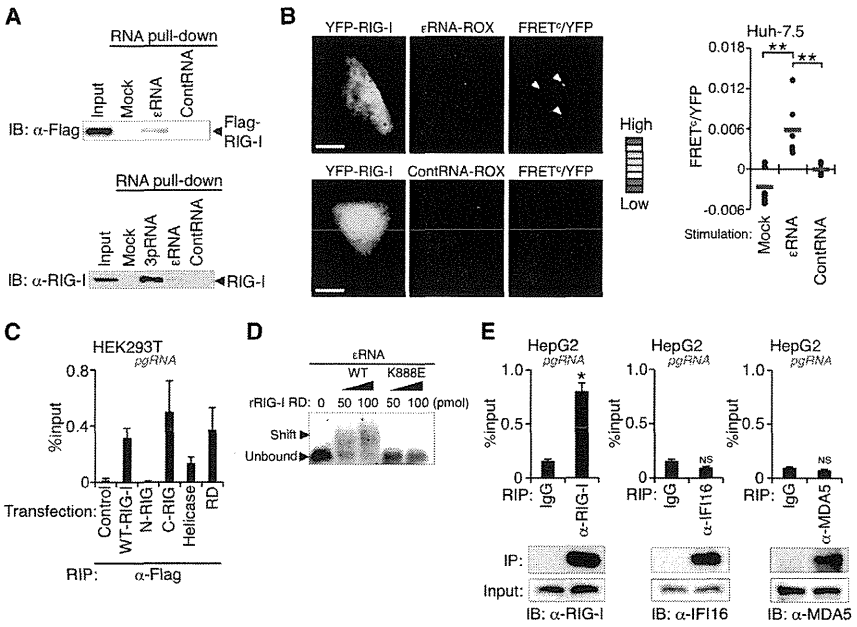

Figure 4. RIG-I Interacts with the ε Region of pgRNA

(A) RNA pull-down assay showing the binding activity of the indicated RNAs to Flag-tagged RIG-I (Flag-RIG-I) in HEK293T cells (top) or endogenous RIG-I in HepG2 cells (bottom).

(B) FRET analysis for the interaction of YFP-tagged RIG-I (YFP-RIG-I) with rhodamine (ROX)-conjugated εRNA (εRNA-ROX) or ContRNA (ContRNA-ROX). Representative fluorescence images of YFP, ROX, and FRET^C/YFP (the ratio of corrected FRET (FRET^C) to YFP). Arrowheads indicate area showing high FRET efficiency. Scale bar represents 20 μm. Right, dot plot of FRET^C/YFP ratio (small horizontal bars, mean).

(C) RIP assay with HEK293T cell lysates expressing several Flag-tagged deletion mutants of RIG-I and pgRNA expression vector by using anti-Flag antibody. Immunoprecipitated pgRNA was quantitated by qRT-PCR and normalized to the amount of immunoprecipated proteins (Figure S4C) and is represented as fraction of input RNA prior to immunoprecipitation (percentage input).

(D) Gel-shift analysis of complex formation between εRNA and recombinant RIG-I RD (WT) or RD (K888E). Arrowheads denote position of unbound RNA and RNA–RIG-I complexes.

(E) RIP assay with HepG2 cell lysates prepared after 48 hr of transfection of the HBV-C genome by using anti-RIG-I, anti-IFI16, anti-MDA5, or control immunoglobulin G. The immunoprecipitated pgRNA was measured by qRT-PCR (top) as described in (C). Whole-cell expression and immunoprecipitated amounts of RIG-I, IFI16, and MDA5 (bottom). Data are presented as mean and SD (n = 3) and are representative of at least three independent experiments. *$p < 0.05$ and **$p < 0.01$ versus control in (B and E). NS, not significant. See also Figure S4.

the 5′-ε region of viral pgRNA functions as an HBV-associated molecular pattern to be specifically recognized by RIG-I and can trigger IFN-λ response.

RIG-I Exerts an Antiviral Activity by Counteracting the Interaction of HBV Polymerase with pgRNA

We next assessed the contribution of RIG-I pathway in antiviral defense against HBV infection. RIG-I knockdown in PHH resulted in a higher HBV genome copy number at 10 days after infection with HBV-C, as compared with PHH treated with control siRNA (Figure 5A). A similar observation was made for RIG-I siRNA-treated HuS-E/2 cells (Figure S5A). These results indicate an implicated role of RIG-I as an innate sensor to activate antiviral response against HBV infection. On the other hand, it has been previously reported that the 5′-ε region of HBV pgRNA is important to serve as a binding site of viral P protein for initiating reverse transcription (Bartenschlager and Schaller, 1992). As consistent with this, we showed that the P protein interacts with εRNA in Huh-7.5 and HEK293T cells, by fluorescence resonance energy transfer (FRET) analysis (Figure 5B) and RNA pull-down assay (Figure S5B), respectively. These findings facilitated us to examine whether RIG-I could block the access of P protein toward the ε region. As we expected, recombinant RIG-I protein suppressed the interaction of P protein with pgRNA in a dose-dependent manner (Figure 5C). Such an inhibitory effect was also observed in Huh-7.5 cells by expression of WT RIG-I, as well as its T55I (Sumpter et al., 2005; Saito et al., 2007) or K270A (Takahasi et al., 2008) mutant (Figures 5D and S5C), both of which are not able to induce ligand-dependent activation of the downstream signaling but retain their RNA-binding activities. On the other hand, the K888E (Cui et al., 2008) mutant could not inhibit the binding of P protein with pgRNA (Figures 5D and S5C). In addition, treatment with recombinant IFN-λ1 in Huh-7.5 cells upregulated the amount of the mutant RIG-I protein (T55I) (Figure S5D), resulting in a partial inhibition of the P protein interaction with pgRNA, and this inhibitory effect was abrogated by RIG-I knockdown (Figure 5E). In fact, FRET analysis showed that the P protein-εRNA interaction was significantly suppressed by expression of the RIG-I RD (WT) alone, but not the mutant RD (K888E) (Figure S5E). Furthermore, HBV replication was also suppressed by expression of the RIG-I RD (WT) in Huh-7.5 cells, wherein any IFN induction is not observed, while the mutant RD (K888E) did not affect viral replication (Figure 5F). These findings revealed another aspect of RIG-I as a direct antiviral factor through its interference with the binding of HBV P protein to pgRNA in an IFN pathway-independent manner.

The εRNA Restricts HBV Replication in Human Hepatocyte-Chimeric Mice

Lastly, based on the above results, we tried to harness the therapeutic potential of the P protein-interacting εRNA for the control of HBV infection. A vector was designed to include a 63 bp DNA oligo, which is transcribed into an εRNA. We confirmed in the in vitro experiments using Huh-7.5 cells that εRNA induced by this vector-driven expression is capable to function as a decoy RNA to interfere with the binding of HBV P protein to pgRNA and to inhibit viral replication in an IFN-independent manner (Figures 6A and 6B, left). On the other hand, εRNA did not show any difference in HCV replication as compared with control (Figure 6B, right). In order to evaluate the therapeutic efficacy of εRNA in vivo, we exploited HBV infection model of human

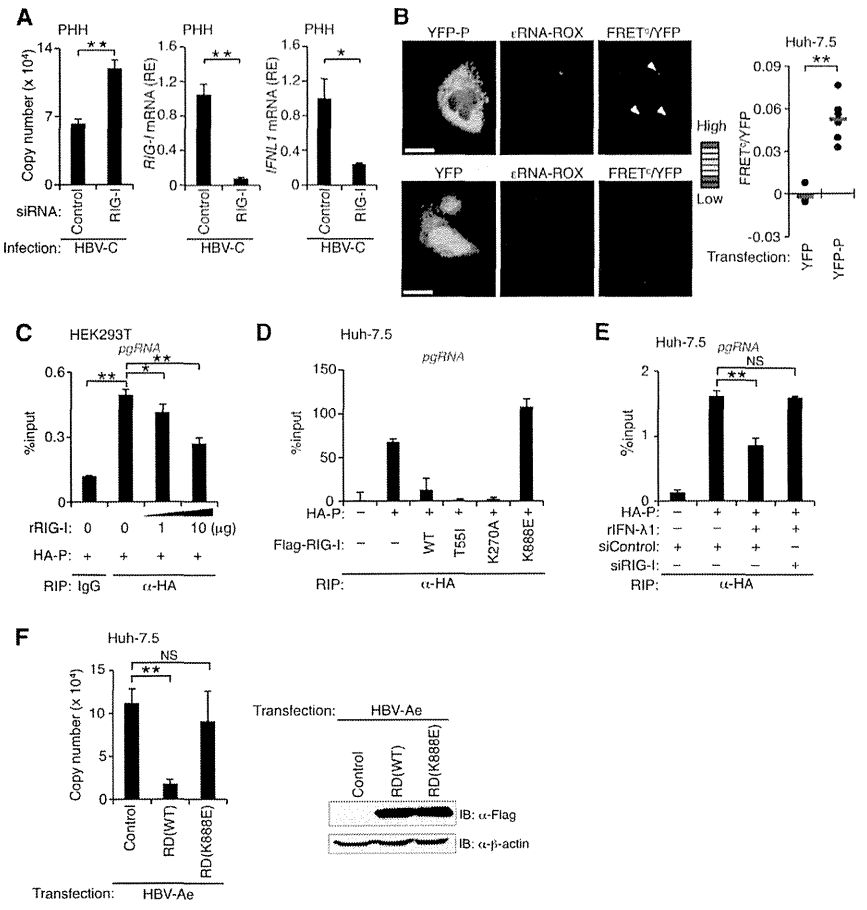

Figure 5. RIG-I Functions as an Antiviral Factor by Counteracting the Interaction of HBV P Protein with pgRNA

(A) qPCR analysis of copy numbers of encapsidated HBV DNA (left) and qRT-PCR analysis of *RIG-I* (middle) and *IFNL1* mRNA (right) in control or RIG-I siRNA-treated PHH after 10 days of infection with HBV-C.

(B) FRET analysis for the interaction between YFP-tagged P protein (YFP-P) or YFP and εRNA-ROX as described in Figure 4B. Scale bar represents 20 μm. Arrowheads indicate area showing high FRET efficiency.

(C) HEK293T cell lysates expressing pgRNA and HA-tagged P protein (HA-P) were incubated with the indicated amount of recombinant RIG-I (rRIG-I). The interaction of pgRNA with HA-P was analyzed by RIP assay and qRT-PCR analysis as described in Figure 4C.

(D) Cell lysates from Huh-7.5 cells expressing HBV pgRNA, HA-P, and Flag-RIG-I or its mutants as indicated were subjected to RIP assay for the characterization of the capability of RIG-I to counteract the interaction of pgRNA with HA-P, as described in Figure 4C.

(E) The effect of rIFN-λ1 treatment on the interaction of pgRNA with HA-P in Huh-7.5 cells was assessed by RIP assay. Huh-7.5 cells expressing both pgRNA and HA-P were treated with rIFN-λ1 (100 ng/ml) for 24 hr, and subjected to RIP assay as described in Figure 4C. RIG-I dependency was also determined by RIG-I knockdown analysis.

(F) Huh-7.5 cells were transfected with an expression vector for RIG-I RD (WT) or RD (K888E), together with the HBV-Ae genome. After 72 hr of transfection, copy numbers of encapsidated HBV DNA were measured (left), as described in (A). Expression of Flag-RIG-I RD (WT) and RD (K888E) (right). *$p < 0.05$ and **$p < 0.01$ versus control. NS, not significant. See also Figure S5.

hepatocyte-chimeric mice. HBV-infected mice underwent intravenous administration with the εRNA expression vector loaded in a liposomal carrier, a multifunctional envelope-type nanodevice (MEND) for efficient delivery, for 2 weeks. Treatment with εRNA-MEND significantly suppressed the elevation of the number of viral genome copies in the sera by less than one tenth of those for control mice (Figure 6C). Consistently, immunofluorescence analyses showed that the expression of HBV core antigen (HBcAg) in the liver tissues of εRNA-MEND-treated chimeric mice was remarkably reduced as compared with those of control mice (Figure 6D).

DISCUSSION

The innate immune system acts as a front line of host defense against viral infection. In this step, PRRs play a crucial role in the recognition of invading viruses. In particular, nucleic acid sensing of viruses is central to the initiation of antiviral immune responses. In this study, we tried to seek for a relevant nucleic acid sensor(s) for HBV and to characterize the IFN response during HBV infection. As a result, we have identified RIG-I as an important innate sensor of HBV to predominantly induce type III IFNs in hepatocytes through its recognition of the 5′-ε stem-loop of HBV pgRNA (Figures 1, 2, 3, and 4). In this respect, there have also been several reports showing that HBV X or P protein interacts with MAVS or competes for DDX3 binding with TBK1, respectively (Wei et al., 2010; Wang and Ryu, 2010; Yu et al., 2010), and inhibits RIG-I-mediated type I IFN pathway, which possibly enables HBV to evade from antiviral innate immune response. This would mirror the important role of RIG-I-mediated signaling for antiviral defense against HBV infection, although further investigation will be required to determine whether other sensing molecules except for RIG-I are engaged in the activation of innate responses in other cell types including dendritic cell subsets. Interestingly, Lu et al. have recently showed that the genotype D of HBV is sensed by MDA5, but not RIG-I, which is based only upon the analyses with HBV genome (2-fold) plasmid transfection in a single cell line Huh-7 (Lu and Liao, 2013). In this respect, we presume that such seemingly contradictory results might arise mainly from the difference in HBV genotype: It has been reported that the genotype D is phylogenetically different from the genotypes A, B, and C, which we analyzed in this study (Kato et al., 2002).

In addition, according to our results (Figure 1C and S1C), HBV-induced type III IFN response does not seem to be efficient as compared with the case with NDV infection. We speculate that

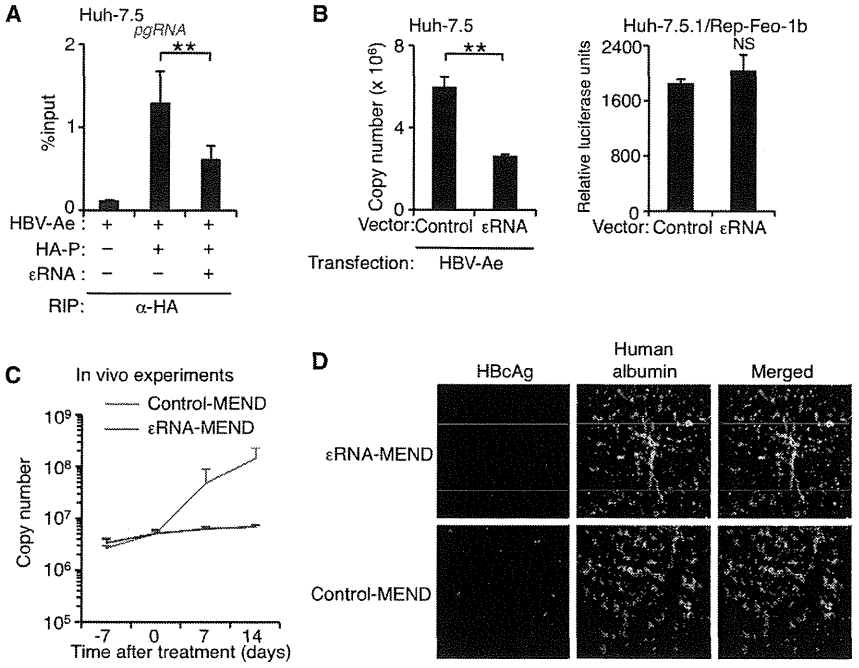

Figure 6. Inhibition of HBV Replication by εRNA

(A) Huh-7.5 cells were transfected with expression vectors for HA-P and εRNA, together with the HBV-Ae genome. RIP assay was performed to evaluate the effect of εRNA on the interaction between HA-P and pgRNA, as described in Figure 5D.

(B) Copy numbers of encapsidated HBV DNA in Huh-7.5 cells expressing the HBV-Ae genome and εRNA, as determined by qPCR (left). HCV replication in Huh-7.5.1/Rep-Feo-1b cells expressing εRNA, as determined by luciferase assay (right).

(C) HBV-infected mice were intravenously administrated with the εRNA expression vector (εRNA-MEND) or empty vector (Control-MEND) loaded in liposomal carrier at a dose of 0.5 mg/kg of body weight every 2 days for 14 days. Serum HBV DNA in HBV-infected chimeric mice was determined by qPCR (n = 3 per group). Day 0 indicates the time of the initiation of administration.

(D) Immunofluorescence imaging was performed for the detection of HBcAg (red) and human albumin (green) in the liver sections of HBV-infected chimeric mice at 14 days after treatment with εRNA-MEND or Control-MEND as described in Experimental Procedures. Data are presented as mean and SD (n = 3) and are representative of at least three independent experiments. **p < 0.01 versus control. NS, not significant.

the weakness of the IFN response during HBV infection might attribute at least in part to these viral evasions from host-cell control, which would be supported by our preliminary data showing that one HBV mutant, which generates viral RNAs including pgRNA but lacks the ability to express whole viral proteins including HBV X and P proteins, can induce higher amounts of IFN-λ1 than intact HBV (Figure S1G). In relevance with this, our present data indicate that the interaction of HBV P protein with the 5′-ε stem-loop affects the RIG-I-mediated recognition of viral pgRNA and the subsequent downstream signaling events, which might likely suppress the induction of IFN-λs. This might provide an aspect of HBV P protein in terms of viral evasion from RIG-I activation. As for the mechanism for the preferential induction of type III IFNs in hepatocytes in response to HBV, as well as HCV (Nakagawa et al., 2013; Park et al., 2012), we might speculate the existence of a hepatocyte-specific factor(s), which is selectively involved in type III IFN gene induction, although this issue merits further investigation including epigenetic evaluation of human hepatocytes. We also found that either of the 5′- or 3′-ε region of pgRNA could interact with RIG-I but it was only the 5′-ε region that contributed to the induction of IFN-λ1 (Figures 3D and S4A). In this respect, we presume that some cofactor(s) might additionally determine the preferential use of the 5′-ε region for RIG-I activation; however, it would be a next interesting issue to be solved. In addition to this, our data demonstrated a hitherto-unidentified function of RIG-I as a direct antiviral factor against HBV infection (Figure 5). Mechanistically, RIG-I was found to counteract the accessibility of HBV P protein to the 5′-ε stem-loop of pgRNA, which is an important process for the initiation of viral replication (Bartenschlager and Schaller, 1992). As is the case with this, several viral PAMPs known to be recognized by RIG-I, for example, the poly-U/UC tract in the 3′ nontranslated region of HCV genome (Saito et al., 2008) and 5′ terminal region of influenza virus genome (Baum et al., 2010) were previously reported to be directly or indirectly critical for viral replication (You and Rice, 2008; Huang et al., 2005; Moeller et al., 2012). In this respect, one could envisage that such an exquisite targeting by RIG-I would confer a unique machinery to ensure efficient antiviral activities of RIG-I. Therefore, RIG-I is likely to play dual roles as an innate sensor and as a direct antiviral effector for host defense during viral infection.

In relation to the evaluation of the experiments shown in Figures 6C and 6D, we additionally analyzed the following points: When we treated HepG2 cells with εRNA-MEND or Control-MEND, in both cases we hardly detected the massive induction of cytokines such as *TNF*, *IL6*, and *CXCL10* (data not shown). This was further confirmed by analyzing SCID mice injected with εRNA-MEND or Control-MEND (data not shown). In addition, εRNA-MEND has the specific effect on the replication of HBV, but not HCV in Huh-7.5 cells (Figure 6B). These data suggest that the results (Figures 6C and 6D) might not be mainly influenced by massive production of antiviral cytokines, although the cross-reactivity of cytokines should be still carefully considered. Therefore, it is presumed that the effect of εRNA might be based on not only its antagonistic activity but also its cytokine-inducing activity. These findings might afford a new therapeutic modality in replace of conventional antiviral drugs that have been reported to have a risk to develop drug-resistance HBV (Song et al., 2012). The present study might provide a better approach to the strategy for development of nucleic acid medicine and offer an attractive clinical option for the therapy against not only HBV but also possibly other virus infections.

EXPERIMENTAL PROCEDURES

Infection of Human Hepatocyte-Chimeric Mice with HBV and In Vivo Treatment with εRNA

To generate human hepatocyte-chimeric mice, uPA$^{+/+}$/SCID mice, were transplanted with commercially available cryopreserved human hepatocytes (a 2-year-old Hispanic female; BD Bioscience) as described previously (Tateno et al., 2004). Chimeric mice were intravenously infected with HBV-C (10^6 copies per mouse) derived from patient with chronic hepatitis (Sugiyama et al., 2006). Total RNAs were isolated from liver tissues at 0, 4, or 5 weeks after infection and subjected to quantitative RT-PCR (qRT-PCR). In preparation for εRNA treatment, HBV infection at 3 weeks after infection was confirmed by measuring the number of viral genome copies in the sera of HBV-infected chimeric mice by qPCR analysis, and at 4-week postinfection, εRNA expression vector or empty vector loaded in a liposomal carrier, a multifunctional envelope-type nanodevice (MEND), was administered intravenously at a dose of 0.5 mg/kg of body weight (n = 3 per group) every two days for 14 days. Serum samples were subjected to qPCR for the quantification of DNA copy numbers of HBV as described previously (Nakagawa et al., 2013). All animal protocols described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of Phoenix Bio Co., Ltd.

HBV Infection in Human Hepatocytes and Quantification of Encapsidated HBV DNA

HBV infection in PHH or HepG2-hNTCP-C4 cells was performed at 10 or 100 genome equivalents per cell, respectively, in the presence of 4% PEG8000 at 37°C for 24 hr as previously described (Sugiyama et al., 2006; Watashi et al., 2013; Iwamoto et al., 2014). Lamivudine (50 μM; Sigma) was added in Huh-7 culture media during HBV production. HBV DNA was purified from intracellular core particles as described previously (Turelli et al., 2004; Fujiwara et al., 2005). Briefly, cells were suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% NP-40. Nuclei were pelleted by centrifugation at 4°C and 15,000 rpm for 5 min. The supernatant was adjusted to 6 mM MgOAc and treated with DNase I (200 μg/ml) and of RNase A (100 μg/ml) for 3 hr at 37°C. The reaction was stopped by addition of 10 mM EDTA and the mixture was incubated for 15 min at 65°C. After treatment with proteinase K (200 μg/ml), 1% SDS, and 100 mM NaCl for 2 hr at 37°C, viral nucleic acids were isolated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation with 20 μg glycogen. Copy numbers of HBV DNA were measured by qPCR with the indicated primers (Table S3).

qRT-PCR Analysis

Total RNAs were isolated from culture cells or frozen liver tissue using Isogen (Nippon Gene) and were treated with DNase I (Invitrogen). cDNA was prepared from total RNAs using ReverTra Ace (TOYOBO). qRT-PCR was performed using SYBR Premix Ex Taq (TAKARA) and analyzed on a StepOnePlus real-time PCR system (Applied Biosystems). Detailed information about the primers used here is shown in Table S3. Data were normalized to the expression of *GAPDH* or HBV RNAs for each sample.

RIP Assay

After 2 hr incubation with the antibody as indicated for immunoprecipitation, cell lysates were mixed with Protein-G Dynabeads (Invitrogen) and further incubated for 1 hr with gentle shaking. After washing three times, the precipitated RNAs were analyzed by qRT-PCR with appropriate primers to detect the target RNA. The amount of immunoprecipitated RNAs is represented as the percentile of the amount of input RNA (percentage input). The detail was described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.12.016.

AUTHOR CONTRIBUTIONS

S.S., K.L., T.K., and T.H. carried out most of the experiments and analyzed data. Y.T. provided materials of HBV and designed the protocol for infection. Y.I. conducted HBV infection in chimeric mice and preparation of human hepatocytes, and S.M., T. Watanabe, S.I., S.T., and Y.T. performed the related analysis and contributed to the interpretation of the results. C.M.R. offered critical advice on the whole manuscript and provided a pair of Huh-7 and Huh-7.5 cell lines. K.W. and T. Wakita provided HepG2-hNTCP-C4 cells. T.K. carried out FRET analysis. Y. Sakurai, Y. Sato, H.A., and H.H. syntheses of plasmid-loaded MEND, and M.K. contributed to establishment of the protocols for in vivo treatment. A.T. supervised the project, designed experiments and wrote the manuscript with critical input from the coauthors, and all authors contributed to discussing the results.

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Postexposure Prophylactic Effect of Hepatitis B Virus (HBV)-Active Antiretroviral Therapy against HBV Infection

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Retrospective study indicates that hepatitis B virus (HBV)-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy reduce the incidence of acute HBV infections in human immunodeficiency virus (HIV)-infected patients. Learning from HIV postexposure prophylaxis (PEP), we explored the possibility of using NAs in PEP following HBV exposure, if preexposure prophylaxis is feasible clinically. Using freshly isolated primary human hepatocytes cultured *in vitro*, we analyzed the effect of HBV-active tenofovir and lamivudine in primary HBV infection and also the effect of treatment with these NAs after HBV infection. HBV-active NAs applied from 24 h before inoculation could not prevent the secretion of hepatitis B surface antigen into the culture medium, and cessation of the NAs after inoculation allowed the cells to establish an apparent HBV infection. In contrast, hepatitis B immune globulin was able to prevent HBV infection completely. NA treatment before infection, however, can control the spread of HBV infection, as detected by immunohistochemistry. Practically, starting NA treatment within 2 days of primary HBV infection inhibited viral spread effectively, as well as preexposure treatment. We demonstrated that preexposure NA treatment was not able to prevent the acquisition of HBV infection but prevented viral spread by suppressing the production of mature progeny HBV virions. The effect of postexposure treatment within 2 days was similar to the effect of preexposure treatment, suggesting the possibility of HBV PEP using HBV-active NAs in HIV- and HBV-susceptible high-risk groups.

Patients infected with human immunodeficiency virus (HIV) are at high risk of hepatitis B virus (HBV) infection, and an estimated 10% of HIV-infected individuals worldwide have chronic hepatitis B (1). Because of the shared transmission routes of the two viruses, i.e., sexual intercourse and blood contact (2), HIV-infected individuals, particularly men who have sex with men (MSM) and intravenous drug users, are at high risk of acute HBV infection.

Two recent retrospective studies from Japan and the Netherlands indicated that HBV-active nucleoside (nucleotide) analogues (NAs) used for antiretroviral therapy (ART) reduced the incidence of acute HBV infections in HIV-infected patients (3, 4). The prophylactic effects of regimens containing NAs as reverse transcription (RT) inhibitors are well accepted against HIV infection (5, 6), because RT is an initial essential step following HIV entry into susceptible cells. However, considering the differences between the HIV and HBV replication cycles, this notion cannot be applied simply to HBV infection.

Unlike HIV, when HBV enters the hepatocyte, its genomic DNA is transported to the nucleus and converted to covalently closed circular DNA (cccDNA), which serves as the template for transcription. One transcript, the pregenomic RNA, is converted to genomic DNA by RT, which is the target of NAs.

Studies of occult HBV infections strongly suggest that HBV cccDNA remains in the hepatocyte nuclei for a long time after resolution of acute infection (7), often leading to reestablishment of infection (HBV reactivation) following immunosuppressive therapy, i.e., anticancer chemotherapy (8). The phenomenon of HBV reactivation suggests that cccDNA remaining in the hepatocytes can produce infectious virions, leading to very rapid viral spread. Therefore, establishment of cccDNA in the nuclei means HBV infection of susceptible cells.

Nonetheless, human clinical studies have shown that HBV-active ART protects against the occurrence of *de novo* HBV infection, indicating that NA-based strategies inhibit the serological changes of HBV markers (hepatitis B surface antigen [HBsAg], hepatitis B surface antibody [anti-HBs], and hepatitis B core antibody [anti-HBc]) that provide evidence of HBV infection and have a clinical prophylactic effect against incident HBV infection. Learning from HIV postexposure prophylaxis (PEP), we consider the possibility of NA-based PEP against HBV exposure, given that preexposure prophylaxis (PrEP) is feasible clinically. Therefore, using freshly isolated primary human hepatocytes (PHH) cultured *in vitro*, we analyzed the effect of HBV-active NAs on primary HBV infection and showed the possibility of prophylaxis by NAs not only as PrEP but also as PEP against acute HBV infection.

MATERIALS AND METHODS

Freshly isolated PHH. Freshly isolated primary human hepatocytes (PHH) from severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator, whose livers were repopulated with human hepatocytes, were purchased from Phoenix Bio Co., Ltd. (Higashihiroshima, Japan), without cryopreservation. The isolation and culture of PHH were described previously (9).

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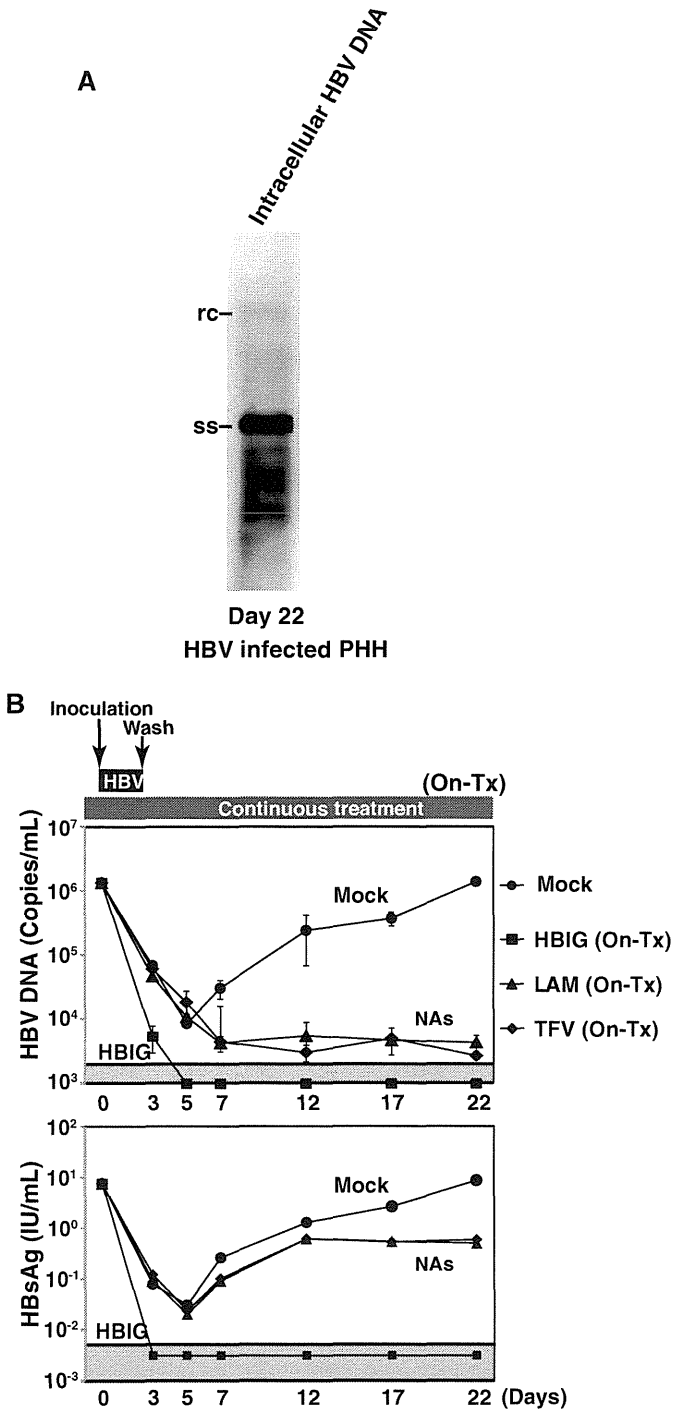

FIG 1 Incomplete prevention of primary HBV infection by NA. (A) Freshly isolated PHH susceptible to HBV infection. Single-stranded HBV DNA (ssDNA), a replicative intermediate in PHH on day 22 postinfection, was visualized by Southern blotting. rcDNA, relaxed-circular, partially double-stranded HBV DNA. ssDNA denotes HBV replication following infection in susceptible cells. (B) The kinetics of HBV DNA (top) and hepatitis B surface antigen (HBsAg; bottom) concentrations in the culture medium of HBV-infected PHH with various treatments. PHH (2.1×10^5 cells/cm^2) were inoculated with HBV (2×10^6 copies/ml) for 48 h at 37°C, and then PHH were washed several times and sampling of the supernatants throughout the course (days 3, 5, 7, 12, 17, and 22 postinfection) was performed. The concentrations of HBV DNA and HBsAg in the culture supernatants, mainly remaining inoculum, decreased until day 5; however, HBV DNA and HBsAg began to increase after around day 5 because of HBV replication in PHH. On-Tx, HBV-infected PHH were treated continuously with lamivudine (LAM), tenofovir (TFV), or hepatitis B immune globulin (HBIG) up to 22 days. Ongoing treatment with LAM or TFV resulted in a continuous decrease in HBV DNA in the supernatant. However, the production of HBsAg increased after day 5. Data are presented as means ± standard deviations (SD) ($n = 3$ experiments). The NAs were LAM and TFV. Mock, the HBV infection control experiment without treatment.

HBV infection experiments. Inoculation of PHH with 10 HBV genome equivalents per cell was carried out in culture medium without polyethylene glycol for 48 h at 37°C. PHH were washed with medium three times on days 2 and 3 to remove the inoculum. Supernatants were collected, and the culture media were replenished on days 3, 5, 7, 12, 17, and 22 postinfection. To specifically block HBV attachment to the PHH, the HBV inoculum (2×10^6 copies/ml) was preincubated with 1,000 mIU/ml of hepatitis B immunoglobulin (HBIG) for 2 h, and the mixture of HBIG and HBV was added to the PHH. Also, 2.5 mM lamivudine (LAM) or 100 μM tenofovir (TFV) was added to the culture medium, starting 24 h before inoculation. According to previous results, using the HBV-expressing cell lines (Hep2.2.15), the 50% effective concentration (EC_{50}) value of LAM (10) is around 0.12 μM and that of TFV (11) is around 1.1 μM. Thus, the doses of LAM and TFV used in our study are 20,000 times and 100 times higher, respectively, than the EC_{50} for HBV inhibition and thus provide sufficient excess for the desired clinical effect. In addition, the high concentration of LAM and TFV could not cause any cytotoxicity on the cells.

Determination of HBsAg and HBV DNA levels. The levels of HBsAg were determined by chemiluminescent enzyme immunoassay as described previously (12). The detection limit of the HBsAg assay is 0.05 IU/ml. HBV DNA was quantified by quantitative PCR as described previously (13). The detection limit of HBV DNA was set to 2.0×10^3 copies/ml. cccDNA was measured by quantitative PCR with primers cccF2 and cccR4, described previously (14), and a fluorescent probe, cccP2 (5′-FAM-CTGTAGGCATAAATTGGT-MGB-3′ [FAM is 6-carboxyfluorescein]). The detection limit of cccDNA was set to 1.0×10^2 copies/μg DNA.

Southern hybridization. Southern hybridization was performed with full-length probes for HBV as described previously (12, 15).

Immunofluorescence assay. Fluorescence staining of intracellular HBsAg was performed by standard methods using goat anti-HBs (Bioss, Inc., MA, USA) and donkey anti-goat IgG conjugated with Alexa Fluor 488 (Life Technologies, Maryland, USA).

RESULTS

Effects of HBV-active NAs against primary infection. Freshly isolated PHH were confirmed to be susceptible to HBV primary infection by using Southern blot analysis (Fig. 1A). We examined the effect of NAs on the establishment of HBV infection in the hepatocytes. PHH were treated with the HBV-active NAs, LAM and TFV, at extremely high concentrations (2.5 mM and 100 μM, respectively) starting 24 h before HBV inoculation to gain full efficacy of NAs. Figure 1B shows the kinetics of HBV DNA (top) and HBsAg (bottom) concentrations in the culture medium of HBV-infected PHH. After day 5, HBV DNA concentrations increased in the supernatants of nontreated cells, indicating HBV replication, i.e., accumulation of cccDNA in the cell and release of progeny virions into the culture medium. Because NAs inhibit RT activity, treatment with LAM or TFV results in a continuous decrease of HBV DNA in the supernatants. In contrast, HBIG, which inhibits viral entry by blocking receptor binding (16), effectively prevented HBV infection, and HBV DNA could not be detected for 22 days. As shown in the bottom panel of Fig. 1B, LAM and TFV treatment have a small effect on the HBsAg concentration in the

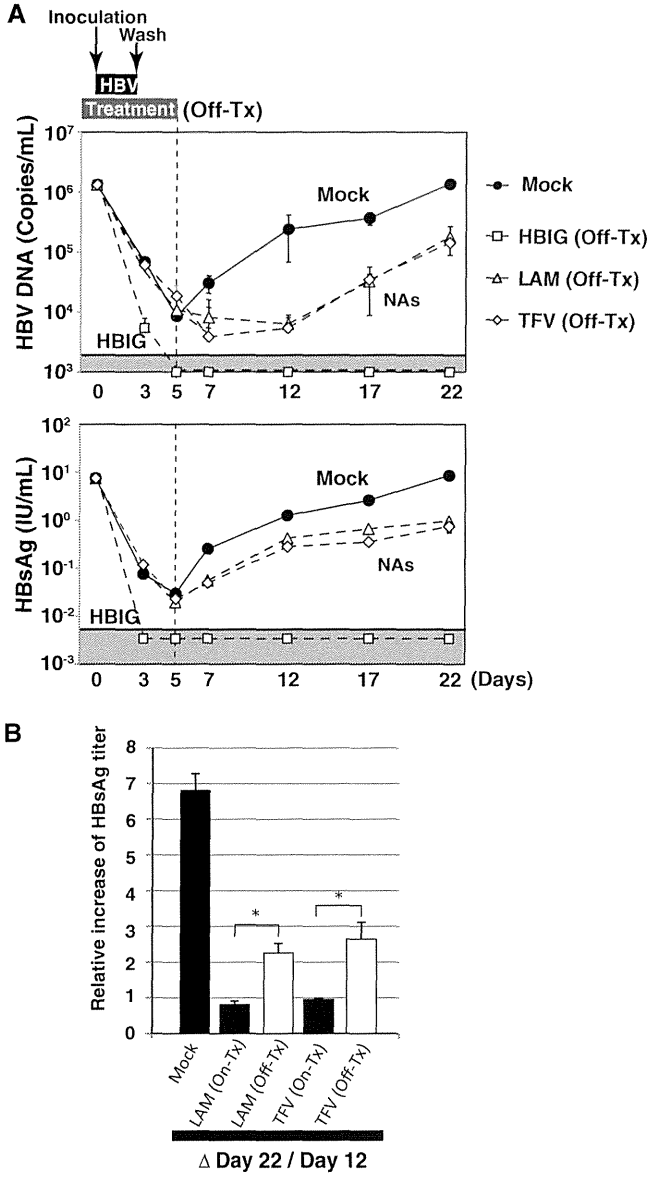

FIG 2 Preexposure NA treatment fails to prevent HBV infection. (A) PHH were inoculated with HBV for 48 h at 37°C and washed several times, and sampling of the supernatants was performed (as described in Fig. 1B). The concentration of HBV DNA increased gradually after day 12 when the LAM or TFV treatment was stopped on day 5, despite the complete cessation of hepatitis B immune globulin (HBIG) treatment. The production of HBsAg in the control experiment increased further after day 12, and HBsAg productions in cases in which NA treatment was stopped on day 5 (Off-Tx) gradually increased in the supernatant. Off-Tx, HBV-infected PHH were treated for only 5 days with lamivudine (LAM), tenofovir (TFV), or HBIG. Data are presented as means ± SD ($n = 3$ experiments). (B) Increasing production of HBsAg in the supernatants during the period from day 12 to day 22. In order to confirm the change of HBsAg production, the HBsAg titer on day 22 was divided by that on day 12. The increase of the rate in the nontreatment experiment was 6.8 from day 12 to day 22; otherwise, the rates of the cells treated continuously with NAs (On-Tx of both LAM and TFV) were maintained at steady levels (the ratio was approximately 1). In the cases with cessation of NA treatment on day 5, the increase of HBsAg production during the period from day 12 to day 22 was observed in the culture medium (Off-Tx of LAM, 2.3; Off-Tx of TFV, 2.6). Data are presented as means ± SD ($n = 3$ experiments). Mock, the HBV infection control experiment without treatment. Statistical analysis of the difference was conducted using the analysis of variance with a nonparametric Mann-Whitney U test. *, $P < 0.05$.

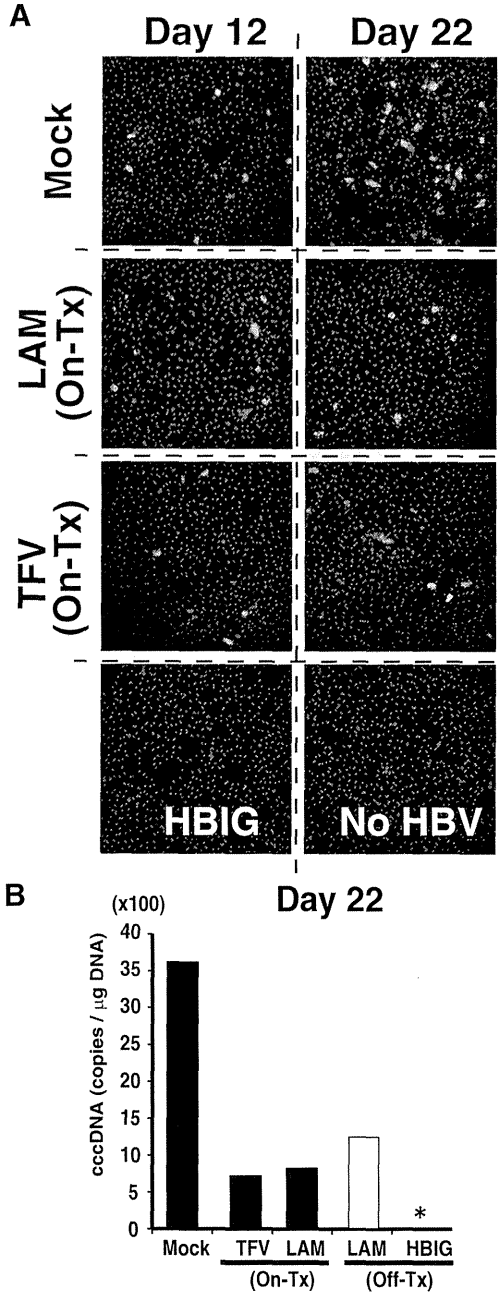

FIG 3 HBV-active NAs can inhibit viral spread after acquisition of HBV infection in freshly isolated PHH. (A) Immunohistochemical staining of HBV-infected PHH with various treatments. Viral spread occurred over 10 days from day 12 to day 22 in the nontreatment experiment (Mock); however, the numbers of cells staining for HBsAg did not change from day 12 to day 22 with continuous treatment by either LAM or TFV. HBIG, treatment with HBIG for 12 days; no HBV, PHH at day 22 without HBV inoculation; green, staining of HBsAg; blue, staining of nuclear DNA. (B) The level of cccDNA in HBV-infected PHH at day 22. Compared to the control experiment (Mock), the levels of cccDNA treated continuously with NAs (On-Tx of both LAM and TFV) were suppressed and that with LAM treatment for only 5 days (LAM Off-Tx) exhibited an increase following the cessation of LAM treatment. The asterisk indicates a value below the detection limit.

supernatant up to day 12; presumably, these NAs could not protect against the formation albeit at high concentration but do inhibit the accumulation of cccDNA as the template for HBsAg. Through the experiments, we did not see evidence of cytotoxicity by 3-(4,5-di-

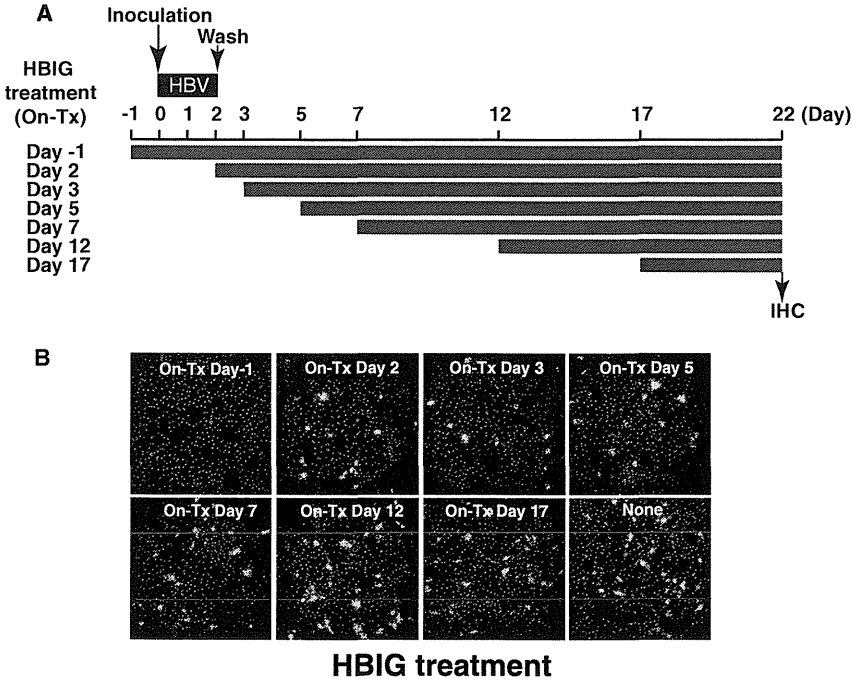

FIG 4 HBIG treatment in freshly isolated PHH post-HBV inoculation inhibits viral spread, while delaying HBIG addition allows viral spread. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with hepatitis B immune globulin (HBIG) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with HBIG treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of HBIG after inoculation did not protect against primary HBV infection (On-Tx Day 2), and delaying the addition of HBIG allowed gradual viral spread (On-Tx Day 2 to Day 17), detected by fluorescence IHC at day 22 postinoculation.

methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays or by microscopic examination of cell morphology after treatment with these high concentrations (data not shown).

Next, to confirm the prophylactic effect of NAs, treatment was stopped at day 5 (Fig. 2A). As expected, HBIG continued to prevent HBV infection. When LAM or TFV treatment was stopped on day 5, however, the concentration of HBV DNA increased gradually after day 12, suggesting that these agents did not protect against the establishment of HBV infection (Fig. 2A). The production of HBsAg in the control experiment also increased after day 12, and HBsAg production in cases in which NA treatment had ceased on day 5 (Off-Tx) also slowly increased. Previously, it was thought that the rapid differentiation of PHH leads to a loss of susceptibility to HBV infection within a few days. However, the PHH used in this study were isolated from living chimeric mice without cryopreservation. We therefore confirmed the susceptibility to HBV infection over 10 days using the increase in HBsAg production as a surrogate marker for the accumulation of cccDNA and thus the increasing number of HBV-infected PHH. Although HBsAg production increased markedly in the nontreatment experiment (the relative increase ratio was 6.8) during the period from day 12 to day 22 (Fig. 2B), there was no increase in cells continuously treated with NA (the ratios of LAM and TFV On-Tx were approximately 1). However, in the cases with cessation of NA treatment on day 5, HBsAg production increased in the culture medium from day 12 to day 22 (the ratios of LAM and TFV Off-Tx were 2.3 and 2.6, respectively). These results indicated that freshly isolated PHH, without cryopreservation, have prolonged susceptibility to HBV infection. Although we did not compare fresh and cryopreserved PHH, our observations indicate that freshly isolated PHH remain susceptible to HBV infection at least for 1 month after preparation (data not shown).

To further demonstrate the susceptibility of freshly isolated PHH to HBV infection, we investigated HBsAg expression and measured PHH cccDNA concentration. Detection of HBV-infected PHH by fluorescence immunohistochemistry (IHC) revealed that the number of infected cells on day 22 was approximately 6 times greater than that on day 12, indicating viral spread during this period (Fig. 3A, Mock). In contrast, no increase in the number of infected cells was observed following continuous LAM or TFV treatment. Although the level of cccDNA in cells treated continuously with NAs (LAM and TFV) was suppressed on day 22, compared to the control experiment, those treated with LAM for only 5 days (LAM Off-Tx) exhibited an increase following cessation of LAM treatment (Fig. 3B). Because NAs inhibit the production of infectious virions at the stage of conversion of pregenomic RNA to genomic DNA, the release of progeny virions is inhibited by NAs. This allows us to suggest that secondary infection of neighboring cells by newly produced HBV virions, viral spread, occurred in the *in vitro* system. From these results and basic mechanisms regarding the HBV replication cycle, we conclude that NAs do not prevent the establishment of cccDNA and acquisition of HBV infection but prevent viral spread by suppressing the production of progeny HBV virions.

Feasibility of NA-based HBV postexposure prophylaxis. Considering that HBV-active NAs act only on viral spread, we explored the possibility that the addition of LAM to the culture medium shortly after HBV inoculation might have an effect similar to that of preexposure LAM treatment, as shown in Fig. 1 and 3. Again, to confirm the susceptibility to HBV infection during the

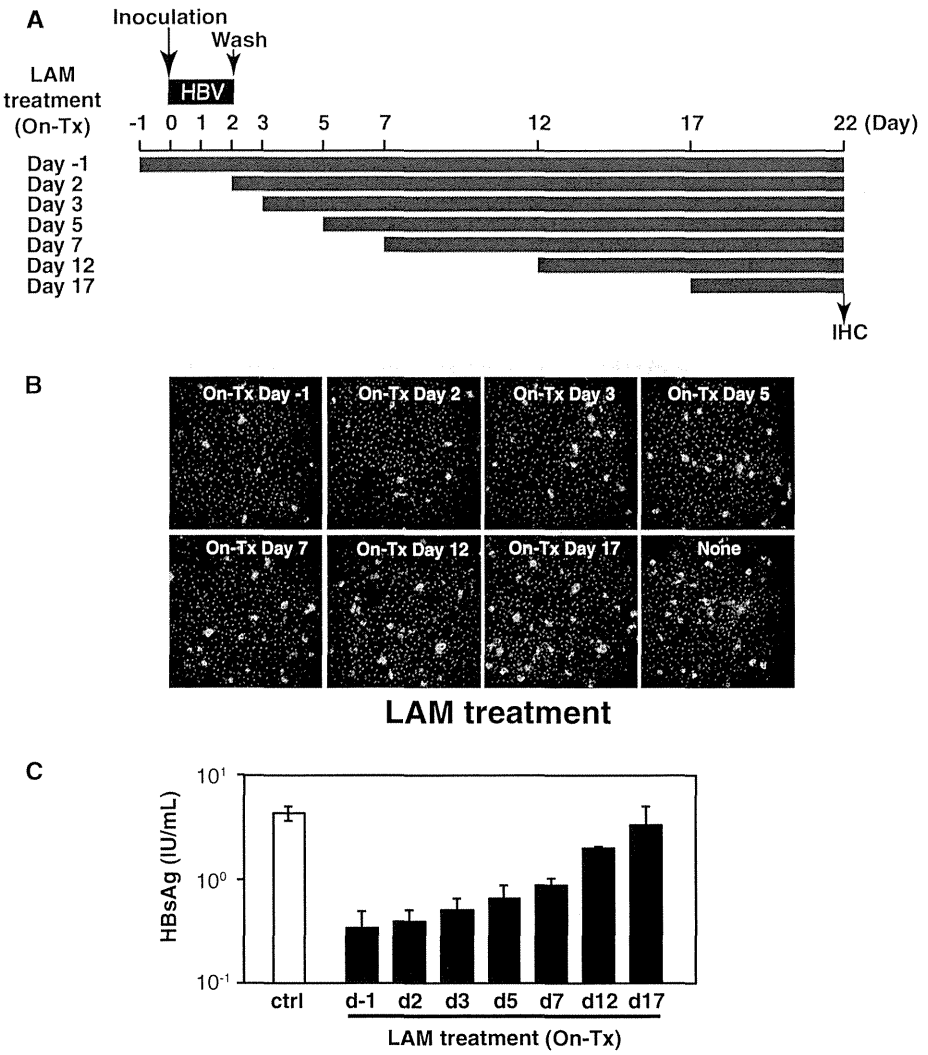

FIG 5 Effects of LAM on post-HBV inoculation *in vitro*. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with lamivudine (LAM) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with LAM treatment started at 2, 3, 5, 7, 12, and 17 days after HBV inoculation, as indicated in panel A. The addition of LAM before and after inoculation did not protect against primary HBV infection (On-Tx Day −1 and Day 2), and the delay in the addition of LAM resulted in viral spread at day 22 postinoculation. (C) The HBsAg concentrations in the culture medium collected on day 22 from the HBV-infected PHH with LAM treatment as shown in panel A. Data are presented as means ± SD ($n = 3$ experiments). ctrl, HBV-infected PHH without treatment.

follow-up periods, the addition of HBIG after inoculation was explored for 22 days (Fig. 4A), because HBIG treatment protects completely against new HBV infection (Fig. 1B, 2A, and 3B). The addition of HBIG after inoculation was not able to protect against primary HBV infection (Fig. 4B, On-Tx Day −1 compared to Day 2), and delaying adding HBIG allowed gradual viral spread, monitored by fluorescence IHC at day 22 postinoculation (Fig. 4B, On-Tx Day 2 to Day 17 compared to None). These results strongly indicate that delaying starting HBIG treatment after HBV inoculation allowed viral spread into the freshly isolated PHH system. Next, the effect of the addition of LAM after inoculation was monitored and quantified, based on the HBsAg titer in the supernatant (Fig. 5). Delaying the addition of LAM (Fig. 5A) allowed gradual viral spread, as observed in the HBIG treatment. However, the addition of LAM on day 2 after inoculation effectively inhibited the viral spread observed on day 22 (Fig. 5B, On-Tx Day 2). Furthermore, the HBsAg levels in the culture medium on day 22 indicate that starting LAM treatment within 2 days of inoculation has a similar effect on viral spread to preexposure treatment (Fig. 5C).

DISCUSSION

Coinfection with HBV and HIV increases the morbidity and mortality beyond those caused by either virus alone, and the burden of coinfection is greatest in developing countries, particularly in Southeast Asia and sub-Saharan Africa (1). Therefore, the assessment of HBV status is warranted for all HIV-positive persons, and hepatitis B vaccination should be offered to all susceptible individuals. Although immunization remains the mainstay of disease prevention, HIV-positive individuals mount poorer antibody responses to hepatitis B vaccine than healthy donors (17, 18). Moreover, as the course of hepatitis B immunization involves injections at 0, 1, and 6 months, a coordinated effort and period of time are required.

Because NA-based PEP has been used as an HIV prevention strategy for nearly 20 years, we explored the possibility of HBV PEP using NAs, with reference to clinical evidence that PrEP may be feasible for *de novo* HBV infection. In Japan, Gatanaga et al. explored NA-based PrEP using sequential serum samples from HIV-infected MSM who had no detectable HBsAg, anti-HBs, and anti-HBc at baseline (3). Evidence of HBV infection was detected in follow-up samples from 43 of the 354 men (12.1%) after a median time period of 1.6 years. The rate of incident infection was approximately 90% lower for patients taking LAM or TFV than for those receiving no ART or a non-LAM/TFV-containing regimen. Moreover, no new HBV infections occurred in patients taking TDF; however, the 7 men who developed HBV infection despite taking LAM were more likely to be infected with LAM-resistant strains (50.0%) than the 36 individuals not receiving HBV-active ART (7.1%). Another serological follow-up study from the Netherlands reported that LAM and TFV protected against the occurrence of *de novo* HBV infection among HIV-infected MSM over a median of 6.8 years and also demonstrated a superior protective effect on HBV seroconversion in the group taking TFV (4). These clinical data indicated that HBV-active NAs can prevent acute HBV infection, defined by the absence of the serological markers HBsAg, anti-HBs, and anti-HBc, and TFV-based PrEP has a significant effect on HBV primary infection. In the case of HIV infection, the prophylactic effects of NAs as RT inhibitors are well accepted, because these block the initial step following virus entry. However, during HBV infection, RT inhibitors act after cccDNA formation and do not prevent HBV infection completely, confirmed by our original HBV-infected PHH system. Our results clearly indicate that the effect of NAs on HBV primary infection was simply prevention or inhibition of viral spread.

It remains largely unknown how NAs act as prophylaxis. As clinical data showed, treatment with NAs protects against the acquisition of HBV without an antibody response. We speculated that acquired immunity, especially humoral immunity, could not be responsible for the clinical outcome of prophylaxis by NAs. Recent reports show that natural killer (NK) cells contribute to protection against acute HCV infection (19–21), and NK cells may play important roles in the eradication of HBV from hepatocytes (22, 23). Continuous treatment with NAs could prevent viral spread and, consequently, the cccDNA would be confined to the cells infected initially. If this is the case, the small number of cccDNA-containing cells could readily be eradicated by the major innate immune cells, i.e., NK cells, without an antibody response, and/or the cccDNA could be diluted during hepatocyte turnover with subsequent diminishment of hepatocytes harboring cccDNA in the absence of immune responses (24).

Although the extent to which the data from *in vitro* studies can be extrapolated to clinical human studies is largely unknown, we postulate that treatment with NAs should be used for longer periods of HBV prophylaxis than of HIV prophylaxis, typically for 4 weeks in HIV PEP (5, 25), because of the evidence from our *in vitro* data regarding acquisition of HBV infection. Correspondingly, the use of NAs for preexposure and postexposure prophylaxis to prevent HBV transmission must also be studied in the context of HIV prevention in areas where there may be problems in obtaining and/or storing HBIG. Despite the fact that our results with higher concentrations of NAs than those in the clinical setting indicate the possibility of NA-based HBV PEP, as well as PrEP shown by retrospective human studies, further research and human clinical trials are needed to evaluate brief screening, drug selection, as well as dose, timing, and duration of NA-based PEP against *de novo* HBV infection in susceptible high-risk groups.

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We have no conflicts of interest to declare.

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RESEARCH ARTICLE

Validation of Cross-Genotype Neutralization by Hepatitis B Virus-Specific Monoclonal Antibodies by *In Vitro* and *In Vivo* Infection

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Abstract

Vaccines based on hepatitis B virus (HBV) genotype A have been used worldwide for immunoprophylaxis and are thought to prevent infections by non-A HBV strains effectively, whereas, vaccines generated from genotype C have been used in several Asian countries, including Japan and Korea, where HBV genotype C is prevalent. However, acute hepatitis B caused by HBV genotype A infection has been increasing in Japan and little is known about the efficacy of immunization with genotype C-based vaccines against non-C infection. We have isolated human monoclonal antibodies (mAbs) from individuals who were immunized with the genotype C-based vaccine. In this study, the efficacies of these two mAbs, HB0116 and HB0478, were analyzed using in vivo and in vitro models of HBV infection. Intravenous inoculation of HBV genotype C into chimeric mice with human hepatocytes resulted in the establishment of HBV infection after five weeks, whereas preincubation of the inocula with HB0116 or HB0478 protected chimeric mice from genotype C infection completely. Interestingly, both HB0116 and HB0478 were found to block completely genotype A infection. Moreover, infection by a genotype C strain with an immune escape substitution of amino acid 145 in the hepatitis B surface protein was also completely inhibited by incubation with HB0478. Finally, in vitro analysis of dose dependency revealed that the amounts of HB0478 required for complete protection against genotype C and genotype A infection were 5.5 mIU and 55 mIU, respectively. These results suggested that genotype C-based vaccines have ability to induce cross-genotype immunity against HBV infection.

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Introduction

Hepatitis B virus (HBV) is a blood-borne, hepatotropic virus that infects an estimated 350 million people worldwide. Besides the manifestations associated with acute hepatitis, chronic HBV infection constitutes a significantly high risk for the development of liver cirrhosis and hepatocellular carcinoma. HBV strains are classified into eight genotypes based on genetic diversity [1,2] and the prevalence of these genotypes varies geographically [3]. Hepatitis B surface antigen (HBsAg) is the key molecule for HBV entry into the hepatocyte [4] and HBV vaccination establishes host immunity by activating B lymphocytes that produce HBsAg-specific antibodies (anti-HBs) with neutralizing activities. The highly immunogenic region of HBsAg, known as the "a" determinant, comprises two peptide loops in which several amino acids vary among the HBV genotypes [5].

Vaccination of high risk individuals and universal infant/childhood vaccination programs have effectively decreased the incidence of acute HBV infection and consequent chronic hepatitis B [6]. Recombinant vaccines containing HBsAg generated from HBV genotype A2 (gt-A2) have been used worldwide. Although these A2-type vaccines are effective in preventing non-A2 HBV infections [7], investigation of cross-genotype protection is limited in the clinical setting. On the other hand, genotype B (gt-B) and genotype C (gt-C) strains are the most prevalent in east Asian countries [1] and some of these countries, including Japan and Korea, have used recombinant vaccines generated from gt-C for immunoprophylaxis against HBV endemic in these communities [8,9]. In the last decade, however, the spread of gt-A strains imported from foreign countries and the subsequent increase of hepatitis caused by HBV gt-A is a growing concern in Japan [10]. Until now, little is known about whether the gt-C HBV vaccine can induce effective immunity against non-C HBV infection.

Previously, we isolated human monoclonal antibodies (mAbs) against HBV from healthy volunteers who had been immunized with a gt-C type recombinant HBV vaccine (Biimugen), using a cell-microarray system [11–13]. A subsequent report revealed that among these mAbs, HB0116 and HB0478, recognize the first N-terminal peptide loop within the "a" determinant and have HBV-neutralizing activities [14]. In this report, whether these mAbs generated by the gt-C type vaccine can protect gt-A strain infections was investigated using in vitro and in vivo HBV infection models, including primary human hepatocytes (PHHs) and severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator, whose livers were repopulated with human hepatocytes (hereafter referred to as chimeric mice) [15–17]. The neutralizing activities of these mAbs against the frequently isolated immune escape mutant, which has an amino acid substitution of arginine for glycine at residue 145 within the second, C-terminal loop of HBsAg (G145R) [18–20], were also investigated.

Materials and Methods

Ethics statement

This study conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by approval by the Ethics Committee of University of Toyama with written informed consent (Permit Number: 14–123). All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the Ethics Committees of PhoenixBio Co., Ltd (Permit Number: 0253). Chimeric mice were housed in specific pathogen—free facilities at the laboratory of PhoenixBio Co., Ltd. Food and water were delivered ad libitum. Chimeric mice were weighed and anesthetized using isofluorane prior to blood collection from the

orbital vein. The chimeric mice were anesthetized using isofluorane and sacrificed by exsanguination from the heart at the end of the experiment.

HBV-specific mAbs and recombinant peptides

Recombinant HB0116 and HB0478 in IgG form were generated as described previously [14]. Synthetic peptides for the first loop of HBsAg gt-C and gt-A (123–137 gt-C: TCTI-PAQGTSMFPSC; 123–137 gt-A: TCTTPAQGNSMFPSC) were generated also as described previously [14].

The binding activity of each mAb for recombinant peptides was examined by ELISA with streptavidin-coated plates (Nunc, Roskilde, Denmark). Plates were coated with the peptides at 10 μg/mL and nonspecific binding was blocked with PBS containing 3% bovine serum albumin (BSA). Each mAb was added to the wells for 2 hours, followed by washing and reaction with alkaline phosphatase-conjugated anti-human IgG (Sigma, Saint Louis, MO). The O.D. value at 405 nm was evaluated after addition of phosphate substrate (Sigma). Control human monoclonal IgG1 (cIgG, Athens Research & Technology, Athens, GA) was added at the same concentration as the control.

Immunoprecipitation assay

1×10^4 copies of HBV of gt-C, gt-A and G145R (gt-C with an amino acid substitution of arginine for glycine at position 145 of HBsAg) were incubated with 1 μg of mAbs diluted in 2% BSA/PBS or cIgG on a rotating wheel overnight at 4°C and then protein A-Sepharose beads (GE Healthcare) were added to the mixture and incubated for a further 4 hours. The beads were centrifuged briefly to remove the supernatants, washed four times with 1 mL 2% BSA/PBS and resuspended in 30 μL sample loading buffer (Tris/HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0·001% bromophenol blue). After boiling for 5 minutes, 15 μL aliquots were applied to 15% SDS-PAGE and the proteins were separated and transferred to a nitrocellulose membrane. HBsAg was detected using 1 μg/mL of a HB0116/HB0478 mixture, followed by anti-human IgG conjugates of horseradish peroxidase (1:5000, Sigma) as the secondary antibody. The bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

HBV-neutralizing assay using HepaRG cells

The HBV-neutralizing capacities of HB0116 and HB0478 were investigated using the HepaRG cell line (supplied by Biopredic International, Rennes, France). The HepaRG cells were cultured and differentiated as described previously [21,22]. 1×10^4 copies of HBV and 1 μg of each mAb were preincubated for 1 hour at room temperature and then added to HepaRG cells in medium containing 4% polyethylene glycol (PEG) 8000 (Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation, the HepaRG cells were washed gently three times with medium and then cultured with fresh medium. On day 7 after infection, cellular DNA was extracted and HBV DNA was quantified as described previously [14].

In vivo HBV-neutralizing assay using chimeric mice

The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan). The HBV inocula used in this experiment were prepared as follows: culture supernatants from cells transfected with plasmids expressing HBV gt-C, gt-A, and G145R contained immature HBV virions [16] and chimeric mice were inoculated with these culture supernatants to obtain the

monoclonal and intact infectious virions. After establishing viremia in these mice, the sera were collected and used as inocula after titration in another experimental chimeric mouse.

Firstly, $1x10^4$ copies of the sera of chimeric mice infected with gt-A, gt-C, G145R were incubated at 37°C for 2 hours in the presence of HB0116 and/or HB0478 and injected intravenously into chimeric mice. Five weeks after injection, serum HBV DNA was measured by quantitative polymerase chain reaction (PCR) as reported previously [23].

In vitro HBV-neutralizing assay using PHHs isolated from chimeric mice

Freshly isolated PHHs were purchased from PhoenixBio Co., Ltd (Higashihiroshima, Japan). Briefly, human hepatocytes were collected from the livers of chimeric mice by collagenase perfusion and plated on collagen-coated 96-well multiplates at a density of 6.7×10^4 cells per well. The cells were then grown in dHCGM medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1 μg/mL of penicillin, 1 μg/mL of streptomycin, 20 mM HEPES, 15 μg/mL of L-proline, 0.25 μg/mL of human recombinant insulin, 50 nM dexamethazone, 5 ng/mL of human recombinant epidermal growth factor, 0.1 mM ascorbic acid, and 2% DMSO).

To investigate HBV kinetics, PHHs were inoculated with serum from HBV gt-C chimeric mice at 5 genomes per cell for 24 hours in the presence of 4% PEG 8000. The sera from chimeric mice contained excess subviral particles including HBs proteins. The cells were then washed three times with the medium to remove the inoculum, and the culture supernatants were collected and replenished with fresh medium on 2, 3, 5, 7, and 12 days post infection (dpi).

To optimize the infectious condition for the analysis of antibody neutralization, HBV gt-C at 10, 3, 1, and 0.3 genomes per cell was preincubated with or without 100 mIU of hepatitis B immune globulin (HBIG) for 2 hours and PHHs were inoculated with the HBV-HBIG mixture for 24 hours with PEG or for 48 hours without PEG. The cells were washed and the supernatants were collected as described above.

Antibody neutralization experiments were performed as follows. HBV gt-C or gt-A inocula at 10 genomes per cells (6.7×10^5 genomes/well) were preincubated with 670, 67, 6.7, or 0.67 ng of HB0478 (corresponding to 550, 55, 5.5, or 0.55 mIU) and exposed to PHHs for 48 hours without PEG. The cells were then washed and the supernatants were collected as described above.

Southern blot analysis of HBV DNA

Southern blot analysis was performed with full-length probes for HBV as described previously [24].

Quantification of HBV DNA, pregenomic RNA and HBsAg

Total RNA and total DNA were extracted from PHHs using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan) and SMITEST EX R&D Kit (Genome Science Laboratories, Tokyo, Japan), respectively. Purified total RNA was then reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Extracellular HBV DNA, intracellular HBV DNA and pregenomic RNA were quantified by real-time quantitative PCR using StepOne Plus and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The samples were denatured by incubating for 10 minutes at 95°C and amplified for 45 cycles (95°C 15 seconds, 60°C 60 seconds) with specific primers and TaqMan fluorescent probes. HBV DNA was amplified using primers HBV-F (5′-CACATCAGGATTCCTAGGACC-3′), HBV-R (5′-AGGTTGGTGAGTGATTGGAG-3′), and TaqMan probe HBV-FT (5′-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-TAMRA-3′).

Primers HBV-PC-F (5′-GGTCTGCGCACCAGCACC-3′), HBV-DN-R (5′-GGAAAGAAGTCAGAAGGCAA-3′) and TaqMan probe HBV-FM (5′-FAM-TCCAAGCTGTGCCTT-MGB-3′) specifically amplify cDNA from precore RNA. Primers HBV-PG-F (5′-CACCTCTGCCTAATCATC-3′), HBV-DN-R and TaqMan probe HBV-FM amplifies cDNA from both precore RNA and pregenomic RNA. The amount of pregenomic RNA was calculated by subtracting the copy number of precore RNA amplification from that of precore/pregenomic RNA amplification [25]. Extracellular HBsAg was quantified by automated ELISA (Fujirebio Inc., Tokyo, Japan). The detection limits are 2 × 103 copies for HBV DNA, 2 × 102 copies for pregenomic RNA and 0.005 IU/mL for HBsAg.

Results

Influence of genotype and amino acid substitutions on recognition by HBV-specific mAbs

The mAbs HB0116 and HB0478 bind to the first loop (amino acids 123–137) of the "a" determinant and strongly inhibit HBV gt-C infection [14]. Therefore, whether the binding capacity of each mAb is affected by amino acid variation within the first loop was examined using recombinant peptides; there is amino acid variation between genotypes C and A at positions 126 (gt-C: I, gt-A: T) and 131 (gt-C: T, gt-A: N). Both HB0116 and HB0478 bound peptides not only corresponding to the first loop with the gt-C sequence but also corresponding to those with the gt-A sequence, indicating their cross-genotype recognition on binding in vitro (Fig. 1A). The binding capacities to the native HBs proteins of gt-A, gt-C, together with gt-C with the substitution G145R located within the second loop of HBsAg extracellular domain, were also examined. Interestingly, immunoprecipitation assays revealed that HB0116 bound to HBsAg of HBV gt-C and gt-A, but not to G145R, whereas HB0478 could bind to all three proteins (Fig. 1B).

Next, the HBV-neutralizing activity of these mAbs was evaluated using HepaRG cells, which support HBV infection, by inoculating them with a high dose of HBV. Fig. 2 shows that HB0116 suppressed the increase of HBV DNA after inoculation of both HBV gt-C and gt-A, but could not inhibit infection by G145R. However, HB0478 could prevent infection by HBV gt-C, gt-A, and also G145R. These results are consistent with the immunoprecipitation results shown in Fig. 1B and indicate that HB0478 can bind to the first loop, regardless of genotype, and also bind to the G145R substituted protein, which is seen as an antibody escape variant in clinical practice.

HB0116 and HB0478 protect against HBV gt-C and gt-A infections but only HB0478 protects against G145R mutant infection in vivo

The in vivo neutralizing activity of the mAbs was investigated using chimeric mice with human hepatocytes. After 1×10^4 copies of HBV gt-C or gt-A were incubated with HB0116 and/or HB0478, the mixtures were injected intravenously into naïve chimeric mice and serum HBV DNA concentrations were measured for the evaluation of HBV infection at five weeks after injection. Although HBV gt-C infection was confirmed in the control experiment (Group 1, 9.8×10^3 and 1.1×10^4 copies/ml) (Table 1), preincubation of the inoculum with either 1 μg or 10 μg of HB0116 or HB0478 completely blocked HBV infection with both gt-C and gt-A (Groups 2–5 for gt-C, Groups 6–9 for gt-A). Meanwhile, inoculation of the HBV G145R strain into naïve chimeric mice resulted in the establishment of infection (Group 10, 1.0×10^4 and 1.4×10^4 copies/ml) and incubation with 10 μg of HB0116 had no impact on infection by G145R (Group 11, 1.1×10^4–4.4×10^4 copies/ml), whereas as little as 1 μg of HB0478 completely blocked G145R infection (Groups 12 and 15). Apparently, a combination of HB0116 and

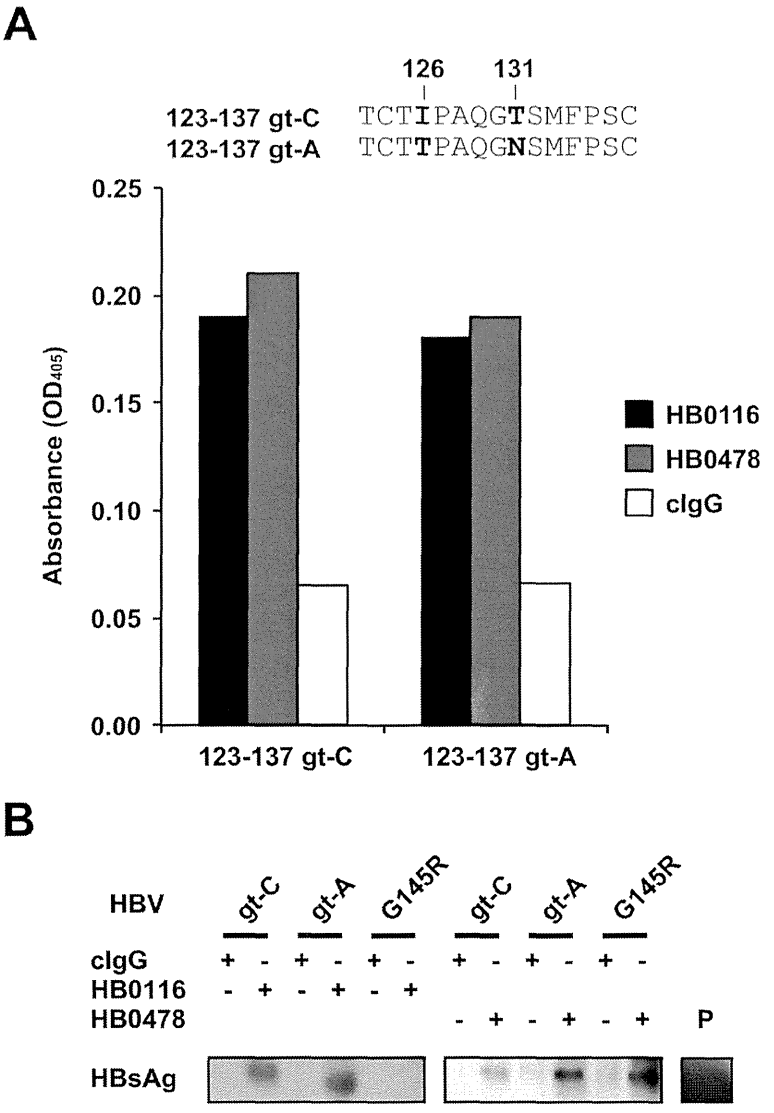

Fig 1. Binding capacity of mAbs HB0116 and HB0478 against with gt-C and gt-A HBsAg and the G145R variant. (A) Binding of mAbs HB0116 and HB0478 to synthetic peptides covering the first external loop of small-HBsAg was demonstrated by ELISA. The sequences of the recombinant peptides used in the analysis are shown above: amino acids which vary between genotype C (gt-C) and genotype A (gt-A) are indicated in bold. The absorbance at 405 nm is shown on the Y axis. Average data of three independent experiments are shown. **(B)** The gt-C, gt-A, and G145R virions were immunoprecipitated with HB0116 or HB0478 and HBsAg in the precipitates was detected by Western blotting. Recombinant HBsAg protein was used as the positive control (P lane). Representative data of three independent experiments are shown.

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HB0478, either at 1 μg or 10 μg protected the chimeric mice from HBV infection (Groups 13 and 14).

Evaluation of PHHs isolated from chimeric mice with human hepatocytes as an in vitro HBV infection model

PHHs isolated from the chimeric mice with human hepatocytes were used to characterize further the neutralizing activity of mAb HB0478. In vitro HBV infection of the PHHs was

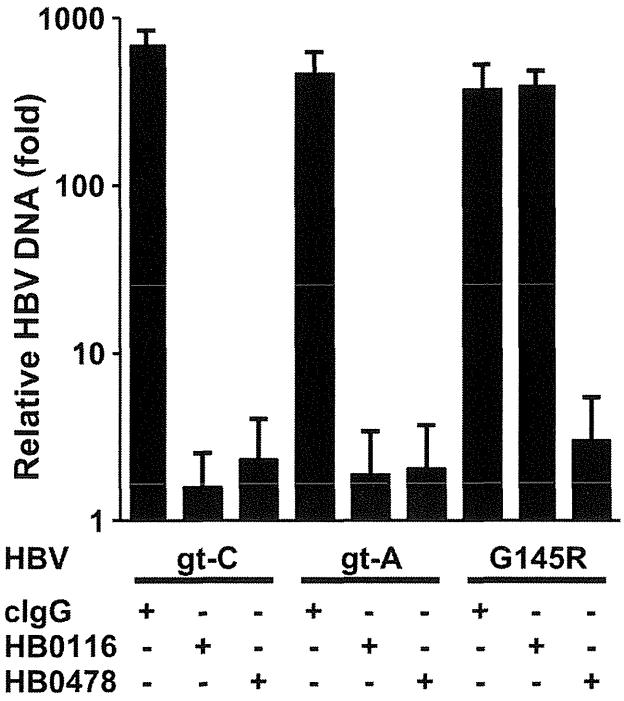

Fig 2. Relative HBV DNA concentrations in the total DNA extracted from HepaRG cells at 7 days after HBV infection. The Y-axis depicts the relative HBV DNA concentrations in the cells, with the concentrations on day 1 set at 1. Mean ± SD of three independent experiments are shown. cIgG, control human monoclonal IgG.

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confirmed by inoculating HBV gt-C at 5 HBV genomes per cell in the presence of 4% PEG 8000. The levels of pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA, and extracellular HBsAg were monitored and it was found that all these viral products gradually increased from 3 to 12 dpi (Fig. 3A). Southern blot analysis of cell lysates revealed the presence of single-stranded HBV DNA as a replication intermediate in the infected PHHs, confirming HBV replication in the cells (Fig. 3B). Furthermore, culture supernatants from HBV-infected donor PHHs were inoculated into newly prepared PHHs. An increase of HBsAg production from the PHHs was observed following exposure of the cells to another culture supernatant containing HBV DNA (Fig. 3C), indicating that the donor PHHs produced infectious HBV virions (also known as Dane particles).

Next, to investigate whether this model can be adapted for the study of neutralizing activities against HBV infection, the effect of HBIG on HBV infection was evaluated in vitro. Fig. 3D shows that HBIG strongly reduced HBV infection but residual infection was detected in the presence of PEG, whereas, in the absence of PEG, the HBV infection was completely blocked by HBIG. These results indicate that, when neutralizing activities against HBV infection were investigated using this PHH system, inoculation without PEG is appropriate for the specificity of the establishment of HBV infection. However, because inoculation without PEG would be less efficient for HBV infection, the efficacy of HBV infection in the absence of PEG was also examined. Various titers of HBV (10, 3, 1, and 0.3 genomes per cell) were inoculated into PHHs and the HBsAg titers in the supernatants were monitored for 22 days (Fig. 3E). Although the HBsAg levels from PHHs infected without PEG were lower than those with

Table 1. In vivo neutralization of HBV infection by monoclonal antibodies (mAbs).

HBV genotype	Group	HB0116 (μg/body)	HB0478 (μg/body)	HBV DNA (copies/mL)
C	Group 1	-	-	9.8×10^3
	(n = 2)	-	-	1.1×10^4
	Group 2	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 3	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 4	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 5	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
A	Group 6	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 7	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 8	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 9	-	10	n.d.
	(n = 2)	-	10	n.d.
G145R	Group 10	-	-	1.4×10^4
	(n = 2)	-	-	1.0×10^4
	Group 11	10	-	4.4×10^4
	(n = 3)	10	-	1.1×10^4
		10	-	3.3×10^4
	Group 12	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
	Group 13	1	1	n.d.
	(n = 2)	1	1	n.d.
	Group 14	10	10	n.d.
	(n = 2)	10	10	n.d.
	Group 15	-	1	n.d.
	(n = 2)	-	1	n.d.

n.d.: not detected.

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PEG, the HBsAg levels in the supernatants were well correlated with the initial input of HBV (10 to 0.3 genomes per cells) in the absence of PEG. These results suggest that, albeit with somewhat lower infectivity, inoculation without PEG is available for neutralization assays using the PHH system.