HBV Tg mice carrying the null mutation for the IFN-γR gene. Thus, by inducing a strong bias toward a Th1 immune response, DNA-based immunization could represent an alternative approach for the treatment of individuals chronically infected with HBV.

Materials and Methods

Mice

The generation and characterization of the HBV envelope Tg mouse lineage E36 has been reported previously (6, 7). These Tg mice were produced on a C57BL/6 × SJL/J background and were backcrossed against C57BL/6 (H-2b) ≥20 times before use. Only 5–8-wk-old female mice that were heterozygous for the HBV envelope transgene (ayw subtype) and their non-Tg littermates were used. The Tg mice sera contain 200–2000 ng/ml of HBsAg; their livers exhibit no pathology.

The construction of the IFN-γR knockout (IFN-γR⁻⁻) mice has been described previously (9). Briefly, these mice are infertile 129/Sv (H-2b) animals for which the IFN-γR has been inactivated by homologous recombination. The mice were mated with the HBV Tg mouse lineage to yield

Copyright © 1998 by The American Association of Immunologists
heterozygous mice (Tg/IFN-γR<sup>−/−</sup>). These animals were subsequently infected with IFN-γR<sup>−/−</sup> mice to yield Tg mice homozygous for the null mutation of the IFN-γR gene (Tg/IFN-γR<sup>−/−/−</sup>).

DBA/2 and C57BL/6 mice were purchased from Iffa-Credo (Lyon, France). All experiments involving mice were conducted in accordance with institutional guidelines.

**DNA immunization**

Mice were injected on a single occasion with 100 μg of recombinant plasmid DNA expressing the S and preS2 domains of the gene encoding the HBV envelope proteins (pCMV-S2.S) (10) or the Escherichia coli LacZ gene encoding β-galactosidase (pCMV-LacZ) (11). DNA was purified by anion-exchange chromatography (Endofree Plasmid Kit, Qiagen, Hilden, Germany) and was injected directly into regenerating tibialis anterior muscles as described previously (12). Purified DNA contains only negligible amounts of endotoxin (<0.1 endotoxin unit/μg plasmid DNA).

**Serology**

Blood was collected from anesthetized mice by retrobulbar puncture using heparinized glass pipettes; HBsAg was measured in the plasma using a commercial ELISA kit (Monolisa AgHBs; Diagnostics Pasteur, Marnes la Coquette, France). Quantitation of mouse anti-hepatitis B surface Ab (HBsAb) was performed by ELISA (13). The serum alanine aminotransferase activity in the plasma was measured using a commercial kit (EnzymeBioMérieux, Lyon, France).

**Histologic procedures**

Tissues were fixed in alcoholic Bouin’s fixative. Sections of paraffin-embedded tissues were cut at 5-μm thickness and stained with hematoxylin-eosin and trichrome.

**Northern Blot analysis**

The total RNA in the liver was extracted from mechanically pulverized tissue by RNA-Plus (Bioprime, Montreuil-sous-Bois, France). The RNA (40 μg) was fractionated on 1% formaldehyde-agarose gels and blotted onto nylon membranes; next, membranes were hybridized with 32P-labeled DNA probes synthesized from HBV DNA fragment or from a 0.2-kb PstI cDNA fragment of the murine 18S ribosomal RNA gene (Valbiotech, Paris, France) using the Rediprime DNA labeling system (Amersham, Les Ulis, France).

**Adoptive transfer of spleen cells**

Splenocytes obtained for adoptive transfer by lysis of RBCs (incubation with Tris-buffered ammonium chloride for 5 min at 4°C). After four washes with RPMI 1640 medium (Life Technologies, Cergy Pontoise, France), the remaining white blood cells were counted and resuspended in 200 μl of PBS. Each recipient mouse was injected with cells obtained from a single spleen. For the transfer of subpopulations, ~3–6 × 10<sup>6</sup> cells were injected into the retroorbital cavity of recipient mice that had been sublethally irradiated (5 Gy) at 2 h before cell transfer.

**Lymphocyte subset fractionation**

T cell subpopulations were isolated from the total spleen cell population using CD4/CD8 subset column kits (R&D Systems, Abingdon, U.K.). The purity of the CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes was confirmed by cytometric analysis using a FACScan (Becton Dickinson, Le Pont de Claix, France). The percentages of undesired cell subsets in the enriched cell population are <4% for CD4<sup>+</sup> T cells, <1% for CD8<sup>+</sup> T cells, and <6% for B cells.

**CTL generation in mice**

Groups of mice were immunized with pCMV-S2.S DNA vector. Splenocytes were removed from immunized mice at 8–12 wk postinjection, and single-cell suspensions were prepared. Cells (10<sup>7</sup> cells/well) were suspended in 2 ml of α-minimum essential medium tissue culture medium supplemented with 10 mM HEPES buffer, 1 mM sodium pyruvate, nonessential amino acids, 5 × 10<sup>−3</sup> M β-mercaptoethanol, antibiotics, and 10% FCS (Life Technologies) in 24-well plates. Responder spleen cells from C57BL/6 or DBA/2 mice were stimulated with 10<sup>5</sup> irradiated RBL5/S or P815/S transfecteds, respectively, expressing the small envelope protein (14). After 5 days in culture, one-half of the medium was replaced with fresh medium; cells were used as effectors in a standard chromium release assay that was performed 2 or 3 days later. Targets were RBL5 or P815 cells infected with recombinant vaccinia virus expressing the HBV envelope and labeled with 51Cr (3.7 Mbq/10<sup>6</sup> cells, reference number CJS4, Amersham). After a 4-h incubation at 37°C, 50 μl of the supernatant was removed from each well and counted on a beta counter as described previously (15). The percentage of specific release was calculated as follows (experimental release − spontaneous release)/(total release − spontaneous release) × 100. Total release was measured by resuspending target cells in lysis buffer. Spontaneous release was obtained from targets incubated with medium alone and is usually <15% of the total release.

**T cell proliferation assay**

Spleen cell suspensions from pCMV-S2.S-immunized mice were cultured in triplicate using 96-well round-bottom plates at 5 × 10<sup>4</sup> cells/ml in 200 μl of RPMI 1640 medium (Life Technologies) containing 2% mouse sera with different concentrations of either HBsAg particles (3, 1, 0.3, and 0.1 μg/ml), preS2 synthetic peptides (10, 3, 1, and 0.3 μg/ml), or medium alone. A set of peptides covering the entire preS2 domain of HBV (glyw subtype) was used to detect minimal epitopes recognized by activated T cells. Spleen cells were stimulated for 96 h, and [3H]thymidine (reference number TRK 120, specific activity 25 Ci/mmol; Amersham) was added (1 μCi/well). Cells were incubated for an additional 18 h, and the [3H]thymidine incorporation into DNA was measured after harvesting.

Depletion of CD4<sup>+</sup> or CD8<sup>+</sup> cells of murine spleen cells was achieved by high-gradient magnetic cell sorting using a MidiMACS (Miltenyi BioTec, Bergisch Gladbach, Germany) (16). Briefly, CD4<sup>+</sup> or CD8<sup>+</sup> cells were magnetically labeled with CD4 (L3T4) or CD8a (Ly-2) microbeads and passed through a separation column that had been placed in the magnetic field of a magnetic-advanced cell sorting separator. The effluant fraction was collected as the depleted fraction. The percentage of undesired T cells was <2%.

**Cytokine assay**

In vivo-primed spleen cells were cocultured with Ags in parallel with the proliferation assays. Culture supernatants were collected each day, and concentrations of IL-2, IL-4, IFN-γ, and TNF-α were determined by ELISA using commercial kits (Genzyme, Cambridge, MA). For these assays, the limit of detection was 5 pg/ml of IL-2, IL-4, or IFN-γ and 15 pg/ml of TNF-α.

**Results**

We have described previously that a single injection of pCMV-S2.S DNA into HBsAg-Tg mice induced a persistent decrease of circulating HBsAg particles and a concomitant appearance of anti-HBsAbs that were maintained over time. We have also shown that this Ag clearance, which persisted over time, was not simply due to the strong Ab response but also to the control of hepatic transgene expression by the HBsAg-specific T cells induced after DNA-based immunization. Both components of the immune response may act in synergy, leading to the clearance of HBsAg within 7 days after adoptive transfer of spleen cells (8). To further determine which T cell subpopulation is implicated in the downregulation of HBV-specific mRNA and in the observed decrease or elimination of the circulating Ag in the Tg mice, we conducted adoptive transfer experiments using fractionated T cells from DNA-immunized non-Tg mice.

**Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells control transgene expression**

Fully immunocompetent C57BL/6 non-Tg mice were immunized once with 100 μg of pCMV-S2.S DNA vector. Total T cells and CD4<sup>+</sup> or CD8<sup>+</sup> T cells were purified from spleens at 8–12 wk after DNA injection. Tg recipient mice were immunosuppressed by sublethal total body irradiation to confine the analysis to mechanisms expressed by the donor cells alone. Injection of the primed CD8<sup>+</sup> T cell suspensions obtained from pCMV-S2.S-immunized non-Tg donor mice into Tg littermate recipients resulted in a rapid clearance of circulating HBsAg in the absence of Ab production. The HBsAg became undetectable in the sera from 12–33 days posttransfer, depending upon the initial HBsAg concentration in the serum (Fig. 1, left panel). This regulation required ≥10<sup>6</sup> CD8<sup>+</sup> T cells to obtain the clearance of the circulating HBsAg (data not shown).
Remarkably, an injection of pCMV-S2.S-primed CD4\(^+\) T cells was also able to induce a similar decrease in the circulating HBsAg (Fig. 1, right panel). For the recipient Tg mice having the highest HBsAg concentration in the sera before transfer, complete clearance was slightly delayed. In mice displaying faster Ag clearance, FACS analysis of the transferred cells showed that contaminant CD8\(^+\) T cells did not exceed 3 \times 10^3. This finding corresponds to a number of CD8\(^+\) T cells that we have shown by dilution experiments not to be sufficient to clear the HBsAg (see above) and indicates that the transferred CD4\(^+\) T cells by themselves are responsible for the observed decrease in HBsAg.

We subsequently investigated the effect of the purified T cell subpopulations on HBV mRNA expression in the livers of Tg mice. As we have reported previously, adoptive transfer of HBsAg- but not β-galactosidase-primed spleen cells was able to induce a complete disappearance of HBV mRNA in livers taken from 17 days posttransfer (Fig. 2, lanes 2 and 3, respectively) up to 5 mo (8). This effect is mediated by pCMV-S2.S-primed T cells, because the transfer of these cells in the absence of B cells is sufficient to completely abolish the detection of HBV mRNA in the livers of recipient Tg mice (Fig. 2, lane 4). Most importantly, both purified HBsAg-primed CD8\(^+\) and CD4\(^+\) T cells were able to reduce HBV mRNA in the livers of Tg mice to an undetectable level by 33 days after adoptive transfer (Fig. 2, lanes 6–8 and 10–12, respectively). Since we could not correlate the observed decrease of HBV mRNA in the liver to a hypermethylation of the transgene and to a subsequent switch-off of gene expression (data not shown), this indicates that each of the T cell subpopulations was responsible for the down-regulation of the transgene expression.

\textbf{Cytolytic activity of CD8\(^+\) T cells primed by pCMV-S2.S immunization is detectable in vitro but very minor in vivo}

C57BL/6 mice have been classified as a nonresponder haplotype to HBsAg at the CTL level in response to immunization with different exogenous or virally expressed HBsAg preparations. Nevertheless, this nonresponse can be circumvented after in vivo priming using DNA-based immunization (17). Spleens from C57BL/6 non-Tg mice immunized by a single injection of pCMV-S2.S were removed at 8 wk postinjection; primed T cells were tested for their lytic activity in vitro. Spleen cells restimulated in vitro with HBsAg-expressing transfected cells (RBL5/S) were able to specifically lyse autologous target cells (RBL5) infected with recombinant vaccinia virus expressing the HBV envelope proteins but not target cells infected with a wild-type vaccinia virus (Fig. 3A, left).

The in vivo pathogenic effect of the pCMV-S2.S-primed precursor T cells was investigated on HBs-expressing liver cells. We performed adoptive transfer experiments using spleen cells derived from C57BL/6 immunized mice and Tg mice as recipients. Direct transfer of HBs-primed spleen cells displaying in vitro cytotoxic activity (see above) into C57BL/6 Tg mice did not result in any change in transaminase activity in the sera tested sequentially for \(\approx 50\) days posttransfer. Histologic examination of livers showed few necrotic foci only on some sections taken 10–17 days posttransfer (Fig. 3A, right).

Because the lytic activity of H-2\(^b\)-restricted CD8\(^+\) T cells is not very strong in vitro, we decided to use T cells derived from H-2\(^d\) mice, which are described as good responders at the CTL level after DNA-based immunization (17). Using a single pCMV-S2.S DNA injection, HBs-specific CTL precursors were efficiently primed in DBA/2 mice, since in vitro-restimulated spleen cells...
efficiently lysed autologous target cells (p815) infected with recombinant vaccinia virus expressing the HBV envelope protein (Fig. 3B, left). The CTL populations generated in H-2d mice expressed a higher specific cytolytic reactivity than the CTL populations from H-2b mice immunized with the same vector (compare Fig. 3, A and B, left panel).

Injection of HBs-primed precursor T cells derived from the spleens of DBA/2 mice immunized with pCMV-S2.S and displaying cytolytic activity were injected directly into F1, hybrid Tg mice that had been backcrossed one generation against DBA-2 mice before transfer (H-2b×d mice). This resulted in a mild and transient increase in the serum glutamic-pyruvic transaminase activity observed only 1 day after transfer (n = 3, pretransfer 29 ± 9 U/L; posttransfer 79 ± 15 U/L).

To further increase the number of specific lytic T cells, spleen cells derived from DBA-2 immunized mice were restimulated in vitro with HBsAg-expressing transfectant cells before transfer. Bulk CTL lines (9 × 105) restimulated in vitro two times with p815/S transfectant (>70% CD8+ cells and 13% CD4+ cells) were injected i.v. into H-2b×d Tg mice; transaminase activity was monitored in the sera at different timepoints posttransfer. Serum glutamic-pyruvic transaminase activity was slightly elevated after transfer (n = 3, 207 ± 45 U/L at day 1; 99 ± 21 U/L at day 2) to above normal values (22 ± 3 U/L), but this activity returned to basal level after 3 days. Histologic examination of thin liver sections taken sequentially from Tg recipients showed only few small foci of mononuclear cells and acidophilic bodies at 2 days posttransfer (Fig. 3B, right). This observation correlates well with the mild elevation in transaminase levels. These results indicate that in our model, although the transferred CD8+ T cells exhibit cytolytic activity in vitro, their major effector function in vivo seems to be the control of transgene expression rather than the killing of hepatocytes.

**T cells primed by pCMV-S2.S immunization have a Th1 phenotype**

Because the transfer of purified HBs-primed CD4+ T cells also induced the clearance of HBsAg and the control of HBV mRNA in the livers of the transferred Tg mice, it was important to characterize the function and specificity of the HBs-primed CD4+ T cells in vitro. Spleen cells that were harvested from C57BL/6 non-Tg mice at 3–5 wk after pCMV-S2.S immunization and cultured in the presence of HBsAg particles or peptides derived from the preS2 domain of the HBV envelope middle protein are specifically stimulated in vitro (Fig. 4). The fine specificity of the Th cells was determined using a panel of synthetic peptides (Fig. 4A). The three preS2-overlapping peptides (109–134, 124–148, and 139–163) used to cover the entire preS2 domain are recognized by T cells. A shorter peptide (126–138) was also recognized by T cells to the same extent and defined a Th epitope generated by DNA-based immunization in C57BL/6 mice. This peptide is included in a larger sequence that has been shown previously to induce T cell proliferation in C57BL/6 mice (18). Depletion experiments showed that only CD4+ T cells but not CD8+ T cells are induced to proliferate after in vitro stimulation with these Ags (Fig. 4B).

Next, the Ag-driven cytokine secretion pattern of in vitro-restimulated T cells was analyzed. A Th1 pattern characterized by the secretion of IFN-γ and TNF-α in the absence of IL-4 was observed. This pattern was not affected by the depletion of CD8+ T cells (Table I). Similarly, CD4+ T-depleted primed splenocytes displaying lytic activity in vitro (data not shown) also produced Th1 cytokines after stimulation with irradiated RBL5/S cells presenting HBV envelope peptides (Table I). These results are in agreement with the isotype profile of secreted IgG present in the sera of pCMV-S2.S-immunized C57BL/6 mice (8,19).

**FIGURE 3.** In vitro and in vivo effector functions of pCMV-S2.S-primed spleen cells. Left panel, In vitro cytolytic function of spleen cells from C57BL/6 (A) or DBA/2 (B) mice immunized with pCMV-S2.S DNA. Groups of mice were immunized once i.m. with 100 μg of DNA. Spleen cells were harvested and stimulated with irradiated RBL5/S or P815/S transfectants, respectively. Target cells were RBL5 or P815 cells infected with either recombinant vaccinia virus expressing the HBV envelope (vv HBV-S) (closed symbols) or wild-type vaccinia virus (vv WT) (open symbols). Two representative mice are shown on each panel. The plotted lysis values at the indicated E:T ratios represent the means of triplicates. Please note that the E:T ratios are different in the two panels. Right panel, Histologic examination of liver sections from C57BL/6 Tg mice (A) or F1 DBA × C57BL/6 Tg mice (B) following adoptive transfer of primed spleen cells (hematoxylin-eosin, ×300 magnification).
As cpm of incorporated [3H]thymidine (mean of triplicates). Results from representative experiments are shown and are expressed the Tg/IFN-γR 0/0 mice was not eliminated after immunization with pCMV-S2.S DNA (Fig. 5A). Nevertheless, a decrease was observed in half of the mice at 4 wk postimmunization; 2 of 10 mice almost completely cleared HBsAg in 12 wk. In contrast, in five of five heterozygous mice (Tg/IFN-γR +/−), a single injection of this plasmid DNA was sufficient to decrease HBsAg titers in 2 wk and to clear the Ag in 4–8 wk (Fig. 5A). Since the clearance of HBsAg correlates in the HBsAg Tg mice with the appearance of Abs (Fig. 5B, Ref. 8), we checked the ability of IFN-γR 0/0 mice to mount an Ab response. Following pCMV-S2.S.S DNA immunization, anti-HBsAbs were induced in non-Tg/IFN-γR 0/0 mice at a level and with a kinetic that were comparable with those induced in C57BL/6 mice (8), whereas only low levels of anti-HBsAbs were detectable in the sera of the Tg/IFN-γR 0/0 mice (Fig. 5B). The lower Ab titers in the Tg/IFN-γR 0/0 mice as compared with the non-Tg/IFN-γR 0/0 mice are due to the persistence of Ag in the serum and to the formation of Ag/Ab complexes. This finding indicates that, despite the efficient production of hepatitis B surface-specific Abs by IFN-γR 0/0 mice that could clear the Ag, the presence of a functional receptor is required for the long-term elimination of HBsAg. At 12 wk after pCMV-S2.S.S immunization, Northern blot analysis of total RNA from the livers of Tg/IFN-γR 0/0 immunized mice showed that HBV mRNA, although variable from animal to animal, was clearly detectable (Fig. 6, lanes 7 and 9) and was decreased in the livers of mice that partially cleared (Fig. 6, lane 8) the envelope Ag from their serum. In contrast, HBV mRNA remained undetectable in the livers taken at the same time from pCMV-S2.S.S-immunized Tg/IFN-γR +/− mice (Fig. 6, lanes 5 and 6). This observation indicates that in these mice, the control of HBV mRNA in the liver operates primarily via signaling through a functional IFN-γR, whereas the decrease of the HBsAg and HBV mRNA observed over time in some of the knockout mice may involve other TH1 cytokines such as TNF-α that could also play a role in this down-regulation.

Discussion

In the present study, the functional importance and relative contribution of DNA-primed CD4 + or CD8 + T cells to the clearance of the transgene-encoded HBsAg from the sera and to the expression of HBV mRNA in the livers of Tg mice were examined using adoptive transfer experiments. It was shown that populations of cells that were predominantly either CD8 + or CD4 + T lymphocytes were sufficient to control viral gene expression in the liver of the recipient Tg mice. This effect was mediated largely by a nonlytic cytokine-dependent mechanism common to both T cell subpopulations that involved the production of IFN-γ. The lytic activity of CD8 + T cells played little or no role.

A single i.m. injection of the plasmid pCMV-S2.S encoding the HBV small and middle envelope proteins was shown previously to be sufficient to break B and T cell unresponsiveness to these antigenic proteins in Tg mice, which express the same envelope coding sequences in their livers. In the experiments reported here, we used animals that had a complete immune response as a source of donor cells to see which populations were capable of regulating transgene expression. In the non-Tg mice after pCMV-S2.S DNA immunization, the HBs-specific CD8 + T lymphocytes were sufficient to control viral gene expression in the liver of the recipient Tg mice. This effect was mediated largely by a nonlytic cytokine-dependent mechanism common to both T cell subpopulations that involved the production of IFN-γ. The lytic activity of CD8 + T cells played little or no role.

Since one of the major cytokines produced by pCMV-S2.S-primed T cells is IFN-γ, we decided to investigate the possible role of this cytokine in the down-regulation of HBV mRNA. For that purpose, we used mice with a deficient IFN-γR (IFN-γR 0/0), which provides an excellent in vivo model to study the regulatory function of endogenous IFN-γ (9). The HBV Tg mouse lineage was mated with IFN-γR 0/0 mice to yield Tg mice that were heterozygous (Tg/IFN-γR +/−) or homozygous for the null mutation of the IFN-γR gene (Tg/IFN-γR 0/0). The HBsAg in the sera from most of the Tg/IFN-γR 0/0 mice was not eliminated after immunization with pCMV-S2.S DNA (Fig. 5A). Nevertheless, a decrease was observed in half of the mice at 4 wk postimmunization; 2 of 10 mice almost completely cleared HBsAg in 12 wk. In contrast, in five of five heterozygous mice (Tg/IFN-γR +/−), a single injection of this plasmid DNA was sufficient to decrease HBsAg titers in 2 wk and to clear the Ag in 4–8 wk (Fig. 5A). Since the clearance of HBsAg correlates in the HBsAg Tg mice with the appearance of Abs (Fig. 5B, Ref. 8), we checked the ability of IFN-γR 0/0 mice to mount an Ab response. Following pCMV-S2.S.S DNA immunization, anti-HBsAbs were induced in non-Tg/IFN-γR 0/0 mice at a level and with a kinetic that were comparable with those induced in C57BL/6 mice (8), whereas only low levels of anti-HBsAbs were detectable in the sera of the Tg/IFN-γR 0/0 mice (Fig. 5B). The lower Ab titers in the Tg/IFN-γR 0/0 mice as compared with the non-Tg/IFN-γR 0/0 mice are due to the persistence of Ag in the serum and to the formation of Ag/Ab complexes. This finding indicates that, despite the efficient production of hepatitis B surface-specific Abs by IFN-γR 0/0 mice that could clear the Ag, the presence of a functional receptor is required for the long-term elimination of HBsAg. At 12 wk after pCMV-S2.S.S immunization, Northern blot analysis of total RNA from the livers of Tg/IFN-γR 0/0 immunized mice showed that HBV mRNA, although variable from animal to animal, was clearly detectable (Fig. 6, lanes 7 and 9) and was decreased in the livers of mice that partially cleared (Fig. 6, lane 8) the envelope Ag from their serum. In contrast, HBV mRNA remained undetectable in the livers taken at the same time from pCMV-S2.S.S-immunized Tg/IFN-γR +/− mice (Fig. 6, lanes 5 and 6). This observation indicates that in these mice, the control of HBV mRNA in the liver operates primarily via signaling through a functional IFN-γR, whereas the decrease of the HBsAg and HBV mRNA observed over time in some of the knockout mice may involve other TH1 cytokines such as TNF-α that could also play a role in this down-regulation.

Figure 4. In vitro proliferative responses of pCMV-S2.S-primed splenic cells. A, Undepleted splenocytes were stimulated with a panel of preS2-derived synthetic peptides (10 μg/ml). Amino acid sequences of peptides start from the first methionine of the HBV ayw subtype preS domain. B, Undepleted, CD4 + -depleted, or CD8 + -depleted splenic cells were cultured with HBsAg particles (3 μg/ml), reactive preS2 peptides (10 μg/ml), or medium alone. Con A (2.5 μg/ml) was used as a positive control. Results from representative experiments are shown and are expressed as cpm of incorporated [3H]thymidine (mean of triplicates).

Tg mice lacking IFN-γR are not susceptible to HBV mRNA down-regulation by T cells

In the present study, the functional importance and relative contribution of DNA-primed CD4 + or CD8 + T cells to the clearance of the transgene-encoded HBsAg from the sera and to the expression of HBV mRNA in the livers of Tg mice were examined using adoptive transfer experiments. It was shown that populations of cells that were predominantly either CD8 + or CD4 + T lymphocytes were sufficient to control viral gene expression in the liver of the recipient Tg mice. This effect was mediated largely by a nonlytic cytokine-dependent mechanism common to both T cell subpopulations that involved the production of IFN-γ. The lytic activity of CD8 + T cells played little or no role.

A single i.m. injection of the plasmid pCMV-S2.S encoding the HBV small and middle envelope proteins was shown previously to be sufficient to break B and T cell unresponsiveness to these antigenic proteins in Tg mice, which express the same envelope coding sequences in their livers. In the experiments reported here, we used animals that had a complete immune response as a source of donor cells to see which populations were capable of regulating transgene expression. In the non-Tg mice after pCMV-S2.S DNA immunization, the HBs-specific CD8 + T lymphocytes were sufficient to control viral gene expression in the liver of the recipient Tg mice. This effect was mediated largely by a nonlytic cytokine-dependent mechanism common to both T cell subpopulations that involved the production of IFN-γ. The lytic activity of CD8 + T cells played little or no role.
controlled hepatic transgene expression via a noncytopathic mechanism. Adoptive transfer experiments of each of these subpopulations resulted in the elimination of the transgene expression product from the sera of the Tg recipient mice; this elimination was first detected at 12 days posttransfer. The rate of clearance could be accelerated to appear by 7 days by cotransfer with pCMV-S2.S-primed B cells (8), because of the synergistic effect of the anti-HBsAbs. Northern blot analysis of total HBV mRNAs from the livers of the recipient Tg mice revealed that HBV mRNA levels were reduced to undetectable levels by 1 mo after transfer of either CD4+ or CD8+ HBs-specific T cells. Nevertheless, HBV mRNAs were still detectable by RT-PCR amplification (data not shown), suggesting that transgene expression is not extinguished but rather controlled. We cannot rule out that all aspects of the immune response are not identical in Tg mice postimmunization. Nevertheless, passive transfer in recipient Tg mouse or direct immunization resulted in loss of HBsAg and mRNA to a similar degree.

The finding that the kinetics of Ag clearance in mice receiving either CD4+ or CD8+ T cells were similar, and that these populations appeared equally effective in transferring the mechanisms of viral RNA elimination, suggested that a function common to both T cell subsets may be involved. It was demonstrated previously in other Tg lineages that HBV gene expression was extremely sensitive to Th1 cytokines (20). In our model, the main cytokines produced by the activated T cells after pCMV-S2.S immunization were IFN-γ and TNF-α. This result is in agreement with previous reports showing that DNA-based immunization is a powerful means of inducing a strong Th1 response in mice (4). IFN-γ has the potential to influence viral infections by a number of mechanisms (reviewed in Ref. 21). IFN-γ affects different aspects of the specific immune response through the up-regulation of class I and class II MHC expression (22) and enhances the expression of the MHC-linked proteins involved in intracellular peptide Ag processing for presentation by the class I Ag presentation pathway (23). In our model, evidence to support an IFN-mediated mechanism was derived from experiments performed in mice with a disrupted IFN-γR gene. The persistence of HBsAg in the sera and the sustained expression of the transgene in the livers of most of the pCMV-S2.S-immunized Tg/IFN-γR0/0 mice confirmed that this

Table I. In vitro cytokine production by activated splenocytes

<table>
<thead>
<tr>
<th>Undeployed and CD8+ -Depleted Spleocytes</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IFN-γ</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
<td>Undeployed</td>
<td>Δ CD8</td>
<td>Undeployed</td>
<td>Δ CD8</td>
</tr>
<tr>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Con A</td>
<td>31 ± 3</td>
<td>296 ± 102</td>
<td>15 ± 3</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>HBsAg</td>
<td>10 ± 4</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>109–134</td>
<td>18 ± 6</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>124–148</td>
<td>61 ± 19</td>
<td>37 ± 19</td>
<td>21 ± 6</td>
<td>&lt;5</td>
</tr>
<tr>
<td>126–138</td>
<td>33 ± 7</td>
<td>29 ± 7</td>
<td>16 ± 7</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>139–163</td>
<td>26 ± 10</td>
<td>7 ± 7</td>
<td>18 ± 8</td>
<td>9 ± 5</td>
</tr>
</tbody>
</table>

*Undeployed and CD8+ -depleted (Δ CD8) pCMV-S2.S-primed splenocytes from non-Tg mice were stimulated in vitro as described in Fig. 4. Undeployed and CD4+ -depleted splenocytes (Δ CD4) were stimulated with RBL5/S transfected cells as described for CTL generation. Ag-specific culture supernatants were harvested, and concentrations of IL-2, IL-4, IFN-γ, and TNF-α were quantified by specific ELISA. Data are expressed in picograms per milliliter as the arithmetic mean ± SEM of at least three spleens tested independently.

![FIGURE 5](image-url) DNA-based immunization of IFN-γR0/0 or IFN-γR+/- mice. Groups of 10 female Tg/IFN-γR0/0 mice (●), 5 Tg/IFN-γR+/- mice (▲), and 5 non-Tg/IFN-γR0/0 mice (▲) were immunized once by i.m. injection of 100 μg of pCMV-S2.S. A group of five Tg/IFN-γR0/0 mice were injected with pCMV-LacZ DNA as a control (●). Mice were bled at weekly intervals, and the sera were analyzed for HBsAg (A) or anti-HBsAbs (B). Anti-hepatitis B surface IgG was expressed as 1/log10 of the Ab titer (determined by serial endpoint dilution analysis). Each point represents the mean titer for the group.

![FIGURE 6](image-url) HBV mRNA content in the livers of Tg mice taken 12 wk after direct injection of DNA (see Fig. 5). Northern blot and hybridization were performed as described in Fig. 2. The IFN-γR status of the Tg mice is indicated at the top. Lane 1, nonimmunized Tg mouse; lanes 2–4, pCMV-LacZ-immunized Tg mice; lanes 5–9, Tg mice immunized with pCMV-S2.S DNA.
cytokine plays an important role in the effenter phase of the immune response to HBV.

No pathogenic effector functions of the HBV-specific T cells primed after DNA-based immunization were found after adoptive transfer of T cell subsets in our Tg lineage. This is in contradiction with another report (24) in which the transfer of a CD4⁺ T cell clone displaying a Th1 phenotype and cytolytic activity resulted in necroinflammatory liver disease in two different transferred HBV Tg lineages. Several differences could account for these discrepancies. The number of transferred HBV-specific T cells and consequently the amount of secreted cytokines (i.e., IFN-γ and TNF-α) following Ag stimulation could account for the recruitment of T and non-T cells observed in the liver. In our model, the number of transferred HBV-specific T cells is much lower and is equivalent to the number present in a single spleen postimmunization. Even if DNA-based immunization is powerful enough to induce strong and sustained class I- and class II-restricted T cell responses, this effect cannot be compared with the transfer of a clonal population. In addition, we detected very rare infiltrates in the liver after adoptive transfer, indicating that the T cells did not need to reach the liver to be activated. The HBsAg present in large amounts in the sera of the Tg mice is sufficient to activate the transferred T cells in the lymphoid organs and to induce these cells to secrete cytokines. Another difference between the two models include the genetic background of the Tg lineage. Finally, the level and the site of Ag expression could greatly influence the sensitivity to IFN-γ, because considerable differences in the severity of IFN-γ-induced liver disease were observed in the two lineages used in this study (24).

At present, little is known about the balance between the limitation of viral infection and the destruction of liver tissue in viral hepatitis. Th1 responses have been reported to be associated with the capacity of the host to resolve or control viral infections (25–27). Because Th1 cells and their cytokines are involved in delayed hypersensitivity, which is a primary defense against intracellular pathogens, the prevalent Th1 pattern of secreted cytokines can be regarded not only as a mechanism contributing to inflammation but also as an appropriate response of the immune system to hamper viral expression and eventually lead to viral elimination.

Acknowledgments

We thank Dr. M. Aguet for the IFN-γ receptor knockout mice, Dr. M. E. Major for p815/S transfectants, and Dr. J. Reimann for RBL5 and RBL5/S cells. We also thank Yves Rivière and Katia Schlienger for helpful discussions and critical reading of the manuscript.

References