Transfer of HBV Genomes Using Low Doses of Adenovirus Vectors Leads to Persistent Infection in Immune Competent Mice

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Studies of mechanisms responsible for the persistence of hepatitis B virus (HBV) infection have been hindered by a lack of appropriate animal models. HBV genomes can be delivered to livers of mice using hydrodynamic injection or high doses of an adenoviral vector; these lead to clearance of HBV. We found that infection of immunocompetent mice with low doses of an adenoviral vector resulted in persistent HBV infection; the mice neither underwent seroconversion nor developed a strong HBV-specific effector T-cell response. As in patients with chronic HBV infection, DNA vaccination failed to generate T cells that cleared infection. This model of persistent HBV infection could be used to study the pathogenesis of chronic HBV infection and develop new therapeutic strategies.

Keywords: Mouse Model; Liver Disease; Virology; Immunity.

Hepatitis B virus (HBV) infection is restricted to its natural hosts (ie, human beings and chimpanzees), but the host barrier can be overcome by generation of HBV-transgenic mice or delivery of HBV genomes into murine hepatocytes in vivo through either adenoviral gene transfer (AdHBV) or hydrodynamic injection (HDI-HBV).1,2 Although both HDI-HBV into immunocompromised mice or HDI of mutant HBV lacking core (HBc) as the key target of cytotoxic T lymphocyte (CTL) immunity led to persistence of HBV,2,3 a model for persistent HBV infection is lacking in immunocompetent mice. Transfer of wild-type HBV genomes by HDI, leading to transfection of approximately 10% of hepatocytes, or high-dose infection with adenoviral vectors targeting more than 90% of hepatocytes (10⁹ infectious units/mouse) resulted in acute self-limiting viral hepatitis.2,4 Nevertheless, adenoviral genome transfer has been shown to allow for persistence of HBV genomes in vivo despite an initial vigorous virus-specific immune response.5 We here examined the course of HBV infection of a nonsusceptible host, the mouse, after low-dose AdHBV. This approach was based on the observation that smaller rather than large virus inocula lead to persistent HBV infection in chimpanzees.5

To our surprise, we observed that low-dose AdHBV infection using 10⁸ infectious units per mouse led to persistent infection that was characterized by persistence of HBsAg and hepatitis B e antigen in serum and lack of seroconversion to antibodies to HBsAg (anti-HBs) until day 98 after infection (Figure 1A–D); increasing the dose of infectious AdHBV to 3 × 10⁹, 10⁹, and 3 × 10⁹ dose-dependently induced anti-HBs seroconversion over time (Figure 1D, inset, and Supplementary Figure 1), indicating that less than 3 × 10⁹ AdHBV should be used for induction of persistent infection. As expected,4 high-dose but not low-dose AdHBV infection led to increased serum alanine aminotransferase (ALT) levels (Supplementary Figure 1). Early after low-dose AdHBV infection (day 4), only a few HBc-expressing hepatocytes were detected but their numbers increased up to day 21 (Figure 1E), and expression persisted up to day 98 after infection. Because there is no evidence for spread of HBV in mice,4,6 the increase in HBc-expressing hepatocytes may result from immunohistochemical detection of accumulating levels of HBcAg. HDI-HBV mice showed strong HBV antigen expression in a higher number of hepatocytes at day 4, reflecting higher levels of antigen expression, which rapidly decreased, becoming undetectable after day 14 (Figure 1A–E). Persistent antigen expression in low-dose AdHBV-infected mice is owing to persistence of the AdHBV inoculum4 and not to establishment of HBV covalently closed circular DNA, the natural transcription template. It correlated with lymphocyte infiltration in the liver, visualized by H&E staining of liver tissue sections (Figure 1E).

Because HBV clearance in HDI-HBV–treated6 as well as in high-dose Ad-HBV–infected mice depends on potent virus-specific CTL immunity, we assumed that low-dose AdHBV infection does not elicit functional HBV-specific CTL. Indeed, neither HBc- nor HBs-specific CTLs were detected in AdHBV-infected mice until day 21, in contrast to rapid generation of such CTLs in HDI-HBV–treated mice (Figure 2A). Although we observed a small yet significant increase in HBV-specific CTLs at day 21 after low-dose AdHBV infection, it apparently was not able to...
control viral antigen expression (Figure 1A and B). CTLs induced by introduction of HBV into hepatocytes via HDI were fully functional: upon antigen re-encounter ex vivo, HBc-specific CTLs up-regulated lysosomal marker CD107a (Figure 2B, left) and produced interferon-γ (Figure 2B, upper right panel), indicating cytotoxic degranulation and effector function. In addition, killing of HBV-peptide–loaded target cells in vivo showed potent cytotoxic activity in HDI-HBV mice (Figure 2B, lower right). In contrast, HBc- and HBs-specific CTLs generated in low-dose AdHBV-infected mice did not show any effector function ex vivo or in vivo.

Absence of an ALT peak at the time of seroconversion despite the presence of significant numbers of functional HBV-specific CTLs in HDI-HBV–treated mice can be explained by our recent observation that CTL-mediated elimination of low numbers of virus-infected hepatocytes escapes detection by measuring serum ALT level. Thus, viral antigenemia in low-dose AdHBV-infected mice resembles the initial stages of natural HBV infection in which significant levels of circulating HBV-antigens but no virus-specific CTL immunity is detected. To determine whether viral persistence in AdHBV-infected mice also reflects exhaustion of CTL
immunity during persistent human hepatic infection, we subjected these mice to therapeutic DNA vaccination with HBc-encoding or control plasmid DNA. Compared with naive mice, we found lower numbers of HBV-specific CTLs after HBc-DNA vaccination in low-dose AdHBV-infected mice (Figure 2C), indicating T-cell tolerance. HBV-specific CTLs generated by HBc-DNA vaccination homed into the livers of low-dose AdHBV-infected animals (Figure 2D), causing mild hepatitis (serum ALT level, <100 U/L). These CTLs, however, up-regulated T-cell immunoglobulin domain and mucin domain 3 (TIM3) and programmed death 1 (PD1) as markers of T-cell dysfunction, and failed to control HBV infection (Figure 2E and F). This recapitulates findings in persistent murine LCMV infection and in persistently HBV-infected human beings.

Figure 2. (A) Flow cytometry analysis of HBc93-100 or HBs190-197–specific hepatic CTLs (left; day 14); numbers of HBc93-100– (middle) and HBs190-197–specific (right) CTLs detected in HDI-HBV– (n = 8) and Ad-HBV– (n = 7) treated mice at indicated time points; naive mice (n = 7) served as control. (B) Liver–associated CTL were stimulated ex vivo with HBV peptides for 5 hours. Left: CD107a surface-expression levels on hepatic HBc93-100–specific or control CTLs at day 14 after HDI-HBV; mean frequency (±SD, n = 4) is indicated; shaded area shows isotype control. Upper right: percentages of interferon-γ–secreting CTLs at day 21 postinfection (n = 3). Lower right: HBV–specific in vivo cytotoxicity in HDI-HBV– or AdHBV–treated mice determined at day 19 postinfection (n = 4). (C)–(F) DNA vaccination with plasmids coding for HBc or a negative control plasmid encoding ovalbumin (OVA) of naive or AdHBV–infected mice (≥2 independent experiments). (C) Peripheral and (D) liver–associated HBc93-100–specific CTLs. (E) CTL phenotype of HBc-DNA vaccinated mice. (F) Serum HBeAg. *P < .05; **P < .01.
Taken together, we describe here a model for persistent HBV infection in immunocompetent mice resembling many of the immunologic features observed in patients with chronic HBV infection, such as lack of HBV-specific B-cell/CTL responses and failure to respond to therapeutic DNA vaccination. Low-dose AdHBV infection seems to mimic underwhelming of the CTL response by low-dose HBV or the stealth strategy of HBV to enter the liver without causing innate immune defense or hepatocyte apoptosis, which may fail to provide sufficient innate immune stimulation to mount CTL immunity. In contrast, HDI elicits danger signals from dying hepatocytes and high-dose AdHBV infection triggers innate immune responses that likely provide sufficient immunogenic stimuli to elicit potent antiviral immunity. Our model suggests that cross-presentation of circulating HBV antigens in the absence of strong innate immune stimulation fails to promote CTL immunity, which remains to be formally proven. Prolonged circulation of HBV antigens did not lead to depletion of HBV-specific CTLs because we were able to induce HBV-specific CTLs by DNA vaccination, which however, became dysfunctional. Because of its similarities with persistent HBV infection in human beings, this model will serve as an important tool for evaluating novel therapeutic approaches aiming to overcome HBV persistence related to immune-mediated mechanisms.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.03.006.

References


Received September 2, 2011. Accepted March 3, 2012.

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Acknowledgments

The authors thank Theresa Asen and the Institute of Virology in Bonn for providing help in measuring viral parameters and Ruth Hillermann and Daniel Kull for help with immunohistochemical staining.

P.A.K. and U.P. contributed equally to this manuscript.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by the Helmholtz Alliance for Immunotherapy (HA-202 to U.P. and P.A.K.); the German Research Foundation (SFB 670, TR57, and 704 to P.A.K., and SFB 576 to U.P.) the European Research Council (starting grant No. 261317 to MH); an institutional grant (BONFOR programme to L.R.H.).
Supplementary Materials and Methods

**HBV Plasmid for HDI and Recombinant Adenovirus Preparation**

Plasmid, pENTRY HBV1.3, contained an 1.3-fold overlength genome of HBV, subtype ayw, with a 5′ terminal redundancy encompassing enhancers I and II, direct repeats DR1 and DR2, the X- and pregenomic/core-promoter regions, the transcription initiation site of the pregenomic RNA, the unique polyadenylation site, a duplication of the entire X open reading frame, and a single copy of every other open reading frame as described previously. The pENTRY HBV1.3 was prepared using the EndoFree plasmid Maxi or Mega Kit (Qiagen, Hilden, Germany) and used for HDI and adenoviral vector preparation, respectively. The HBV1.3 genome from pENTRY HBV1.3 was inserted into the E1 region of adenovirus (Ad5ΔE1/E3) backbone plasmid pAd/PL-DEST through Gateway recombination following the manufacturer’s instructions (Gateway System; Invitrogen, Karlsruhe, Germany) and used for HDI and adenoviral vector preparation, respectively. The HBV1.3 genome from pENTRY HBV1.3 was inserted into the E1 region of adenovirus (Ad5ΔE1/E3) backbone plasmid pAd/PL-DEST through Gateway recombination following the manufacturer’s instructions (Gateway System; Invitrogen, Karlsruhe, Germany). The recombinant adenoviral genome was transfected into human embryonic kidney 293 cells for production of AdHBV. The AdHBV was purified twice by CsCl gradient centrifugation and further dialyzed and quantified for its titer as described previously.

**Mice Experiments and HBV Infections**

C57BL/6j mice were obtained from Janvier (St Berthevin Cedex, France) and maintained under specific pathogen-free conditions according to the guidelines of the Federation of Laboratory Animal Science Associations. All the animal experiments were performed in accordance with German legislation governing animal studies and the Principles of Laboratory Animal Care guidelines (National Institutes of Health publication 85–23, 1996 revision). For HDI of pENTRY HBV1.3 plasmid in mice, 6- to 8-week-old male C57BL/6 mice were anesthetized with ketamine (Medistar Arzneimittelvertrieb, Ascheberg, Germany) and xylazine (Sigma, Munich, Germany). Ten micrograms of HBV plasmid DNA was injected into the tail veins of mice in a volume of phosphate-buffered saline equivalent to 8% of the mouse body weight. The total volume was delivered within 5 seconds. For AdHBV infection, the indicated dose of AdHBV in 200 μL phosphate-buffered saline was delivered into mice intravenously. The serum or blood specimens for assays of hepatitis B surface antigen, hepatitis B e antigen, anti-HBs, and serum ALT were collected at the indicated time points after infections. The livers of mice were fixed in 10% buffered formalin (Sigma) for 24 hours for histologic and immunohistochemical analysis.

**Detection of HBV Antigens, Anti-HBs, and Serum ALT**

Serum levels of HBsAg and hepatitis B e antigen and anti-HBs of the infected mice were determined using the Architect System (for antigens) or the AXSYM system (for anti-HBs) (Abbott Laboratories, Abbott Park, IL). Serum alanine aminotransferase activity was measured using specific bioreaction strips on a Reflovet Plus reader (Roche Diagnostics, Mannheim, Germany).

**Immunohistochemical Analysis**

Fixed, pretreated, 2-μm thick, paraffin-embedded liver sections were decorated with a monoclonal antibody against HBCAg (NCL-HBCAg-506; clone #LF161, dilution 1:200; Leica Microsystems, Wetzlar, Germany). Pretreatment (H2O2) and staining were performed on a Leica Bond-Max, and detection was performed with a Bond Polymer refine kit (DS9800) using a rabbit anti-mouse secondary antibody, coupled to hors eradish peroxidase and 3,3′-diaminobenzidine tetra hydrochloride as a substrate.

**Detection of HBV-Specific CD8 T-Cell Responses Ex Vivo**

Livers were harvested from infected mice after perfusion with phosphate-buffered saline via portal vain for 30 seconds at a flow rate of 4 mL/min at indicated time points after infections and minced through a 250-μm cell strainer. Cell suspension from one liver was resuspended twice by Percoll and centrifugation and further dialyzed and quantified for its titer as described previously.

**Liver-associated lymphocytes (5 × 10⁵) were cultured in RPMI medium 1640 (Invitrogen) with 8% fetal calf serum in the in the presence of 10 μmol/L of peptides, HBc⁵³-¹⁰₀ and HBS¹⁹₀–₁⁹⁷ (Pineda, Berlin, Germany), respectively, for 5 hours. For intracellular interferon-γ staining, Brefeldin A and Monensin (0.1% for each; e-Bioscience, San Diego, CA) in the presence of anti-Fc receptor antibody (clone 2.4G2) for another 20 minutes, and subjected to flow cytometric analysis for detection of HBV-specific CD8 T cells. During flow cytometric analysis, a specific number of CountBright absolute counting beads (Invitrogen) were added to all the samples for calculation of the absolute number of Ag-specific CD8 T cells in the liver.

**Analysis of T-Cell Function Ex Vivo**

Liver-associated lymphocytes (5 × 10⁵) were cultured in RPMI medium 1640 (Invitrogen) with 8% fetal calf serum in the in the presence of 10 μmol/L of peptides, HBc⁵³-¹⁰₀ and HBS¹⁹₀–₁⁹⁷ (Pineda, Berlin, Germany), respectively, for 5 hours. For intracellular interferon-γ staining, Brefeldin A and Monensin (0.1% for each; e-Bioscience) was added to the culture 1 hour after the culture had been set up. The cells were harvested for CD8α surface staining as described previously, subjected to intracellular staining of interferon-γ using allosphycocya-
nin-conjugated anti–IFN-γ antibody (clone XMG1.2; e-Bioscience) and a cell fixation/permeabilization kit (BD Biosciences, Heidelberg, Germany), and flow cytometric analysis. For degranulation assay, the cells were restimulated with peptide in the presence of Monensin and phycoerythrin-conjugated anti-CD107a antibody (clone 1D4B; BD Biosciences) for 5 hours and subjected to dextramer and surface marker staining of CD8α as described previously. All the samples were fixed in 4% paraformaldehyde before flow cytometric analysis and dead cells were excluded from the analysis using the LIVE/DEAD Fixable Violet Dead cell stain kit (Invitrogen).

**In Vivo Cytotoxicity Assay**

Splenocytes from C57BL/6 mice were loaded with 20 μmol/L HBc93-100 and labeled with high-dose carboxyfluorescein succinimidy ester (CFSE) (2 μmol/L; Invitrogen), with controls labeled with low-dose CFSE (0.2 μmol/L). The other target cells were loaded with 20 μmol/L HBs190-197 and labeled with high-dose CellTrace Far Red (0.5 μmol/L; Invitrogen), with controls with low-dose CellTrace Far Red (0.05 μmol/L). The conditions for peptide pulse and fluorescence label were at 37°C for 30 minutes and for 15 minutes, respectively. The 4 labeled cell batches were mixed equally and 2 × 10⁷ of the mixed cells were injected intravenously into each mouse previously infected with HBV by HDI or AdHBV at day 19 after infection. Cytotoxicity was determined after 14 hours using splenocytes from the recipients for flow cytometry. The percentage of specific killing was calculated using the following equation: 100*[1 – (dye<sub>high</sub>⁄dye<sub>low</sub>)<sub>infected mouse</sub>⁄(dye<sub>high</sub>⁄dye<sub>low</sub>)<sub>control mouse</sub>].

**DNA Immunization**

Naive or AdHBV-infected C57BL/6j mice at day 11 after infection were injected intramuscularly into the quadriceps muscle with 100 μg of pCI/HBcAg or pCI/ovalbumin plasmid dissolved in 50 μL of phosphate-buffered saline, followed by in vivo electroporation to increase the expression level of the injected plasmids. Serum hepatitis B e antigen and ALT levels in these mice were monitored at 4 days before DNA immunization, and at 1, 7, 14, and 28 after DNA immunization. Liver-associated lymphocytes and splenocytes were isolated from the mice at days 14 and 28 after immunization following the protocol mentioned earlier for the detection of HBc<sub>93-100</sub>–specific CTLs. The expression of PD-1 and TIM-3 on these CTLs from livers were detected by flow cytometric analysis using fluorescein isothiocyanate–conjugated anti–PD-1 antibody (clone J43; e-Bioscience) and phycoerythrin-conjugated anti–TIM-3 antibody (clone RMT3-23; e-Bioscience).

**Statistical Analysis**

The Student t test was used for analysis. Data are depicted as the mean ± SD, and P values less than .05 were considered significant.

**Supplementary References**

Supplementary Figure 1. Titration of Ad-HBV infection. C57BL/6J mice (n = 6) were infected with different doses of Ad-HBV. (A) Time-course of HBsAg and hepatitis B e antigen (HBeAg) serum levels after infection with different amounts of Ad-HBV at indicated time points. (B) Anti-HBs seroconversion after infection with different amounts of Ad-HBV was determined at day 57 postinfection. (C) As reported previously, high-dose Ad-HBV infection resulted in acute hepatitis at day 6 after infection. Importantly, no increase in serum ALT activity was observed in low-dose Ad-HBV infection performed in parallel. Numbers of anti-HBs positive mice are indicated. *P < .05, **P < .01, ***P < .001.