A Mouse Model for Studying the Clearance of Hepatitis B Virus In Vivo Using a Luciferase Reporter

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Abstract

Hepatitis B virus (HBV) infection remains a global problem, despite the effectiveness of the Hepatitis B vaccine in preventing infection. The resolution of Hepatitis B virus infection has been believed to be attributable to virus-specific immunity. In vivo direct evaluation of anti-HBV immunity in the liver is currently not possible. We have developed a new assay system that detects HBV clearance in the liver after the hydrodynamic transfer of HBV DNA into hepatocytes, followed by bioluminescence imaging of the reporter gene (Fluc). We employed bioluminescence detection of luciferase expression in HBV-infected hepatocytes to measure the Hepatitis B core antigen (HBcAg)-specific immune responses directed against these infected hepatocytes. Only HBcAg-immunized, but not mock-treated, animals decreased the amounts of luciferase expression, HBcAg and viral DNA from the liver at day 28 after hydrodynamic injection with over-length HBV DNA, indicating that control of luciferase expression correlates with viral clearance from infected hepatocytes.

Introduction

Hepatitis B virus (HBV) is a hepatotropic, noncytopathic virus that causes acute and chronic liver diseases and, subsequently, hepatic cirrhosis and hepatocellular carcinoma. Although an effective preventive vaccine is available, there are currently approximately 300 million people who are chronically infected with HBV. Worldwide, the number of deaths from liver cancer caused by HBV infection likely exceeds one million per year. Although an effective vaccine was introduced more than 20 years ago, vaccination is not a treatment for established infections. The resolution of Hepatitis B virus infection has been believed to be attributable to virus-specific immunity. In vivo direct evaluation of anti-HBV immunity in the liver is currently not possible. We have developed a new assay system that detects HBV clearance in the liver after the hydrodynamic transfer of HBV DNA into hepatocytes, followed by bioluminescence imaging of the reporter gene (Fluc). We employed bioluminescence detection of luciferase expression in HBV-infected hepatocytes to measure the Hepatitis B core antigen (HBcAg)-specific immune responses directed against these infected hepatocytes. Only HBcAg-immunized, but not mock-treated, animals decreased the amounts of luciferase expression, HBcAg and viral DNA from the liver at day 28 after hydrodynamic injection with over-length HBV DNA, indicating that control of luciferase expression correlates with viral clearance from infected hepatocytes.

Materials and Methods

Ethics Statement

All animal studies were carried out according to the guidelines established by the ethics committee of the National Beijing Center for Drug Safety Evaluation and Research (Permit No. 11–1725). The mice were anesthetized with ketamine and xylazine for the procedures, including the intramuscular injections, in vivo electroporation, and hydrodynamic injections. The mice were sacrificed by ether anesthesia.
Figure 1. HBV persistence in mice induced by hydrodynamic injection of pGL3/Fluc-HBV1.2. A. Titre of serum HBsAg in C57BL/6 or BALB/c mice after HBV plasmid injection. The detection limit is 0.05 IU/ml. B. Serum HBV DNA levels in C57BL/6 or BALB/c mice after HBV plasmid injection. The detection limit is 100 copies/ml. C. Bioluminescence image of C57BL/6 (H-2b) or BALB/c (H-2d) mice after hydrodynamic injection. D. Fluc expression in the liver and both serum HBsAg and HBV DNA levels showed strong linear correlation. E. Immunohistochemical staining for HBcAg in hepatocytes of HBsAg-positive C57BL/6 mice (magnification, 200x). In C57BL/6 mice, HBcAg-positive cells were maintained at stable levels on day 3 and day 135. F. Positive rate of serum HBsAg in C57BL/6 (n = 10) or BALB/c (n = 9) mice receiving injection with pGL3/Fluc-HBV1.2 at different time points after injection.

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Animals
C57BL/6 and BALB/c mice (males, 4–6 weeks old) were purchased from the National Beijing Center for Drug Safety Evaluation and Research and housed in cages in a controlled environment (22–25°C, 50% humidity, 12 h light/dark cycle).

Plasmid Construction
HBV 1.2 full-length DNA was subcloned from the plasmid pAAV/HBV1.2, which was kindly provided by Pei-Jer Chen (National Taiwan University College of Medicine, Taipei, Taiwan), encoding an HBV fragment longer than the full length of the HBV genome [11,16]. This fragment was subcloned into the Xhol/Mlu site downstream of the firefly luciferase gene in the pGL3-CP-Fluc vector [17] to produce pGL3/Fluc-HBV1.2 (Fig. S1). Plasmid pGL3-HBV1.2 was generated by deleting the Fluc gene from the pGL3/Fluc-HBV1.2 plasmid between the Ncol and Xbal sites. For the generation of pVAX1-HBc, the HBV core sequence was amplified by PCR using pAAV/HBV1.2 as the template and was cloned into the EcoRl/BomHI site of the pVAX1 vector (Invitrogen, Carlsbad, CA). All constructs were sequenced to confirm their identities and insert orientations.

Cell Cultures, DNA Transfections, and Luciferase Assays
Hepa1-6 and HuH-7 cells were purchased from ATCC (Manassas, VA) and cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (HyClone, South Logan, UT) at 37°C in 5% CO₂/air. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), starting with approximately 1x10⁵ cells/well in 24-well dishes with 0.6 µg of plasmid DNA mixture, following the manufacturer’s instructions.

Immunization of Mice with Recombinant Proteins or Plasmids
Mice used for studying the effects of preexisting HBcAg-specific immunity were injected intramuscularly in the tibialis anterior muscle with 100 µg of pVAX1-HBc or pVAX1 dissolved in 50 µl of PBS, thrice within a 2-week interval. Both injections were followed by in vivo electroporation to increase the expression levels of the injected plasmids [20,21]. For the protein immunizations, mice were injected subcutaneously (s.c.) in the back with 2 µg of rHBsAg (GlaxoSmithKline Biologicals S.A.), thrice within a 2-week interval.

Analysis of T-Cell Function Ex Vivo
Livers and spleens were harvested from immunized mice at 21 dpi. Cell suspensions of each liver were resuspended in 4 mL of 40% Percoll (GE Healthcare, Munich, Germany) and underlaid with 4 mL of 70% Percoll. After centrifugation for 20 minutes at 1400 xg at room temperature, liver-associated lymphocytes were collected from the interface between the 40% and 70% Percoll layers. Cell suspensions of each spleen were resuspended in 4 mL of 40% Percoll (GE Healthcare, Munich, Germany) and underlaid with 4 mL of 70% Percoll. After centrifugation for 20 minutes at 1400 xg at room temperature, spleen-associated lymphocytes were collected from the interface between the 40% and 70% layers. Lymphocytes (5x10⁵) were cultured in RPMI medium 1640 (Invitrogen) with 8% fetal calf serum in the presence of 10 µmol/L peptides, HBc93-100 and HBs190–197, respectively, for 5 hours. For intracellular interferon-γ staining, monensin (0.1%; e-Bioscience) was added to the culture 1 hour after the culture had been set up. The cells were harvested for CD8α surface staining, subjected to intracellular staining of interferon-γ using PE-Cy-7.
conjugated anti-IFN-γ antibodies (clone XMG1.2; BD Pharmin-gen), cell fixation/permeabilization using a kit (BD Biosciences, Heidelberg, Germany), and flow cytometric analysis.

Histology

For routine histological analysis, formalin-fixed paraffin-embedded liver samples were cut into sections 4 µm thick, deparaffinized in xylene, and dehydrated through a series of decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin.

Statistics

All data were reported as the mean±SD. For statistical comparisons, parametrical data were compared using a Student’s t-test. Differences were considered significant at P<0.05.

Results

Hydrodynamic Injection of pGL3/Fluc-HBV1.2 Leads to Reporter Gene and HBV Persistence in vivo

Chen and colleagues have shown that a single hydrodynamic injection of a replication-competent HBV DNA, pAAV/HBV1.2, into mice could result in HBV persistence for greater than 1 year in a significant proportion of recipients [11]. Thus, the HBV DNA sequences in pAAV/HBV1.2 were cloned into a different vector, pGL3-CPluc, to construct pGL3/Fluc-HBV1.2, encoding the HBV genes and a reporter gene (Fluc). To determine whether these constructs could express both the HBV gene and Fluc, pGL3-HBV1.2, pGL3-CPluc and pGL3/CPluc-HBV1.2 were transfected into HuH-7 cells. Forty-eight hours after the transfections, Fluc activity and HBsAg were measured. Although Fluc activity was lower in cells transfected with pGL3/Fluc-HBV1.2 than in those transfected with pGL3-CPluc, HBsAg levels were lower in cells transfected with pGL3/Fluc-HBV1.2 than in those transfected with pGL3-CPluc-HBV1.2 (Fig. S2). We verified that pGL3/Fluc-HBV1.2 could sustain HBV gene and reporter gene expression in cells. Next, we investigated whether these constructs could express HBV genes and Fluc in the mouse liver. Plasmid pGL3/Fluc-HBV1.2 was injected hydrodynamically into the tail veins of C57BL/6 (H-2b) or BALB/c (H-2d) mice. After the injection, the mice were regularly bled to monitor the serum levels of HBsAg and HBV DNA. In the sera of BALB/c and C57BL/6 mice, HBsAg accumulated to an average concentration of 1.16×10^4 and 1.19×10^4 IU per milliliter, respectively, on day 4 postinjection (dpi) (Fig. 1A). After this peak, levels of HBsAg in the sera of the BALB/c mice dropped by nearly two orders of magnitude, reaching a concentration of 4.86×10^2 IU per milliliter by day 49. In C57BL/6 mice, HBsAg levels declined much more gradually, at approximately one order of magnitude greater than that in C57BL/6 mice throughout the length of the experiment. For the Fluc reporter gene to be used for monitoring HBsAg and titers of HBV DNA in serum, it was essential to demonstrate a correlation between the expression of the reporter gene and serum HBsAg or HBV DNA. A strong positive linear correlation was present between Fluc expression in the liver and both serum HBsAg and HBV DNA levels on days 4 to 49 (r = 0.998 and 0.942, respectively, for BALB/c mice and r = 0.883 and 0.973, respectively, for C57BL/6 mice) (Fig. 1D). These data demonstrate the ability to use reporter gene expression as a measure of serum HBsAg and titers of serum HBV DNA.

We continued to monitor serum HBsAg in BALB/c and C57BL/6 mice receiving pGL3/Fluc-HBV1.2. In approximately 90% of the C57BL/6 mice, serum HBsAg persisted for 10 weeks, whereas only 40% of the BALB/c mice remained HBsAg positive at 10 weeks postinjection (Fig. 1E). These results demonstrated that the host genetic background influences HBV persistence in the mouse liver, a finding that had been previously reported by Chen Pei-Jer et al. [11]. Liver tissues were collected from serum HBsAg-positive and HBsAg-negative C57BL/6 mice at 3 and 135 dpi and were assayed for levels of HBsAg (Fig. 1F). In BALB/c mice, HBsAg-positive C57BL/6 mice expressed high levels of HBsAg, whereas only low levels of HBsAg were detected in the livers of HBsAg-positive mice at two different time points.

In vivo Bioluminescence Imaging Detects HBcAg-specific Immunity and Clearance HBV

The immune response to the HBV nucleocapsid Ag is believed to play a critical role in the control of HBV infection. HBc-specific CD8+ T cells contribute to viral clearance by killing infected cells (cytolytic HBV control) and/or inhibiting viral replication by producing cytokines such as IFN-γ and TNF-α (noncytolytic HBV control) [4,5,22]. Next, we aimed to demonstrate the usefulness of our assay system for monitoring HBc-specific immunity in the liver after vaccination. Naive C57BL/6 mice were immunized with pVAX1-HBc or pVAX1 thrice intramuscularly within a 2-week interval and subsequently given a hydrodynamic injection of pGL3/Fluc-HBV1.2. After being challenged with pVAX1-HBc, a vigorous antibody response to HBV core protein was observed in C57BL/6 mice (Fig. S5). Secretion of HBsAg and HBV DNA into
the blood were monitored over time (Fig. 2A and B). The levels of serum HBsAg and HBV DNA in HBcAg-immunized mice were similar to those in the mock-immunized mice, from the 1 day to the 14th day after hydrodynamic injection. At later time points, the serum HBsAg and HBV DNA levels in the HBcAg-immunized mice declined rapidly and were undetectable at 28 dpi but remained high in the mock-immunized mice. Next, we used in vivo imaging to characterize the real-time kinetics of clearance of hepatocytes expressing the HBV genome following hydrodynamic injection (Fig. 2C). Fig. 2C clearly shows that only HBcAg-immunized mice, but not mock-immunized mice, controlled luciferase expression in the liver. Longitudinal measurements of bioluminescence demonstrated that vaccinated animals had cleared most of the HBV-Fluc co-expressing hepatocytes between 14 dpi and 28 dpi, whereas mock-treated animals failed to clear the transfected hepatocytes. Therefore, we tried to address whether HBcAg-specific immunity is associated with HBV clearance in C57BL/6 mice. We examined the frequency of CTLs and HBcAg-specific IFNγ-producing CTLs in the spleenocytes and hepatocytes of HBcAg- or mock-immunized C57BL/6 mice at 21 dpi using a FACS assay (Fig. 2D). High frequencies of HBcAg-specific IFNγ-producing CTLs were detected both in the spleenocytes and hepatocytes of HBcAg-immunized mice, indicating greater HBcAg-specific immunity induced by pVAX1-HBc vaccination. Scattered infiltrating CTLs were also observed in HBcAg-immunized mice, but livers of mock-immunized mice showed no obvious inflammatory response (Fig. 2E). To further characterize liver tissue toxicity during the antigen clearance process, ALT values at various time points were also measured (Fig. 2F). There was a transient and rapid increase in the serum ALT values immediately after the hydrodynamic injection in both mice, but these values approached the normal range at 4 dpi. We observed increased ALT serum levels only in HBcAg-immunized mice, but not in mock-immunized mice, at 14 dpi and 21 dpi. These findings suggest that infiltrating CTLs may exert effector functions against infected hepatocytes.

Currently, the commercially recombinant Hepatitis B surface antigen vaccine is widely used to prevent HBV infection by inducing effective humoral immunity [23]. This vaccine elicits relatively weak cell-mediated immune responses, particularly regarding the antigen-specific CTL response. Therefore, prior immunization with this vaccine is unable to facilitate clearance of the virus from infected cells. Here, we used our assay system to monitor HBs-specific humoral and cell-mediated immunity in mice after vaccination. Naïve C57BL/6 mice were immunized with rHBsAg or NaCl thrice subcutaneously in the back within a 2-week interval and subsequently given hydrodynamic injection of pGL3/Fluc-HBV1.2. After being challenged with rHBsAg, C57BL/6 mice had already developed protective levels of anti-HB antibodies (Fig. S4). Most of the mock-immunized C57BL/6 mice maintained antigenaemia for both HBsAg and HBV DNA after pGL3/Fluc-HBV1.2 injection (Fig. 3A and B). However, all rHBsAg-immunized mice cleared HBsAg from their sera within 7 dpi (Fig. 3A). This finding demonstrates that strong antibody responses induced by rHBsAg protein succeeded in clearing the serum of HBsAg. Additionally, serum HBV DNA levels started to decline after the clearance of HBsAg in the rHBsAg-immunized mice but continued to remain at a high level (>1×10^6 copies/ml) at all time points (Fig. 3B). Next, bioluminescence imaging was performed to examine Fluc activity. The result showed that luciferase activity was lower in rHBsAg-immunized mice than that in mock-immunized mice at 21 dpi (Fig. 3C). The frequency of CTLs and HBsAg-specific IFNγ-producing cells in the spleens and livers of C57BL/6 mice at 21 dpi was examined. Greater numbers of CTLs and HBsAg-specific IFNγ-producing CTLs were detected only in the hepatocytes from HBsAg-immunized mice (Fig. 3D). Although we observed a small yet significant increase in HBV-specific CTLs in the livers of the rHBsAg-immunized mice at 21 dpi, immunization apparently failed to clear the HBV infection from the transfected hepatocytes. H&E staining showed small infiltrates of inflammatory cells (Fig. 3E). We also observed slightly increased ALT serum levels in HBsAg-immunized mice at 21 dpi (Fig. 3F).

**Discussion**

Previously, bioluminescence imaging was successfully used to unravel important pathophysiological mechanisms of infection and persistence of various microorganisms [24–26]. Here, we employed bioluminescence detection of luciferase expression in HBV-expressed hepatocytes to follow HBV protein and DNA levels in these cells. In our animal model, a single hydrodynamic injection of a replication-competent HBV DNA, pGL3/Fluc-HBV1.2, into mice resulted in HBV persistence for more than 15 weeks in a significant proportion of recipients. Huang and colleagues have shown that HBV persistence is determined by the mouse genetic background and plasmid backbone [11]. Their group found that the AAV vector favors long-term transgene expression in hepatocytes, whereas injection of pGEM4Z/HBV1.2 (in which the HBV DNA sequence from pAAV/HBV1.2 was cloned into pGEM4Z) into mice produced only transient antigenemia. We injected pGL3/Fluc-HBV1.2, in which the pGL3 vector was substituted for the AAV vector, into mice also produced persistent antigenemia. This result showed that the pGL3 vector can also facilitate HBV persistence in the mouse liver. We also determined that the mouse genetic background can affect HBV persistence. Ten micrograms of pGL3/Fluc-HBV1.2 DNA was injected hydrodynamically into the tail veins of male C57BL/6 (H-2b) or BALB/c (H-2d) mice. In C57BL/6 mice, the HBsAg levels declined much more slowly than those in BALB/c mice. Of the C57BL/6 mice, approximately 80% remained HBsAg-positive at 10 weeks postinjection. In contrast, only approximately 40% of the BALB/c mice remained HBsAg-positive at 10 weeks postinjection. The serum HBV DNA levels in C57BL/6 mice were similar to those in the BALB/c mice. Fluc expression in the livers of all of the mice was sustained for many days at a stable level. But Fluc activity in the C57BL/6 mice was one order of magnitude lower than that in BALB/c mice. It is thought that this difference is partly attributable to the black hair of the C57BL/6 mice absorbing the light.

It is now believed that HBV-specific CD8+ T cells play a critical role in the control of HBV replication and in the pathogenesis of
the disease via the destruction of infected liver cells. HBV core antigen (HBcAg) is recognized as the most efficient agent that primes human leukocyte antigen (HLA) class II-restricted CTL responses, which correlate with viral clearance in acute and self-limiting HBV patients; furthermore, the adoptive transfer of HBcAg-reactive T cells is associated with the resolution of chronic HBV infection [27–29]. In the present study, we employed bioluminescence detection of luciferase expression in HBV-infected hepatocytes to measure HBcAg-specific immune responses directed against these infected hepatocytes. Our data demonstrated that Fluc expression in the livers of the majority of HBcAg-immunized mice gradually became undetectable, accompanied by the rapid clearance of serum HBsAg and HBV DNA within 28 days after hydrodynamic injection of pGL3/Fluc-HBV1.2. Most of the mock-immunized mice maintained antigenemia for both HBsAg and HBV DNA, and additionally, these mice also sustained Fluc expression in the liver. The HBc-specific humoral and cellular immune responses were further analyzed. The results demonstrated that HBc DNA-based vaccination induced both strong antigen-specific T cell and high titer antibody responses systematically and in the liver. Furthermore, immunized mice showed strong cytotoxic responses that eliminated the HBV transfected hepatocytes. These responses were consistent with an increase in ALT and lymphocyte infiltration in the liver, visualized by H&E staining of liver tissue sections. Altogether, our data indicated that CTL-induced killing of HBV-infected hepatocytes occurred and that this process could be detected in vivo by bioluminescence imaging of luciferase expression, a similar procedure developed by Stabenow et al. for studying T cell-mediated immunity in the liver using adenoviral transfer system [12]. But adenoviral system needs transfection, virus packaging, virus immunity in the liver using adenoviral transfer system [12]. But adenoviral system needs transfection, virus packaging, virus amplification and purification. Thus our hydrodynamic injection model is simpler and more accessible than adenoviral system.

We also used our assay system to monitor rHBsAg-mediated immunity in the mice after vaccination. Our data suggested that these recombinant S protein elicited protective HBs-specific antibody responses and a weak T-cell immune responses in the vaccinated mice. The HBs-specific humoral-mediated immunity could clear serum HBsAg and reduce HBV DNA levels. But the weak HBsAg-specific CTL response at 21dpi apparently failed to clear the transfected hepatocytes.

In conclusion, the present study demonstrates that HBV clearance can be detected in vivo by bioluminescence imaging of reporter gene expression. Direct in vivo determination of HBV clearance provides a new tool to characterize the principles underlying antiviral immunity in the liver. This assay system will become a powerful tool for the study of HBV-specific immunity in the liver.

Supporting Information

Figure S1 Schematic diagram of plasmids pGL3-HBV1.2 and pGL3/Fluc-HBV1.2. (TIF)
Figure S2 HBV gene and Fluc expression in HuH7 cells. A. Titer of HBsAg in the supernatant of pGL3-HBV1.2, pGL3-CP-Fluc and pGL3/Fluc-HBV1.2 transfected HuH-7 cell. B. Fluc expression was detected by Luciferase Assay. (TIF)
Figure S3 Serum anti-HBc in immune C57BL/6 mice before and 21 days after hydrodynamic injection of HBV. (TIF)
Figure S4 Serum anti-HBs in immune C57BL/6 mice before and 21 days after hydrodynamic injection of HBV. (TIF)

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Author Contributions

Conceived and designed the experiments: JD LaZ. Performed the experiments: SQ LHY JD YJ D Y Zheng Y Ze Z. Analyzed the data: JD LsZ. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Analyzed the data: JD LsZ. Conceived and designed the experiments: JD LsZ. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Analysed the data: JD LsZ. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Performed the experiments: SqL HY JD YJ D Y Zheng Y Ze Z. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP. Contributed reagents/materials/analysis tools: QxF XhW JcP Nq L. Contributed data or analysis tools: JcP.

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