An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection

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An animal model for human hepatitis B virus (HBV) tolerance is needed to investigate the mechanisms. This model will also facilitate therapeutic strategies for the existing 350 million patients with chronic hepatitis B. We established a mouse model by hydrodynamic injection of an engineered, replication-competent HBV DNA into the tail veins of C57BL/6 mice. In 40% of the injected mice, HBV surface antigenemia persisted for >6 months. Viral replication intermediates, transcripts, and proteins were detected in the liver tissues of the injected mice for up to 1 year. The tolerance toward HBV surface antigen in this model was shown to be due to an insufficient cellular immunity against hepatitis B core antigen, as was documented in humans. This animal model will accelerate further genetic and mechanistic studies of human chronic hepatitis B infection.

surface antigen tolerance | hydrodynamic injection | DNA | HBV persistence

Hepatitis B virus (HBV) is a noncytopathic, enveloped virus with a circular, double-stranded DNA genome. It causes acute and chronic necroinflammatory liver diseases and, subsequently, hepatic cirrhosis and hepatocellular carcinoma. Although a highly effective preventive vaccine is now available, it does not help the estimated 350 million people who have already been infected chronically and are at risk of developing end-stage liver disease and hepatocellular carcinoma.

Although the chronicity of HBV infection is the result of impaired HBV-specific immune responses that cannot eliminate or cure the infected hepatocytes efficiently, many issues remained unsettled (1). It is thus crucial to the advancement of our understanding to have a suitable laboratory animal to study the immunopathogenesis of HBV infection and the mechanisms of HBV persistence. The mouse is the most suitable laboratory animal for immunological studies; however, it cannot be infected with HBV. Thus, most of the studies on immunopathogenesis of HBV have been approached by using HBV-transgenic mice which are inherently tolerant to transgene products (2–5). In these models, manipulation of the animals that are not in normal physiological conditions is required. Thus, many researchers attempted to establish an HBV model in nontransgenic mice. So far, only acute hepatitis B can be demonstrated by using these models (6, 7). These existing animal models have provided invaluable information on the mechanisms of immunopathogenesis of hepatitis B. However, they have limitations in addressing what happens at the onset of HBV infection that determines HBV persistence. Therefore, a mouse model of HBV persistence enabling scientists to study the mechanisms of HBV chronicity is desperately needed.

To meet this challenge, we created a nontransgenic model of persistent HBV infection in immunocompetent mice. We took advantage of the liver-targeting manner of hydrodynamic injection (8). A single hydrodynamic injection of a replication-competent HBV DNA, pAAV/HBV1.2, into mice could result in HBV persistence for >1 year in a significant proportion of recipients. The HBV carrier mice expressed hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), hepatitis B core antigen (HBcAg), and high levels of serum HBV DNA, but with normal levels of serum alanine aminotransferase and without significant inflammation in the liver. The characteristics of this mouse model for HBV persistence are analogous to those of human chronic HBV infections in the immune tolerant stage (9, 10). This animal model will help to further understand the mechanisms of HBV tolerance and to explore new treatments of chronic HBV infections.

Results

HBV Persistence Is Determined by the Mouse Genetic Background and Plasmid Backbone. Milich and colleagues (11–13) have shown that MHC haplotypes affected the host immune responses against HBV antigens at both cellular and humoral levels. Accordingly, we used the strong (H-2^d haplotype) and intermediate (H-2^b haplotype) responders as recipients of HBV-containing constructs to address the influence of host genetic backgrounds on HBV persistence in mice. Ten micrograms of pAAV/HBV1.2 DNA was injected hydrodynamically into the tail veins of male C57BL/6 (H-2^b) or BALB/c (H-2^d) mice. After injection, the mice were regularly bled to monitor the serum levels of HBsAg, HBeAg, HBV DNA, hepatitis B core antibody (anti-HBc), and hepatitis B surface antibody (anti-HBs). In BALB/c mice, the HBsAg level increased promptly within 1 week after pAAV/HBV1.2 injection but dropped quickly thereafter (Fig. 1*A*). All of the mice developed anti-HBs within 14 days (Table 1). In C57BL/6 mice, the HBsAg level declined much more slowly after injection of the same plasmid (Fig. 1*A*). Eighty percent of the injected C57BL/6 mice were still HBsAg-positive at 35 days postinjection (dpi) (Fig. 1*B*). None of the C57BL/6 mice receiving pAAV/HBV1.2 developed anti-HBs at 28 dpi, although all of them developed anti-HBc at 7 dpi, similar to the kinetics of anti-HBc production in BALB/c mice (Table 1). Thus, host genetic backgrounds indeed influence HBV clearance in mice.

The HBV DNA sequences in pAAV/HBV1.2 were cloned into another vector, pGEM4Z, which resulted in pGEM4Z/HBV1.2. Injection of pGEM4Z/HBV1.2 into C57BL/6 mice produced only transient antigenemia (Fig. 1*C*), and all of the mice developed anti-HBs within 28 days (Table 1). These results showed that the host genetic background is not the only factor affecting HBV persistent expression by hepatocytes, but instead the AAV vector also helps to maintain HBV persistence in the mouse liver.

We continued to monitor the serum HBsAg of the mice receiving pAAV/HBV1.2 or pGEM4Z/HBV1.2. Interestingly, in ≈40% of the mice receiving pAAV/HBV1.2, serum HBsAg

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; anti-HBc, hepatitis B core antibody; anti-HBs, hepatitis B surface antibody; dpi, days postinjection; ELISPOT, enzyme-linked immunospot; CFA, complete Freund's adjuvant.

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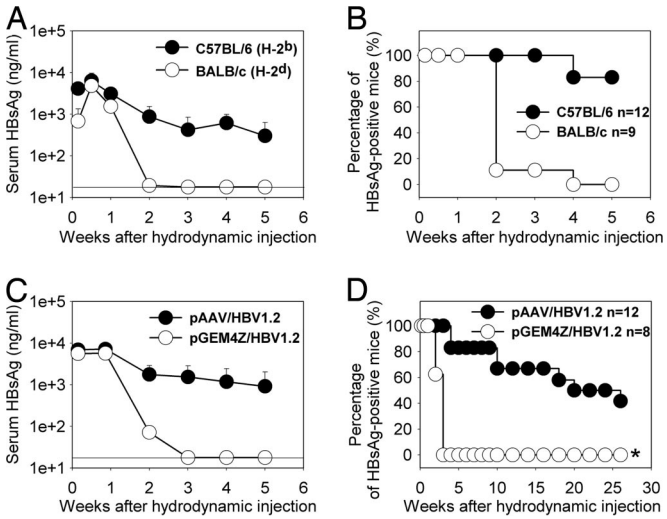

Fig. 1. HBV persistence in mice induced by hydrodynamic injection of HBV plasmids was determined by the genetic background of the recipients and the plasmid backbone. C57BL/6 or BALB/c mice were injected hydrodynamically with 10 μg of HBV plasmid. The HBsAg titer in the mouse serum was determined at the indicated time points by an EIA (Abbott Diagnostics), and the obtained S/N ratio was converted to nanograms per milliliter by applying a standard curve with known concentrations of HBsAg. (*A*) Titer of serum HBsAg in C57BL/6 or BALB/c mice after pAAV/HBV1.2 injection. The detection limitation is 17.76 ng/ml. (*B*) Positive rate of serum HBsAg in C57BL/6 ($n = 12$) or BALB/c ($n = 9$) mice receiving pAAV/HBV1.2 injection at different time points after injection. The data were analyzed by Kaplan–Meier analysis, and the difference was statistically significant ($P < 0.00001$). (*C*) Titer of serum HBsAg in C57BL/6 mice receiving pAAV/HBV1.2 or pGEM4Z/HBV1.2 hydrodynamic injection. (*D*) Positive rate of serum HBsAg in C57BL/6 mice receiving pAAV/HBV1.2 ($n = 12$) or pGEM4Z/HBV1.2 ($n = 8$) injection. The data were analyzed by Kaplan–Meier analysis, and the difference was significant ($P < 0.00001$). The cutoff value for determining HBsAg-positivity is 22 ng/ml. *, mice that experienced anti-HBs seroconversion for >3 weeks were not examined for the appearance of their serum HBsAg.

persisted for >6 months (Fig. 1*D*), meeting the usual definition of persistent HBV infection in humans. These mice failed to develop neutralizing antibodies, anti-HBs, even at 26 weeks after injection (data not shown).

HBV Replication and Transcription in the Livers of Injected Mice. HBV replication intermediates, including relaxed circular DNA, ssDNA, and episomal input DNA, were detected in the livers of both C57BL/6 and BALB/c mice during 3–14 dpi (Fig. 2*A* and *B*). The levels of HBV DNAs started to decrease from 14 dpi in the livers of injected BALB/c mice and became undetectable at 22 dpi, which correlated with the expression of HBsAg in serum or with 3.5-, 2.4-, and 2.1-kb HBV transcripts in the liver (Figs. 1*A* and 2*B*). In contrast to BALB/c mice, the HBV replication intermediates and RNA transcripts as well as serum HBsAg remained detectable in C57BL/6 mice at 22 dpi (Figs. 1*A* and 2*A*).

Liver tissues were collected from serum HBsAg-positive and HBsAg-negative C57BL/6 mice at 70, 93, 222, and 381 dpi and were assayed for the HBV replication intermediates, RNA transcripts, HBsAg, and HBcAg. The HBV replication intermediates and RNA transcripts could be detected in the liver of HBsAg-positive C57BL/6 mice on days 70, 93, and 222 after injection. In addition, the input DNA was also noted at 222 dpi (Fig. 2*C*). Moreover, cytoplasmic HBsAg was positive in 1% to ≈5% of the hepatocytes in HBsAg-positive mice at 381 dpi (Fig. 2*D*). Both cytoplasmic and nucleic HBcAg were also detected in the livers of HBsAg-positive mice (Fig. 2*E*).

Histological Analysis. We further examined whether the HBV clearance in the injected BALB/c mice was associated with stronger immune responses compared with those of the C57BL/6 mice. The livers from injected C57BL/6 and BALB/c mice at 5, 7, 10, and 14 dpi were stained with hematoxylin and eosin. The livers from injected BALB/c mice at 5 dpi showed multiple foci of mononuclear cell infiltration and returned to normal architecture at 14 dpi. The livers from injected C57BL/6 mice showed normal architecture and no obvious inflammatory responses at all time points including 70 or 222 dpi (Fig. 7, which is published as supporting information on the PNAS web site). Long-term expression of HBV in these carrier mice did not cause liver damage, as also evidenced by normal serum alanine aminotransferase levels (ranging from 23 to 31 units/liter).

Detection of HBV DNA in Serum. The serum samples from hydrodynamically injected C57BL/6 mice were also assayed for the presence of encapsidated HBV DNA by real-time PCR. Viral titer at 1 dpi was on average 5.38×10^3 copies per milliliter of sera. The viral titers were peaked during 3–7 dpi and were on average 8.63×10^6 and 6.985×10^6 copies per milliliter of sera, respectively. Then the viral titers dropped and were undetectable in some serum samples at later time points but remained high in the serum samples from HBsAg-positive carrier mice. The titers of viremia and surface antigenemia showed a high correlation (data not shown). The average viral load of serum samples from six high-titer HBsAg-positive mice at >180 dpi was 4.32×10^6 copies per milliliter of sera.

The HBsAg-positive carrier mice constantly produced HBV antigens and virions from their plasmid-bearing hepatocytes. Although these mice produced anti-HBc, the first serological marker for acute HBV infection in humans, they failed to generate anti-HBs, the protective neutralizing antibody against HBV. Overall, the status of the long-term HBsAg-positive mice in our study is similar to that of healthy human HBV carriers in the immune tolerant phase (9, 10).

Tolerance Toward HBsAg in the HBV Carrier Mice. We then tried to address whether the sustained HBsAg antigenemia in carrier C57BL/6 mice is associated with tolerance toward HBsAg. After being challenged with HBsAg, carrier C57BL/6 mice continued to express HBsAg ($n = 5$) without anti-HBs formation at day 14 even after boosting. This is in contrast to all of the control naïve C57BL/6

Table 1. Anti-HBc or anti-HBs in C57BL/6 or BALB/c mice after hydrodynamic injection of HBV plasmids

		Anti-HBc		Anti-HBs		
DNA	Strain	Day 1	Day 7	Day 3	Day 14	Day 28
pAAV/HBV1.2	C57BL/6	0/9	9/9	0/9	0/9	0/9
pAAV/HBV1.2	BALB/c	0/9	9/9	0/9	9/9	9/9
pGEM4Z/HBV1.2	C57BL/6	0/4	4/4	0/4	0/4	4/4

C57BL/6 or BALB/c mice were injected hydrodynamically with 10 μg of HBV plasmids. Shown is number of animals for each cohort showing anti-HBc or anti-HBs positivity.

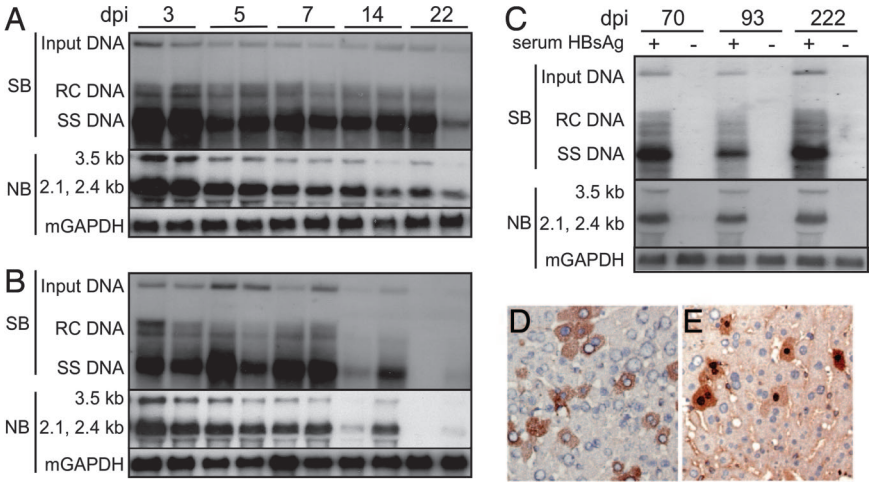

Fig. 2. Long-term expression of HBV replication intermediates, transcripts, and proteins was observed in the livers of HBV carrier mice. Southern blotting (SB) and Northern blotting (NB) showed the input HBV DNA, relaxed-circle (RC) DNA, ssDNA, and 3.5-kb pregenomic and 2.4/2.1-kb surface mRNAs in the livers of C57BL/6 mice (*A*) and BALB/c mice (*B*) at day 3, 5, 7, 14, and 22 after hydrodynamic injection of pAAV/HBV1.2. (*C*) Southern and Northern blotting in the livers of HBsAg-positive or -negative C57BL/6 mice at day 70, 93, or 222 after hydrodynamic injection of pAAV/HBV1.2. (*D* and *E*) Immunohistochemical staining for HBsAg (cytoplasm) (*D*) and HBcAg (cytoplasm/nucleus) (*E*) in hepatocytes of HBsAg-positive mice at 381 dpi. (Original magnification: ×200.)

mice ($n = 4$) that had already developed protective levels of anti-HBs (>10 milliunits/ml) at this time after immunization (Fig. 3*A*). In both immune C57BL/6 and BALB/c mice, titers of anti-HBs in their sera increased dramatically because of the booster effect within 14 days after the rHBsAg challenge (Fig. 3*B*). In carrier C57BL/6 mice, s.c. injection of rHBsAg in complete Freund's adjuvant (CFA) failed to induce production of neutralizing anti-HBs antibodies, indicating a tolerance state toward HBsAg.

Impaired HBcAg-Specific Immunity in C57BL/6 Mice During Primary Activation. Both HBcAg and HBsAg have been suggested to play critical roles in viral clearance (14–16). Therefore, we tried to address whether HBcAg/HBsAg-specific immunity during the acute phase is associated with the HBV persistence/clearance in C57BL/6 and BALB/c mice after hydrodynamic injection of pAAV/HBV1.2. We examined the frequency of HBcAg/HBsAg-specific IFNγ-producing cells in the splenocytes of C57BL/6 and BALB/c mice at 3 and 10 dpi by using the IFNγ enzyme-linked immunospot (ELISPOT) assay.

At 3 dpi we could not detect any significant levels of HBcAg-specific IFNγ-producing cells in the splenocytes in both C57BL/6 and BALB/c mice receiving pAAV/HBV1.2 (Fig. 4*A Left*). These cells started to appear at 10 dpi. The average frequency was 284 and 334 in 10^6 splenocytes in C57BL/6 and BALB/c mice, respectively (Fig. 4*A Right*). Although there was no significant difference between the frequency of HBcAg-specific IFNγ-producing cells of the two strains, the mean spot size of HBcAg-specific IFNγ-producing cells of BALB/c mice was 1.6-fold larger than that of C57BL/6 mice (22.6 vs. 36.2 × 10^{-3}/mm^2; $P = 0.0262$) (Fig. 4*B*).

After stimulation with rHBcAg in culture, a low level of IFNγ mRNA (approximately nine copies per 10^4 GAPDH mRNA) was expressed by splenocytes of C57BL/6 and BALB/c mice receiving PBS. For mice receiving pAAV/HBV1.2, splenocytes from BALB/c mice expressed significantly higher levels of IFNγ mRNA than those from C57BL/6 mice. The average copy number of IFNγ mRNA is 24 and 77 copies per 10^4 GAPDH mRNA for C57BL/6 and BALB/c mice, respectively ($P = 0.0084$) (Fig. 4*C*). These data clearly demonstrated that, upon rHBcAg stimulation, splenocytes from BALB/c mice expressed higher amounts of IFNγ than those from C57BL/6 mice at both the RNA and the protein levels.

Nevertheless, we could not detect HBsAg-specific IFNγ-producing cells in splenocytes from both BALB/c and C57BL/6 mice receiving pAAV/HBV1.2 at 10 dpi or later time points (data not shown). These data are similar to the observations of early HBV infection in humans, in which a strong HBcAg-specific cellular immune response is detected without the generation of a comparative HBsAg-specific cellular immune response (14). The quality and quantity of the HBcAg-specific immune response may thus play a key role in clearing HBV infection in the acute phase, when no HBsAg-specific immunity is detected.

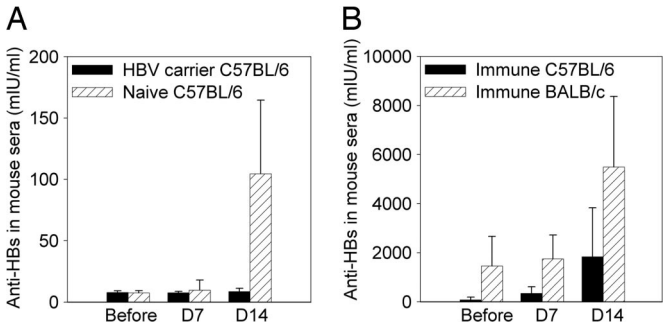

Fig. 3. Tolerance toward HBsAg was noted in HBV carrier mice. (*A*) Serum anti-HBs in HBV carrier ($n = 5$) or naïve C57BL/6 ($n = 4$) mice before or after immunization with 5 μg of rHBsAg formulated in CFA (1.5 or 2 months after hydrodynamic injection of pAAV/HBV1.2 or PBS). (*B*) Serum anti-HBs in immune C57BL/6 ($n = 4$) or BALB/c ($n = 6$) mice before or after immunization with 5 μg of rHBsAg formulated in CFA once (1.5 or 2 months after hydrodynamic injection of pAAV/HBV1.2).

Preexisting HBcAg-Specific Immune Response Protects the C57BL/6 Mice from HBV Persistence After Hydrodynamic Injection. An impaired HBcAg-specific immunity in C57BL/6 mice was postulated to be a major cause for HBV persistence after the hydrodynamic injection of pAAV/HBV1.2. Thus, we immunized the naïve C57BL/6 mice with pCDNA3.1(+)/HBc twice intramuscularly within a 2-week interval and then performed hydrodynamic injection of pAAV/HBV1.2. The resulting mice were bled or killed at the indicated time points. Most of the mock-immunized C57BL/6 mice maintained antigenemia for both

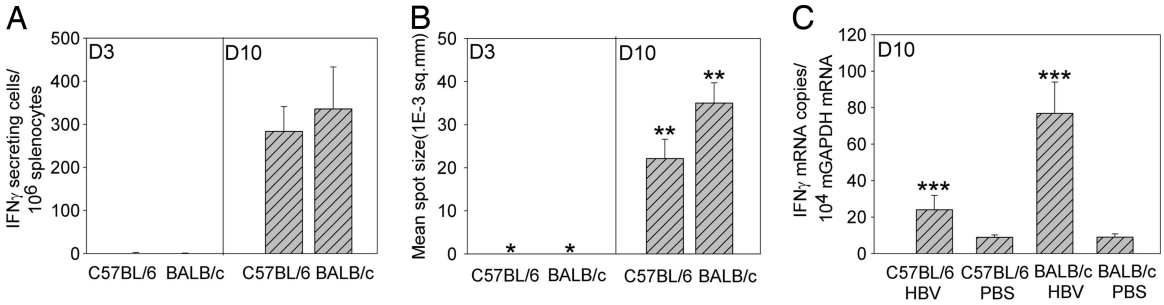

Fig. 4. BALB/c mice produced more IFNγ than C57BL/6 mice after hydrodynamic injection of HBV plasmids. Shown are the numbers (*A*) and mean spot sizes (*B*) of HBcAg-specific IFNγ-producing cells in 1×10^6 splenocytes from C57BL/6 or BALB/c mice receiving pAAV/HBV1.2 at day 3 or day 10 after hydrodynamic injection assayed by IFNγ ELISPOT in the presence of 0.3 μg/ml rHBcAg. (*C*) The copy number of IFNγ mRNA produced by splenocytes from C57BL/6 ($n = 3$) or BALB/c ($n = 3$) mice receiving pAAV/HBV1.2 or PBS at day 10 after hydrodynamic injection in the presence of 0.3 μg/ml rHBcAg in cultures. Copy number of mGAPDH mRNA was used for normalization. The experiments were repeated, and the results were consistent. The data were analyzed by *t* test, and the differences were statistically significant (*, not detectable; **, $P = 0.0262$; ***, $P = 0.0084$).

HBeAg and HBsAg after hydrodynamic injection of pAAV/HBV1.2 (Fig. 5 *A* and *B*). However, all HBcAg-immunized mice cleared HBeAg in their sera within 7 dpi (Fig. 5*A*). In addition, they also cleared HBsAg in their sera by 14 dpi (Fig. 5*B*). The anti-HBs started to rise after the clearance of HBsAg in the HBcAg-immunized mice but remained undetectable in the mock-immunized mice (Fig. 5*C*). The rapid clearance of HBeAg and HBsAg in all of the HBcAg-immunized mice suggested that HBcAg-specific immunity could also help HBsAg clearance and anti-HBs production.

We also assayed the frequencies of IFNγ- and IL-2-producing cells in the splenocytes from HBcAg- or mock-immunized C57BL/6 at 10 dpi. High frequencies of HBcAg- or $HBc_{129-140}$-specific IFNγ- or IL-2-producing cells were detected in the splenocytes from HBcAg-immunized mice, indicating a successful DNA immunization in the generation of HBcAg-specific immunity (Fig. 5*D*).

HBV replication intermediates, transcripts, and proteins in the livers of HBcAg-immunized and mock-immunized mice were also assayed. The input DNA, HBV replication intermediates, and HBV transcripts were detected in the liver samples from mock-immunized C57BL/6 mice at 1, 3, 5, 7, and 14 dpi. In contrast, HBV replication in the livers from HBcAg-immunized mice was barely detectable after hydrodynamic injection of pAAV/HBV1.2 (Fig. 5*E Top*, lanes 2 and 5). We noted that the input DNA that served as a template for transcription and the HBV transcripts could be detected at 1 and 3 dpi, after which they decreased to undetectable levels, while HBV replication intermediates also could not be detected (Fig. 5*E*). These data suggested that HBcAg-specific immunity inhibited HBV replication at the posttranscriptional stage through noncytolytic pathways, because the input DNA was still detectable at 1–3 dpi, indicating that plasmid-bearing hepatocytes still existed. Transcription was subsequently blocked because of the shortage of its template, the input DNA, which may be cleared by HBcAg-specific immunity through noncytolytic or cytolytic pathways. Because transcription was inhibited, neither HBeAg nor HBsAg could be produced in these mice.

Adoptive Transfer of HBcAg-Specific Immunity Helps HBV Clearance in Carrier Mice. We also transferred splenocytes from HBcAg-immunized C57BL/6 mice to the carrier C57BL/6 mice. After adoptive transfer, the carrier mice could eliminate serum HBsAg within 10 days whereas those receiving mock-immunized splenocytes could not (Fig. 6*A*). The anti-HBs started to appear in the sera of HBV carrier mice receiving HBcAg-specific immunity at day 14 after adoptive transfer (Fig. 6*B*).

Our data suggested that HBcAg-specific immunity is crucial for the clearance of HBV persistence during both the acute phase and the chronic carriage phase in our model. Moreover, our aforementioned data have revealed the induction of immune tolerance toward HBsAg in the HBV carrier C57BL/6 mice (Fig. 3*A*). Once the HBsAg in their sera decreased, the tolerance stage seemed to be converted and the anti-HBs could be generated rapidly in these mice (Figs. 5*C* and 6*B*). Similar findings have been reported in some chronic hepatitis B patients receiving bone marrow transplants from HBV-immune donors. In addition, the clearance correlated with the detection of HBcAg-specific $CD4^+$ T cell responses (17).

Discussion

In our animal model there are two crucial factors for successfully establishing HBV persistence in immunocompetent mice. First, we found that the AAV vector favors long-term transgene expression in the hepatocytes. It has been documented that the covalent linkage of bacterial DNA silenced transgene expression in episomal vectors in mouse livers (18). Excision of the expression cassette from plasmids helps to increase the maintenance and persistence of the transgene *in vivo* (19). A hepatic control region from apolipoprotein E locus has been proved to induce long-term and high expression levels of a transgene in the livers (20). Accordingly, long-term maintenance of the input DNA delivered by hydrodynamic injection is regulated by the nontranslational regulatory sequences. The information provides a possible explanation for the influence of the plasmid backbone on HBV expression in the hepatocytes in our model. It is likely that the unknown sequences in the backbone of pAAV/HBV1.2 regulate the long-term maintenance or expression of the transgene in the livers whereas there are no such sequences in the pGEM4Z. On the contrary, it is also likely that the pGEM4Z has negatively regulatory sequences that silence the expression of the transgene. Although the detailed mechanisms for the regulation are not clear, the information still facilitates future experimental design with animal models of liver-specific pathogens.

Second, the genetic background of recipients, which correlates with the strength of immune responses against HBV antigens during primary activation, also determines the outcome after hydrodynamic injection. It is noteworthy that the HBcAg-specific immune responses play a key role in clearing HBV antigens or antigen-bearing hepatocytes during the acute and tolerant phases. In HBV carrier C57BL/6 mice, impaired HBcAg-specific immune responses failed to clear HBV antigens as well as the input DNA, which mimics HBV covalently closed circular DNA in natural HBV infections. Yang *et al.* (6) have observed persistent expression of HBV antigens in the hepatocytes of immunocompromised mice after hydrodynamic injection of HBV plasmid DNA. These data

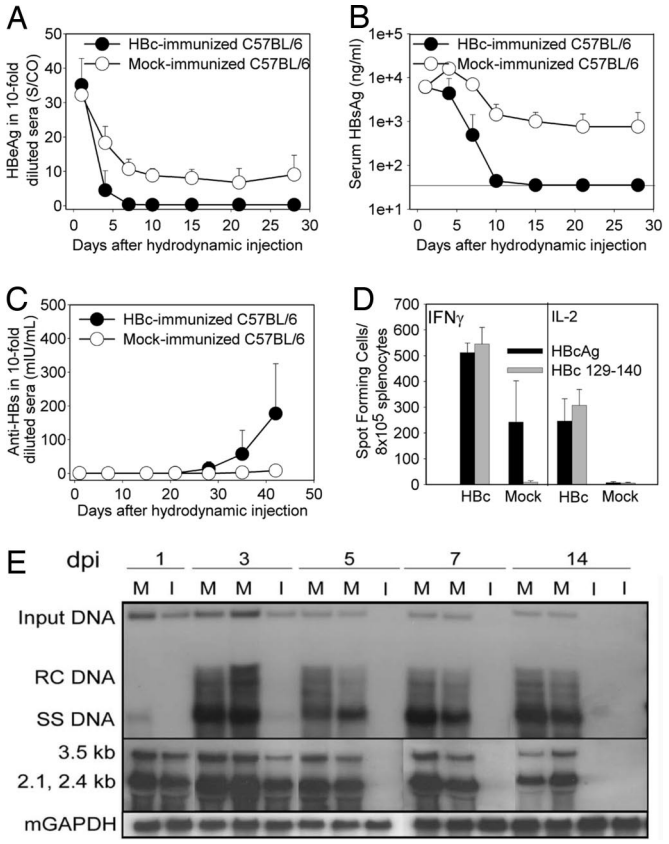

Fig. 5. Preexisting HBcAg-specific immunity could prevent HBV persistence in these mice. C57BL/6 mice were immunized with 100 μg of pCDNA3.1(+)/HBc or PBS twice within a 2-week interval. Then, the HBcAg- or mock-immunized mice were injected hydrodynamically with pAAV/HBV1.2 at day 14 after boost. Shown are titers of serum HBeAg (*A*), HBsAg (*B*), or anti-HBs (*C*) in the HBcAg- or mock-immunized C57BL/6 mice after hydrodynamic injection. (*D*) Number of HBcAg-specific (black bar) or $HBcAg_{129-140}$-specific (I-A^b-restricted peptide) (gray bar) IFNγ or IL-2 spot-forming cells in 8×10^5 splenocytes from HBcAg- or mock-immunized C57BL/6 mice receiving pAAV/HBV1.2 at day 10 after hydrodynamic injection assayed by IFNγ or IL-2 ELISPOT in the presence of 0.3 μg/ml rHBcAg. Experiments depicted in *A–D* were performed on five mice per group and were repeated. (*E*) Southern and Northern blotting by using the livers samples from HBcAg-immunized (I) or mock-immunized (M) C57BL/6 mice at days 1, 3, 5, 7, and 14 after hydrodynamic injection of pAAV/HBV1.2.

also suggested that impaired immune responses in injected mice would allow input HBV DNA persistence.

The HBV nucleocapsid or HBcAg is highly immunogenic during natural infection and after immunization (14, 21). Studies with mice and humans have shown that HBcAg could bind and activate B cells in a T cell-independent manner (22, 23), which may explain the rapid production of anti-HBc after HBV infection. It has also been reported that HBcAg-specific T cells support anti-HBs as well as anti-HBc antibody production (24). In our model, we also found that defective HBcAg-specific immunity could impede anti-HBs production and allow persistent surface antigenemia.

As shown in Fig. 3*A*, the inability to develop anti-HBs *in vivo* after rHBsAg immunization suggested that the tolerance of HBsAg was generated in the HBV carrier mice. Previously, it was reported that the antigen-presenting cells, especially the hepatic dendritic cells, of HBV-transgenic mice were impaired to induce both innate and adaptive immune responses (25, 26). A tolerogenic effect of HBsAg on the $CD4^+$ T cells also has been noted (27). The underlying mechanisms for the tolerance formation

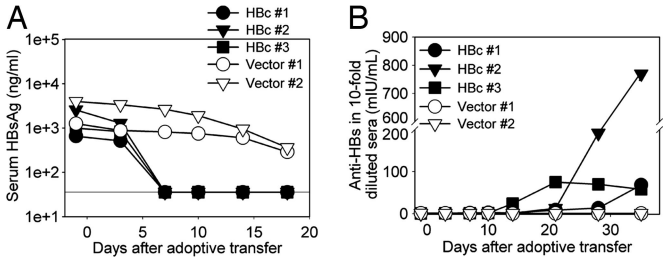

Fig. 6. Adoptive transfer of HBcAg-specific immunity to HBV carrier mice could cure HBV persistence in these mice. Shown are titers of serum HBsAg (*A*) or anti-HBs (*B*) in the HBV carrier C57BL/6 mice adoptively transferred with splenocytes from HBcAg-immunized or vector-immunized C57BL/6 mice.

toward HBsAg at both T cell and B cell levels in our model remain to be determined. Using cell sorting, adoptive transfer, genetic-manipulated mice, and *in vivo* depletion in our animal model, we should be able to address which cell population is responsible for HBV persistence/clearance *in vivo*.

In the past, HBeAg has been reported to be a toleragen during HBV infection (28, 29). Approximately 90% of infants born to HBeAg-positive carrier mothers become chronic carriers (30). However, the reasons why the tolerance toward HBeAg/HBcAg correlates with HBV persistence remain unclear. We have provided an explanation for the above phenomenon. Infants born to HBeAg-positive mothers may acquire neonatal tolerance toward HBeAg/HBcAg so that they are less competent to clear or cure the HBcAg-bearing hepatocytes at the onset of HBV infection and subsequently develop persistent HBV infection.

Our nontransgenic mouse model for HBV persistence provides opportunities to investigate the mechanisms of HBV persistence. By using site-directed mutagenesis to alter specific HBV genes, HBV mutants could be made and easily tested *in vivo* for their influence on HBV persistence. The results will help in identifying viral genes and putative epitopes responsible for tolerance induction and clearance of HBV in mice. Finally, the technique could also be applied to generate mouse models for chronic hepatitis C and hepatitis D viruses (31).

Materials and Methods

Constructs. The HBV 1.2 full-length DNA was subcloned from the plasmid pHBV-48, containing a greater-than-genome-length HBV fragment (32), to a rAAV vector, pAAV-GFP (33), which was provided kindly by Y.-P. Tsao (Mackay Memorial Hospital, Taipei, Taiwan). A BamHI/EcoRI-digested fragment (1.8 kb) and a EcoRI/BglII-digested fragment (2.0 kb) of pHBV-48 were cloned into the BglII site of the AAV-GFP vector. The resulting pAAV/HBV1.2 contains the HBV fragment spanning nucleotides 1400~3182/1~1987 flanked by inverted terminal repeats of AAV. The HBV expression cassette (located inside of the two inverted terminal repeats) was excised by a SmaI digestion of the pAAV/HBV1.2 and was subcloned into the SmaI site of pGEM4Z (Promega, Madison, WI) to result in pGEM4Z/HBV1.2. The map of plasmid pAAV/HBV1.2 is described in Fig. 8, which is published as supporting information on the PNAS web site.

Animal Study. C57BL/6 or BALB/c mice (male, 6–8 weeks old, from the breeding colonies of National Taiwan University or The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ketamine and xylazine. Ten micrograms of HBV plasmid DNA was injected into the tail veins of mice in a volume of PBS equivalent to 8% of the mouse body weight. The total volume was delivered within 5 s. The serum specimens were assayed for HBsAg, HBeAg, anti-HBs, or anti-HBc at the indicated times after injection. The livers of mice were preserved in OCT for immunohistochemical analysis.

All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the National Taiwan University College of Medicine.

Detection of HBV Antigen, Antibody, and Serum Alanine Aminotransferase. Serum levels of HBsAg, HBeAg, anti-HBc, and anti-HBs of the mice were determined by using the AXSYM system kit (Abbott Diagnostika, Wiesbaden Delkenheim, Germany). For HBsAg detection, the obtained S/N ratio was converted to nanograms per milliliter by applying a standard curve with known concentrations of *rHBsAg* (subtype *adw*; HyTest, Turku, Finland). Serum alanine aminotransferase was measured on automated clinical chemistry analyzer TBA-200FR (Toshiba, Tokyo, Japan) by using ALT/GPT reagent (Denka Seiken, Tokyo, Japan).

Immunohistochemistry. Liver tissues were collected from mice killed at the indicated time points. Intrahepatic HBcAg or HBsAg was visualized by immunohistochemical staining of tissues embedded in OCT by rabbit anti-HBc or anti-HBs antibodies (DAKO, Carpinteria, CA; Biomeda, Foster City, CA) and Envision_System, HRP (diaminobenzidine) (DAKO). The liver sections were also stained with hematoxylin.

Detection of Serum HBV DNA. Serum samples were collected at the indicated time points after hydrodynamic injection of pAAV/HBV1.2. The total DNA of the serum samples was extracted and detected for HBV DNA by real-time PCR as described previously (34).

Southern and Northern Hybridization. HBV viral RNA and replicative DNA intermediates were detected by Northern and Southern blot analysis of total genomic liver RNA and DNA, respectively, as described previously (35).

Immunization of Mice with Recombinant Proteins or Plasmids. Mice hydrodynamically injected with pAAV/HBV1.2 or PBS at 30 dpi or later were immunized s.c. with 5 μg of rHBsAg (subtype *adw*; HyTest) formulated in CFA.

Mice used for studying the effects of preexisting HBcAg-specific immunity on HBV persistence or used as donors for adoptive transfer were injected intramuscularly in the tibialis anterior muscle with 100 μg of pCDNA3.1(+)/HBc or pCDNA3.1(+) dissolved in 50 μl of PBS, or they were injected with PBS twice within a 2-week interval. Both injections were followed by *in vivo* electroporation to increase the expression level of the injected plasmids (36). At day 3 after the first immunization, all of the mice were intramuscularly injected with 100 μg of pB-CpG20 dissolved in 50 μl of PBS as adjuvant in the same site of tibialis anterior muscle where the previous injection was given. The pB-CpG20 plasmid was kindly provided by K. Okuda (Yokohama City University, Yokohama, Japan) (37). All mice used for immunization were anesthetized with ketamine and xylazine.

Preparation of Splenocytes and the ELISPOT Assay. At the indicated time points after hydrodynamic injection or immunization, mice were killed. The separated splenocytes were cultured and assayed for the frequencies of antigen-specific IFNγ- or IL-2-secreting cells by using ELISPOT as described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

RNA Extraction and Real-Time PCR. Total RNA from splenocytes cultured with rHBcAg was extracted and reverse-transcribed. The RT product was used for analyzing the copy number of murine IFNγ or mGAPDH cDNA by using real-time PCR as described in *Supporting Materials and Methods*.

Adoptive Transfer of Immune Splenocytes to HBV Carrier Mice. Splenocytes from pCDNA3.1(+)/HBc- or pCDNA3.1(+)-immunized C57BL/6 mice were prepared as single-cell suspensions and treated with ACK buffer (0.83% NH_4Cl/0.1% $KHCO_3$) to lyse the RBCs. The resulting cells were washed once with RPMI medium 1640 supplemented with 2.5% FCS, then washed twice with RPMI medium 1640 without a supplement and resuspended in RPMI medium 1640. A total of 1×10^8 splenocytes were injected intravenously into each of the HBV carrier C57BL/6 mice.

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