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Ongoing Murine T1 or T2 Immune Responses to the Hepatitis B Surface Antigen Are Excluded from the Liver that Expresses Transgene-Encoded Hepatitis B Surface Antigen

Reinhold Schirmbeck,* Jens Wild,* Detlef Stober,* Hubert E. Blum,† Francis V. Chisari,‡ Michael Geissler,§ and Jörg Reimann2* 

Different protein- or DNA-based vaccination techniques are available that prime potent humoral and cellular, T1 or T2 immune responses to the hepatitis B surface Ag (HBsAg) in mice. T1 and T2 are immune responses with isotype profile indicating Th1 and Th2 immunoregulation. We tested whether HBsAg-specific immune responses can be established in transgenic mice that express HBsAg in the liver (HBs-tg mice) using either these different vaccination techniques or an adoptive transfer system. HBsAg-specific responses could not be primed in HBs-tg mice with the established, potent vaccine delivery techniques. In contrast, adoptive transfers of T1- and T2-type HBsAg-immune spleen cells into congenic HBs-tg hosts (that were not conditioned by pretreatment) suppressed HBsAg antigenemia and gave rise to HBsAg-specific serum Ab titers. The establishment of continuously rising anti-HBsAg serum Ab levels with alternative isotype profiles (reflecting T1 or T2 polarization) in transplanted HBs-tg hosts required donor CD4+ T cell-dependent restimulation of adoptively transferred immune cells by transgene-derived HBsAg. Injections of HBsAg-specific Abs into HBs-tg mice did not establish stable humoral immunity. The expanding T1 or T2 immune responses to HBsAg in HBs-tg hosts did not suppress transgene-directed HBsAg expression in the liver and did not induce liver injury. In addition to priming functional antiviral effector cells, the conditioning of the liver microenvironment to enable delivery of antiviral effector functions to this organ are therefore critical for effective antiviral defense. A major challenge in the development of a therapeutic vaccine against chronic hepatitis B or C virus infection is thus the efficient targeting of specifically induced immune effector specificities to the liver. The Journal of Immunology, 2000, 164: 4235–4243.

Therapeutic vaccination to control chronic infection or cancer is a fascinating concept in clinical medicine. Unfortunately, such attempts have been largely unsuccessful in clinical trials up to now. Chronic infection with hepatitis B virus (HBV) is an important medical problem. More than 350 million chronically HBV-infected carriers world-wide represent the reservoir for human infections. Chronic liver disease and hepatocellular carcinoma associated with chronic HBV infection are among the most important human health problems in high-prevalence regions. Available therapeutic approaches to control chronic HBV-associated hepatitis are unsatisfactory. The available evidence indicates that the specific cellular immune response to HBV-encoded proteins is decisive in determining the outcome of the infection (reviewed in Ref. 1). Patients with HBV-associated chronic hepatitis would greatly benefit from a therapeutic vaccine that could control the persistent virus infection. Because HBV is a small, stable, and well-characterized virus and preclinical animal models for the study of the pathogenesis of chronic HBV infection and its therapy are available, studies in this system could contribute generic principles for the development of therapeutic vaccines that can control other chronic virus infections. Hence, success in the design of an efficacious therapeutic vaccine against HBV may reveal new options for the therapeutic vaccination in other chronic infections (e.g., hepatitis C virus or HIV).

Efficient delivery systems for hepatitis B surface Ag (HBsAg) have been developed that prime potent humoral and cellular immune responses in preclinical animal models. These vaccination approaches comprise protein-based as well as DNA-based systems. These candidate vaccines can be delivered in ways that stimulate strictly polarized responses depending on the dose, route, and technique of delivery of the Ag or expression plasmid, and the codelivery of immunostimulating or -modulating reagents (2–24). Most of the vaccination studies have been performed in mice because this species represents the immunologically best-defined preclinical system available, although mice have disadvantages for the study of the immunopathology associated with chronic HBV infection. Another attractive preclinical model for the study of hepatitis virus patholology is the woodchuck hepatitis virus-infected woodchuck, although this system is not well-defined immunologically.

Transgenic (tg) mouse lines have been constructed that express replication-competent HBV genomes or subgenomic HBV fragments in the liver (25–33). We used two alternative experimental strategies to test antiviral therapeutic vaccination approaches. In the first approach, tg line C57BL/6J-TgN(Alb1HBV)44Bri mice...
expressing large, middle, and small HBsAg in the liver (HBs-tg) were vaccinated. This was unsuccessful using many alternative vaccination techniques that were very efficient in congenic, non-tg control mice. Therefore, we focused on an alternative approach in which immune cells (primed and boosted in non-tg, congenic donor mice by different vaccination protocols) were transferred into normal (nonconditioned) HBs-tg hosts. The aim of this experimental design was the establishment of a long-lasting, HBsAg-specific cellular and humoral immune response in the tg host and the investigation of its targeting to the liver.

Materials and Methods

Mice

C57BL/6J mice (H-2b) were kept under standard-pathogen-free conditions in the animal colonies of Ulm University (Ulm, Germany). B6-tg mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 10–16 wk of age.

Recombinant HBsAg

Nonglycosylated HBsAg, subtypes ayw and adw, containing the small HBsAg (S) protein of HBV were produced in the Hansenula polymorpha host strain RB10 (34). HBsAg particles purified from crude yeast extracts by adsorption to silica gel, column chromatography, and isopycnic ultracentrifugation were obtained from Dr. K. Melber (Rhein Biotech, Düsseldorf, Germany) (34).

HBsAg-encoding plasmid DNA used for nucleic acid vaccination

The HBsAg-encoding XhoI/BamHI fragment of HBV (subtype ayw) was cloned into the XhoI/BamHI-cut pCI vector (catalog no. E1731; Promega, Madison, WI). In the generated plasmid pCI/S, the HBsAg is expressed under control of the human CMV immediate early promoter.

In vivo suppression of CD4+ T cells in mice

CD4+ T cells were suppressed in mice by two i.p. injections of 200 μl PBS containing 100 μg of the anti-CD4 mAb YTS 191.1, as described previously (35). Flow cytometric analyses of PBMC populations demonstrated that >99% of the CD4+ T cells expressing the respective phenotype were deleted.

Oligodeoxynucleotides (ODN) with immune-stimulating sequences (ISS)

In some experiments, HBsAg was mixed with either 50 μg ODN TCAT TGGAAAAACGTTCTTCGGGGCG containing a CpG-ISS or 50 μg ODN TCATTGAAAAAGTTCTTCGGGGCG (ISS*) containing no ISS (36). The phosphorothioate-modified ODN were produced by MWG-Biotech (Ebersberg, Germany). HBsAg mixed with the ODN was injected into mice without adding further adjuvants.

Vaccination of mice

Adult mice were immunized i.m. into the tibialis anterior muscle or s.c. (at the base of the tail) with the indicated amounts of plasmid DNA or recombinant HBsAg particles as described previously (17, 37, 38). Alternatively, mice were inoculated intradermally (into the lateral abdominal skin) with particle-coated plasmid DNA using the Helios Gene Gun system (catalog no. 165-2431,2432; Bio-Rad, Munich, Germany). The manufacturer’s instructions were closely followed to coat DNA to gold particles and to operate the “gene gun.” Briefly, 1 μg plasmid DNA was coated onto 0.5 mg microcarrier particles of 1-μm diameter and fixed in a cartridge using 0.05 mg/ml polyvinylpyrrolidone. Mice were intradermally inoculated with DNA-coated gold particles using a helium pressure of 200 psi.

Spleens were obtained from C57BL/6 (B6) mice primed and boosted with the indicated vaccines. Single-cell suspensions were prepared from these spleens in PBS/BSA. A total of 2–5 × 10^7 spleen cells were injected i.p. or i.v. into HBs-tg or non-tg B6 hosts.

HBsAg-specific CTL

Single-cell suspensions were prepared from spleens of immunized mice in MEM-α tissue culture medium supplemented with 10 mM HEPES buffer, 5 × 10^{-7} M 2-ME, antibiotics, and 10% v/v FCS (Life Technologies, Eggenstein, Germany). A selected batch of Con A-stimulated rat spleen cell supernatant (2% v/v) was added to the culture medium. Responder cells (3 × 10^5) were cocultured with 1 × 10^9 irradiated, syngeneic RBL/S transfectants. Coculture was performed in 10 ml medium in upright 25-cm² tissue culture flasks in a humidified atmosphere/7% CO2 at 37°C. After 5 days, the culture, CTL, were harvested, washed, and assayed for HBsAg-specific cytolytic reactivity. All CTL lines generated displayed the CD3+ CD4+ CD8+ TCRαβ² phenotype.

Serial dilutions of effector cells were cultured with 2 × 10^{12}–13 Cr-labelled targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in short-term 1-h Cr-release assays against RBL5, RBL5/BMG (transfected with the BMGneo vector without insert), or RBL/S transfectants (carrying the HBsAg-encoding BMGneo vector) (23). After a 3.5-h incubation at 37°C, 50 μl of supernatant was collected for gamma-radiation counting. The percentage specific release was calculated as ([experimental release – spontaneous release]/total release – spontaneous release) × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always less than 15% of the total counts. Data shown are the mean of triplicate cultures. The SEM of triplicate data was always less than 20% of the mean.

Detection of IFN-γ

IFN-γ released into the supernatants of spleen cells (10^6/ml) stimulated for 40 h with 10 μg/ml HBsAg was detected by double-sandwich ELISA. For detection and capture of IFN-γ, the mAb RA-6A2 and biotinylated mAb XMG1.2 (both from Pharmingen, Hamburg, Germany) were used. Extinction was analyzed at 405/490 nm on Spectra-Max equipment (Molecular Devices, Sunnyvale, CA) using the Softmax Pro software (Molecular Devices).

Determination of serum Ab levels

Serum samples were repeatedly obtained from individual, immunized, or control mice by tail bleedings at different time points postinjection. Abs against HBsAg were detected in mouse sera using the commercial IMX-AUSAB test (catalog no. 7A39-20; Abbott, Wiesbaden, Germany). Abs were quantified using six standard sera. The tested sera were diluted so that the measured OD values were between standard serum one and six. Values presented in this paper are calculated by multiplying the serum dilution by the measured Ab level (μIU/ml).

HBsAg-specific IgG, IgG1, and IgG2a serum Abs were determined by an end-point dilution ELISA assay. MicroELISA plates (Nunc-Maxisorb; Nunc, Wiesbaden, Germany) were coated with 150 ng recombinant HBsAg per well in 50 μl 0.1 M sodium carbonate buffer (pH 9.5) at 4°C. Serial dilutions of the sera in loading buffer (PBS supplemented with 3% BSA and 2% Tween 20) were added to the Ag-coated wells. Serum Abs were incubated for 2 h at 37°C before four washes with PBS supplemented with 0.05% Tween 20. Bound serum Abs were detected using HRP-conjugated rat anti-mouse IgG1 or IgG2a Abs (catalog nos. 02237E and 02017E; Pharmingen) at a dilution of 1:2000 before incubation with o-phenylenediamine × 2 HCl (catalog no. 6172-24; Abbott) in PBS (pH 6.0). The reaction was stopped by 1 M H₂SO₄, and the extinction was determined at 492 nm. End-point titers were defined as the highest serum dilution that resulted in an absorbance value three times greater than that of negative control sera (derived from nonimmunized B6 mice).

HBsAg expression in the liver (at the protein and DNA level)

The HBsAg concentrations present in the livers of immunized and adoptively transferred mice were determined by a commercial ELISA (AUSZYME II; Abbott). Liver lysis and total RNA extraction and purification were performed using the QIAshredder and RNeasy kits, respectively (Qiagen, Köln, Germany), and a subsequent step of digestion with 20 units DNase I. RNA was analyzed by formaldehyde/1.5% agarose gel electrophoresis. Nucleic acids were transferred to Hybond-N membranes (Amersham, Arlington Heights, IL). Hybridization with recombinant full-length HBV 1.4 DNA labeled to high specific activity (2 × 10^{9} cpm/μg) was performed for 16 h at 65°C in 50% (v/v) formamide, 5 × SSC-phosphate/EDTA, 2.3 × Denhardt’s solution, 0.1% SDS, and 200 μg/ml denatured calf thymus DNA/tube after 4 h of prehybridization at 42°C. After hybridization, high-stringency washes were performed. Membranes were exposed to x-ray film at −80°C.

Results

Inducing polarized immunity to HBsAg in B6 mice

B6 mice were injected with 1) 100 μg HBsAg-encoding pCI/S plasmid DNA i.m., 2) 1 μg particle-coated pCI/S plasmid DNA...
intradermally with the gene gun, 4–3) 1–20 μg nonadjuvanted HBsAg lipoprotein particles i.m. (c), or 20 μg HBsAg particles adjuvanted with 50 μg ISS-containing ODN i.m. (d). All vaccinated mice were assayed for MHC-I-restricted, HBsAg-specific CTL reactivity 3 wk postvaccination (upper panel) and for anti-HBsAg serum Ab titers 8 wk postvaccination (lower panel). In the cytotoxic assay, HBsAg-expressing, syngeneic RBL5/S targets and control RBL5 targets (that do not express Ag) were used. Mean IgG or IgG isotype-specific Ab titers or the isotype ratios of four mice/group (±SEM) are shown.

FIGURE 1. Priming polarized murine immune responses to HBsAg by protein- or DNA-based vaccination. B6 mice were injected once with 100 μg HBsAg-encoding pCI/S plasmid DNA i.m. (a), 1 μg particle-coated pCI/S plasmid DNA intradermally with the gene gun (b), 20 μg nonadjuvanted HBsAg lipoprotein particles i.m. (c), or 20 μg HBsAg particles adjuvanted with 50 μg ISS-containing ODN i.m. (d). All vaccinated mice were assayed for MHC-I-restricted, HBsAg-specific CTL reactivity 3 wk postvaccination (upper panel) and for anti-HBsAg serum Ab titers 8 wk postvaccination (lower panel). In the cytotoxic assay, HBsAg-expressing, syngeneic RBL5/S targets and control RBL5 targets (that do not express Ag) were used. Mean IgG or IgG isotype-specific Ab titers or the isotype ratios of four mice/group (±SEM) are shown.

mice immunized with 100 μg nonpackaged pCI/S DNA or with HBsAg particles coadministered with ISS-containing ODN (Fig. 1). In contrast, HBsAg-specific CTL, were not detectable in mice vaccinated with either 1 μg pCI/S plasmid DNA using the gene gun or nonadjuvanted HBsAg particles (Fig. 1). The polarization of these murine immune responses to HBsAg was further confirmed by their specific IFN-γ release pattern (Table I). Thus, different protein- or DNA-based vaccines efficiently prime T1 or T2 immune responses to HBsAg.

Immunization of HBs-tg mice with experimental vaccines does not prime immune responses to HBsAg

HBs-tg B6 mice primed and boosted with each of the four vaccines developed no detectable immune responses to HBsAg. We found no evidence for the stimulation of a cellular or humoral immune response to HBsAg in these mice. The HBsAg antigensemia in vaccinated HBs-tg mice persisted unchanged, no HBsAg-specific serum Abs appeared, HBsAg-specific CTL were not found, and evidence for liver injury (rise in serum transaminases) was not detected (data not shown). Many variants of these vaccination protocols were tested, but none of them could prime an immune response in the HBs-tg host (data not shown; some protocols are described in the Discussion section). Therefore, it is difficult to

induce an immune response against this immunogenic viral surface Ag in mice that express secreted HBsAg in liver and kidney cells early in life and contain high levels of this Ag in all body fluids.

HBsAg antigenemia is suppressed and HBsAg-specific serum Ab titers appear in HBs-tg hosts reconstituted with HBsAg-immune cells

Immune B6 donor mice primed and boosted by i.m. injections of 100 μg pCI/S plasmid DNA or HBsAg particles mixed with ODN showed HBsAg-specific CTL reactivity and high specific serum Ab titers. Within 3–5 days after the adoptive transfer of spleen cells from these donors into congeneric HBs-tg hosts (1–5 × 10^7 cells/host), HBsAg antigenemia was suppressed and anti-HBsAg serum Abs appeared (Fig. 2). The HBsAg-specific serum Ab titers increased for weeks in the transplanted HBs-tg hosts, suggesting restimulation of the immune response in the adoptive host. Furthermore, this adoptive T1 immunity established in the tg host was detectable as specific IFN-γ release of spleen cells from transplanted tg mice in response to HBsAg stimulation (data not shown). The suppression of antigenemia and the high anti-HBsAg

Table 1. The adoptive transfer of immune T1 or T2 cells specific for HBsAg into congeneric HBs-tg hosts stably engrafts polarized Ab immunity

<table>
<thead>
<tr>
<th>Group</th>
<th>Technique*</th>
<th>Vaccine</th>
<th>Dose</th>
<th>Route</th>
<th>CTL*</th>
<th>IgG1/IgG2a*</th>
<th>IFNγ*</th>
<th>Polarization bias</th>
<th>Serum HBsAg (ng/ml)</th>
<th>IgG1/IgG2a*</th>
<th>Polarization bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA-based</td>
<td>pCI/S</td>
<td>100</td>
<td>i.m.</td>
<td>±</td>
<td>1944 ± 512</td>
<td>T1</td>
<td>4</td>
<td>0.3 ± 0.15</td>
<td>54 ± 19</td>
<td>T1</td>
</tr>
<tr>
<td>2</td>
<td>DNA-based</td>
<td>pCI/S</td>
<td>1</td>
<td>i.d.</td>
<td>±</td>
<td>104 ± 36</td>
<td>T2</td>
<td>4</td>
<td>&lt;0.2</td>
<td>64 ± 16</td>
<td>T2</td>
</tr>
<tr>
<td>3</td>
<td>DNA-based</td>
<td>pCI/S</td>
<td>1</td>
<td>i.d.</td>
<td>±</td>
<td>62 ± 58</td>
<td>T2</td>
<td>4</td>
<td>&lt;0.2</td>
<td>57 ± 8</td>
<td>T2</td>
</tr>
<tr>
<td>4</td>
<td>Protein-based</td>
<td>HBsAg</td>
<td>20</td>
<td>i.m.</td>
<td>±</td>
<td>83 ± 21</td>
<td>T2</td>
<td>8</td>
<td>&lt;0.2</td>
<td>0.2 ± 0.08</td>
<td>T1</td>
</tr>
<tr>
<td>5</td>
<td>Protein-based</td>
<td>HBsAg/ODN</td>
<td>20</td>
<td>i.m.</td>
<td>±</td>
<td>0.13 ± 0.1</td>
<td>T1</td>
<td>8</td>
<td>&lt;0.2</td>
<td>1944 ± 512</td>
<td>T1</td>
</tr>
</tbody>
</table>

* Normal H-2b B6 mice were immunized. In Group 1, nonimmune B6 spleen cells were transferred into HBs-tg hosts.
* Congenic Alb-HBs tg hosts (that express HBsAg in liver).
* Immunization with either the plasmid DNA pCI/S or HBsAg particles at the dose (μg/mouse) and route (i.m., intramuscular; i.d., intradermal) indicated.
* MHC-I-restricted, HBsAg-specific CTL reactivity detectable in donor immune cell populations by a 4-h 51 Cr-release assay.
* Mean isotype ratios ± SEM of anti-HBsAg serum Abs of four to eight transplanted HBs-tg mice/group determined 6 wk postvaccination or 1 wk posttransfer.
* IFN-γ release by spleen cells from primed mice specifically restimulated in vitro for 40 h.
* HBsAg antigenemia determined 1 wk posttransfer in the adoptive host.
* HBsAg particles mixed with 50 μg/mouse immune-stimulating ODN.

FIGURE 2. Transfers of T1 or T2 immune (but not nonimmune) cells into HBs-tg hosts establish anti-HBsAg Ab responses that suppress HBsAg antigenemia. B6 donor mice were primed and boosted with either 100 μg pCI/S plasmid DNA i.m. (T1 immune cells) or 1 μg particle-coated pCI/S plasmid DNA intradermally with the gene gun (T2 immune cells). Spleen cells from these donor mice were injected i.p. into HBs-tg hosts (5 × 10^7 cells/mouse). Mean Ab titers (mIU/ml) and serum HBsAg levels (ng/ml) ± SEM of five transplanted HBs-tg hosts/group are shown.
serum Ab levels persisted for at least 5 mo after the transfers. Thus, the adoptive transfers of T1 HBsAg-immune cells into HBs-tg hosts lead to stable engraftment of at least one component of the anti-HBsAg immune reactivity.

A HBsAg-specific, MHC-I-restricted CTL reactivity was present in the immune cell populations used for the transfers (Fig. 3A). This specific CTL reactivity was detected in the spleens of syngeneic, non-tg naive hosts injected 5 days previously with these immune spleen cells (Fig. 3B). In contrast, this HBsAg-specific and MHC-I-restricted CTL reactivity was not found in spleens of transplanted tg hosts that were assayed for this specific immune reactivity at different time points posttransfer (Fig. 3C).

The following experiments were designed to address the questions 1) can T1- as well as T2-biased immune responses be engrafted into the adoptive tg host, 2) is the engraftment of the adoptively transferred immunity to HBsAg in the tg host T cell-dependent, and 3) can the transferred T1 or T2 immunity to HBsAg suppress Ag expression in the liver.

We primed and boosted B6 donor mice with one of the four vaccines that generate stable and potent T1 or T2 immunity to HBsAg. When immune cells from these donors were transferred into HBs-tg hosts, all tested spleen cell populations established rising HBsAg-specific serum Ab titers that suppressed HBsAg antigenemia for weeks (Fig. 2 and Table I). The isotype profile of the serum Abs in the adoptive tg host reflected the polarization prevalent in the transferred immune cell population, i.e., T1 and T2 immune cells were equally well-engrafted and maintained their polarization pattern (imprinted during priming) in the tg host (Table I).

**HBsAg expressed in HBs-tg hosts restimates adaptively transferred immune cells with different polarization profiles in vivo**

HBsAg-immune cells from B6 donor mice were transferred into naive, syngeneic B6 hosts or congenic HBs-tg mice. Fig. 4 (left panel) shows data from adoptive transfers of immune cells primed by ODN-adjuvanted HBsAg particles. After transfer of HBsAg-immune cells into syngeneic, naive hosts, HBsAg-specific serum Abs were detectable for only a few weeks posttransfer (Fig. 4, left panel). In contrast, the transfer of HBsAg-immune cells into HBs-tg hosts established HBsAg-specific serum Ab titers rising for weeks posttransfer (Fig. 4, left panel). Thus, endogenous (transgene-derived) HBsAg restimulates the adoptively transferred immune cells in the HBs-tg hosts without changing their polarization profile (Table I).

**Injection of HBsAg-specific Abs into HBs-tg mice does not establish stable humoral immunity**

We tested whether the transfer of HBsAg hyperimmune sera is as efficient as the adoptive transfer of immune cells in establishing anti-HBsAg immunity in HBs-tg mice. Xenogenic, polyclonal anti-HBsAg Abs (from rabbit or human) were injected into either normal B6 mice or HBs-tg B6 mice (Fig. 4, right panel; and data not shown). This passive serotherapy leads to the appearance of HBsAg-specific Abs in the serum of non-tg B6 mice and HBs-tg mice. HBsAg antigensemia was suppressed in HBs-tg mice for some weeks but always reappeared after 3–12 wk. Transferred anti-HBsAg Abs were more rapidly cleared from the serum of HBs-tg mice than from non-tg mice (data not shown). Binding of xenogenic anti-HBsAg Abs to circulating HBsAg in HBs-tg mice (suppression of HBsAg antigensemia) did not provide “help” for priming HBsAg-specific immune responses in HBs-tg mice. Similar data were obtained when anti-HBsAg monoclonal IgG1 or IgG2b Abs or polyclonal antisera from vaccinated mice were injected into HBs-tg mice (Fig. 4, right panel; and data not shown). Hence, the transfer of immune cells but not that of Abs of different isotypes (and of different species origin) stably suppressed HBsAg antigensemia.

**Engraftment of anti-HBsAg immunity into HBs-tg hosts is CD4+ T cell-dependent**

Different mechanisms may contribute to establish stable humoral immunity to HBsAg in HBs-tg mice by immune cell transfer. These include 1) transfer of long-lived, Ab-producing plasma cells
from primed donor mice, 2) donor T cell-dependent stimulation of engrafted HBsAg-specific donor B cell memory, or 3) donor T cell-dependent stimulation of HBs-tg host-derived B cells. Bone marrow cell transfers from immune donors did not engraft HBsAg immunity in HBs-tg hosts (data not shown). Because long-lived, Ab-producing plasma cells mainly reside in bone marrow, it is unlikely that transfer of long-lived donor plasma cells established humoral immunity in the adoptive host. We investigated the T cell dependence of the HBsAg-specific Ab responses in transplanted HBs-tg mice, which expand over many months and suppress antigenemia. Evidence for the critical role of donor CD4$^+$ T cells in supporting restimulation of anti-HBsAg immune responses in the adoptive host was obtained when immune donor B6 mice were CD4$^+$ T cell-depleted in vivo before the cell transfer by repeated injections of a depleting anti-CD4 mAb (35, 41). Normal numbers of CD19$^+$ B cells and CD8$^+$ T cells but $\sim$1% of the CD4$^+$ T cell population were found in immune spleen cell populations of CD4$^+$ T cell-depleted donor mice. Transfer of CD4$^+$ T cell-depleted immune spleen cells into HBs-tg hosts failed to suppress HBsAg antigenemia and did not lead to the appearance of anti-HBsAg serum Abs (Fig. 5). When CD4$^+$ T cells were depleted from HBs-tg hosts by mAb treatment in vivo shortly before transfer of

FIGURE 4. Transfer of immune cells but not Abs establishes stable HBsAg immunity in HBs-tg hosts. Left panel, Spleen cells were obtained from B6 mice primed and boosted with 20 $\mu$g HBsAg particles delivered with 50 $\mu$g ISS$^+$ ODN i.m. (immune cells). These cells ($5 \times 10^7$ cells/mouse) were injected i.p. into either normal syngeneic B6 hosts or HBs-tg B6 hosts. Right panel, Xenogeneic (rabbit) polyclonal anti-HBsAg IgG Abs or anti-HBsAg IgG1 or IgG2b mAbs were injected into HBs-tg mice. Mean Ab titers (mIU/ml) ± SEM of four transplanted (left panel) or Ab-injected (right panel) mice/group measured at different time intervals postinjection are shown.

FIGURE 5. Establishment of stable HBsAg immunity in adoptive HBs-tg hosts is CD4$^+$ T cell-dependent. B6 mice primed and boosted to HBsAg by pCl/S plasmid DNA injections were depleted in vivo of CD4$^+$ T cells by repeated injections of the anti-CD4 mAb YTS 191.1 (35, 41). Injection i.p. of these immune spleen cells (containing $\sim$1% of the CD4$^+$ T cell population) into HBs-tg hosts failed to suppress HBsAg antigenemia and did not lead to the appearance of anti-HBsAg serum Abs (Fig. 5). When CD4$^+$ T cells were depleted from HBs-tg hosts by mAb treatment in vivo shortly before transfer of

Mean Ab titers (mIU/ml) and serum HBsAg levels (ng/ml) ± SEM of four mice/group.

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immune cells from donors with an intact CD4⁺ T cell compartment, the appearance of serum Abs was delayed but not suppressed (data not shown). This is readily explained by the persistence of depleting anti-CD4 Abs in the host that delete a fraction of the transplanted CD4⁺ T cells. The almost complete depletion of CD4⁺ T cells from HBs-tg mice by repeated in vivo Ab treatment did not lead to the appearance of anti-HBsAg serum Abs or the suppression of HBsAg antigenemia. Thus, primed, donor-derived CD4⁺ T cells (and not the absence of host-derived “suppressive” CD4⁺ T cells) are critical for the establishment of HBsAg-specific Ab responses in the HBs-tg host. This is to be expected because the polarization profile of the Ab response (that usually results from CD4⁺ helper T cell regulation) established in the adoptive tg host always corresponded to that imprinted during priming and boosting in donor mice.

Established immunity to HBsAg in HBs-tg mice does not suppress transgene-directed HBsAg expression in the liver

Liver tissue was obtained from nontreated HBs-tg mice (Table II, group 1), nontreated B6 mice (data not shown), HBs-tg mice reconstituted with immune spleen cells (i.m. 100 μg pCI/S plasmid DNA-vaccinated) from congenic donor mice (Table II, group 2), or HBs-tg mice injected 6 days previously with 3 × 10⁹ mlU rabbit anti-HBsAg antiserum (Table II, group 6). HBsAg concentrations of 15–28 ng/mg tissue were measured in the liver of HBs-tg mice that were nontreated, injected with anti-HBsAg antiserum, or reconstituted with HBsAg immune cells. HBsAg was not detectable in the liver of non-tg B6 mice. Despite the rising anti-HBs Ab titers and the concomitant decline of HBsAg antigenemia in HBs-tg mice reconstituted with immune cells, no decrease in HBsAg content of the liver was demonstrated (Table II, group 2). Furthermore, no differences in the amounts of intrahepatic, pregenomic 2.1/2.4-kb mRNA was observed between nontreated and treated HBs-tg mice of groups 1, 2, and 6 (data not shown). Thus, ongoing HBsAg-specific immune responses in the HBs-tg hosts do not suppress transgene-directed HBsAg expression in the liver.

Discussion

Unsuccessful vaccination of tg mice

Vaccination techniques that efficiently primed humoral and CTL responses with T1 or T2 polarization profiles to HBsAg in H-2d and H-2b mice proved spectacularly inefficient in HBs-tg mice. This may reflect induction of neonatal or peripheral tolerance, exhausted T cell responses, Ag expression in immune-privileged sites, or presentation by CD95L⁺ cells (reviewed in Ref. 1). The described data confirm a previous report in which the direct vaccination of HBs-tg mice was shown to be very difficult (42). Only repeated infections with large numbers of HBsAg-encoding recombinant vaccinia virus or repeated injections of HBsAg adjuvanted in CFA were reported to stimulate low anti-HBsAg Ab responses but no CTL responses in HBs-tg mice. Our data differ from the reported success of therapeutic vaccination with HBsAg-encoding plasmid DNA in a different HBs-tg lineage (31, 43, 44). The reported successful therapeutic vaccination in this system might be related to the fact that HBsAg transgene expression in this particular lineage is extinguished by methylation (45–48). It is unlikely that a deficient anti-pre-S immunity was critical for the failure of our therapeutic vaccination. We vaccinated HBs-tg mice with constructs encoding the large (pre-S1, pre-S2, S) surface Ag or the “mixed” (plasma- or Chinese hamster ovary-derived) HBsAg lipoprotein particles (that contain large, middle, and small HBsAg proteins); these vaccines stimulate efficiently anti-pre-S immunity in normal mice. None of these candidate vaccines suppressed HBsAg antigenemia and induced seroconversion in the HBs-tg hosts. Thus, the data we describe in this paper agree with the conclusion obtained with DNA vaccination in tg lineages in which expression of HBV genes is not methylation-sensitive, i.e., DNA immunization does not break tolerance to HBV Ags (49).

We used additional immunization protocols in HBs-tg mice to provide heterologous T cell help or to bypass T cell help requirements in an attempt to prime humoral and CTL responses to HBsAg. Several approaches were tested: 1) HBsAg-encoding plasmids were mixed with OVA- or hepatitis B core Ag-encoding plasmids to provide heterologous carriers, 2) HBsAg of the transgene-encoded serotype (ayw) as well as HBsAg of a related but different serotype (adw2) were delivered as recombinant lipoprotein particles, 3) murine cytokines (IL-7, IFN-γ, and GM-CSF) were mixed as plasmid DNA with HBsAg-encoding plasmid DNA, and 4) ODN with ISS were codelivered with HBsAg particles to bypass T helper cell requirements (35). None of these experimental strategies established stable anti-HBsAg immunity in HBs-tg mice.

Adoptive transfer of HBsAg-immune cells

Because the direct immunization experiments of HBs-tg mice were unsuccessful, we used adoptive transfer systems to engrraft immune cells into HBs-tg hosts that produce transgene-encoded viral Ag in almost all liver cells. By transferring immune cells from non-tg, congenic donor mice into HBs-tg hosts, we asked whether the Ag-bearing HBs-tg host supports, modulates, or suppresses engraftment of humoral and/or cellular immunity to HBsAg.

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Table II. Adoptive transfer of T1 or T2 HBsAg-immune cells neither induces liver injury nor suppresses transgene-encoded HBsAg expression in the liver of HBs-tg hosts

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Polarization Bias</th>
<th>n</th>
<th>HBs-tg Mice Transplanted with Immune Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum HBsAg (ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>None</td>
<td>3</td>
<td>45 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>pCI/S 100 μg i.m.</td>
<td>T1</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>pCI/S 1 μg i.d.</td>
<td>T2</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>HBsAg 20 μg i.m.</td>
<td>T2</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>HBsAg 20 μg/ODN⁶</td>
<td>T1</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>Rabbit anti-HBsAg antiserum</td>
<td>T1</td>
<td>3</td>
<td>ND</td>
</tr>
</tbody>
</table>

⁶ B6 mice were primed and boosted using the vaccination approaches shown in Table I. ND, not detectable; NT, not tested; i.d., intradermally.

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B6 mice were primed and boosted using the vaccination approaches shown in Table I. ND, not detectable; NT, not tested; i.d., intradermally.
Immune cells (from congenic, non-tg mice primed and boosted with HBsAg) were engrafted into the HBs-tg host after the i.v. or i.p. injection of 1–5 x 10^7 spleen cells. Engraftment of immunity was evident by the appearance of anti-HBsAg serum Ab titers and the concomitant suppression of HBsAg antigenemia. Antigenemia seems to be suppressed because Western analyses detected HBsAg in serum of nontransplanted but not transplanted HBs-tg mice. This makes masking of epitopes required for detection unlikely in that the HBsAg proteins were denatured under reducing conditions and detected with a polyclonal rabbit antiserum that detects denatured, sequential determinants of this viral protein. HBsAg-specific CTL did not establish a functional memory population in the spleen of the adoptive host. They may be selectively anergized, eliminated in the tg host, or trapped in lymph nodes draining hepatic tissues. In ongoing studies, we investigate the fate of primed CD8+ CTL in the adoptive HBs-tg host. Thus, transferred HBsAg-specific CD8+ CTL subset and the CD4+ helper T cell subset seem to have different fates in the adoptive host. While the former subset is functionally silent after its transfer into the tg host, the latter subset supports expanding B cell responses for many months.

Donor CD4+ T cells were required to support long-lasting anti-HBsAg Ab responses restimulated by tg-encoded HBsAg in the adoptive host. When CD4+ T cells were depleted from transferred immune cell populations, engraftment was completely prevented. HBsAg-immune T1 or T2 CD4+ T cells are restimulated by transgene-derived HBsAg in the HBs-tg host, maintain their polarization profile, and (re)stimulate a HBsAg-specific B cell response for months. Because the transfer of purified, immune CD4+ T cells into HBs-tg hosts did not stimulate the appearance of anti-HBsAg serum Ab titers (data not shown), most responding HBsAg-specific B cells are donor-derived.

Targeting antiviral immune cells to the liver

Adoptive transfers of high numbers of MHC-I-restricted, HBsAg-specific CTL lines into irradiated HBs-tg hosts induce liver injury in a well-characterized multistep process (26, 28, 50–53). Injury can also be triggered in these mice by injecting LPS, cytokines, or liver infection with unrelated viruses (33, 42, 54, 55). In our transfer experiments, we used nonirradiated hosts and injected lower numbers of immune cells i.p. to establish an immune response in the host. In the weeks that followed the T1 or T2 immune cell transfers into HBs-tg hosts, the serum transaminase levels did not rise, i.e., no evidence for liver injury was evident. Expression of transgene-encoded HBsAg in the liver (at the RNA and protein level) was not influenced by the establishment of this immunity in the tg host. Northern analyses indicated that neither the level of the (heterologous albumin promoter-driven) 2.4-kb transcript nor that of the (HBV-promoter-driven) 2.1-kb transcript of HBsAg differed between HBs-tg mice that were nontransplanted, transplanted with syngeneic nonimmune spleen cells, or transplanted with syngeneic HBsAg-immune spleen cells. Furthermore, no evidence for suppression of HBsAg expression at the protein level was detectable in the liver of adoptive hosts reconstituted with immune cells. Hence, although a vigorous and expanding antiviral immune response is going on for months, this does not affect Ag expression in the main target organ.

The problem of organ-specific microenvironment for the access and development of activated T cells

The fate of antiviral CTL that rapidly disappear after transfer is under study. CD4+ T cells are engrafted and can be detected for months posttransfer. IFN-γ-producing T1 CD4+ T cells (that supported the development of continuously rising anti-HBsAg serum IgG2a Ab responses) with antiviral activity apparently do not accumulate in the HBsAg-producing liver and cause injury or suppression of Ag expression. These T cells may either not migrate to the liver or home to the liver to be functionally silenced or physically eliminated. In the C57BL/6J-TgN(Alb1HBV)44Bri tg line of HBs-tg mice used in this study, virtually all hepatocytes express HBsAg (25); furthermore, HBsAg is expressed in the kidney epithelium and the epithelium of the choroid plexus of these mice. Restricted CTL access to HBsAg-expressing renal and neural tissues in vivo has been described in this model (8, 9). Our data suggest that a similar failure to accumulate at the site of Ag expression operates in the liver. This indicates a major problem for designing a specific, T cell-based immunotherapy for chronic HBV or HCV infection, i.e., delivering primed antiviral effector cells to the target organ.

As is evident from some experimental systems, breaking peripheral tolerance of MHC-I-restricted CTL in vivo does not directly result in autoimmunity. A well-characterized model demonstrating this point is a TCR tg mouse system displaying peripheral tolerance against a liver-specific MHC-I rb Ag (56–59). Peripheral tolerance to this liver-specific Ag can be broken in vivo by appropriate vaccination strategies employing costimulatory cytokines. However, the activated rb-specific CTL do not induce liver injury. It is only through a (undefined) change in the tissue microenvironment (e.g., triggered by an unrelated infection) that this organ supports activated T cells to specifically deliver their effector functions to the Ag-expressing tissue and thereby cause damage (60). The mechanism underlying this phenomenon remains to be elucidated. The intact tissue environment may restrict access of activated T cells, may suppress in situ T cell responses, or may fail to support clonal expansion and differentiation (61, 62). This observation may not be specific for the liver but may also operate in targeting activated T cells to, e.g., pancreas or nervous system. Many rules remain to be discovered in this field of the tissue-specific regulation of autoaggressive and antiviral T cell responses.

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References


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