Table 3. Quasispecies Complexity and Diversity Among Patients With an Optimal or Partial Response to Entecavir

Variable	Optimal Responders (n = 34)	Partial Responders (n = 63)	*P*
Complexity-normalized Shannon Entropy			
Nucleotide level	0.9673 (0.6868–1)	0.9316 (0.3324–1)	.036
Amino acid level	0.8668 (0.4930–1)	0.7869 (0.2192–1)	.087
Diversity			
d (nucleotide level; 10^{-3} substitutions)	8.7083 (3.2747–36.4403)	5.2904 (0.8906–56.1354)	.019
d (amino acid level; 10^{-3} substitutions)	14.0147 (3.3561–66.2545)	8.6151 (0.8923–94.822)	.032
dS (10^{-3} substitutions/site)	12.4076 (3.8811–42.0139)	7.4706 (0.6533–78.0112)	.015
dN (10^{-3} substitutions/site)	6.6121 (1.6396–30.8776)	3.9649 (0.4040–43.8353)	.039

Abbreviations: d, mean genetic distance; dN, no. of nonsynonymous substitutions per nonsynonymous site; dS, no. of synonymous substitutions per synonymous site.

DNA, while HBV DNA became undetectable in the majority of patients (41/55 [75%]). rt124N variant was found in 9 of 14 patients (64%) with a detectable year 3 HBV DNA level and in 18 of 41 patients (44%) with an undetectable year 3 HBV DNA level ($P = .314$).

We further investigated the role of rt124N in patients who tested positive for HBeAg and had a high HBV DNA level at baseline, the 2 risk factors for suboptimal responses at year 1 that were identified in the present study, and also for a slower 3-year entecavir response, which was identified by a previous study [25]. Among the 55 suboptimal responders with year 3 HBV DNA data available, 32 (58%) were HBeAg positive and had an HBV DNA level of > 8 logs at baseline. Of these 32 HBeAg-positive patients with a high baseline HBV DNA level, 9 (28%) and 23 (72%) had detectable and undetectable HBV DNA at year 3, respectively. The proportion of patients with rt124N was higher in those with detectable year 3 HBV DNA (7/9 [78%]) than in those with undetectable year 3 HBV DNA (8/23 [35%]; $P = .049$).

DISCUSSION

The present study showed that 21% of patients still had detectable HBV DNA after 1 year of therapy, which is comparable to the detectability rate of HBV DNA at year 1 in other studies [3, 5, 6, 25–27]. Although HBV DNA may become undetectable in some partial responders upon long-term treatment, it has been shown that patients with detectable HBV DNA after 1 year of entecavir treatment have a lower probability of having undetectable HBV DNA at year 3 [3]. This partial treatment response also has a long-term clinical implication, as it has been demonstrated that patients with intermediate levels of HBV DNA still carry an increased risk of development of hepatocellular carcinoma and cirrhosis-related complications [28]. A recent study has also demonstrated that a posttreatment cutoff HBV DNA level of < 2000 IU/mL is not sufficient to indicate a reduced probability of disease progression, pinpointing the importance of complete HBV DNA suppression (to a level of <12 IU/mL) as the so-called optimal response [29]. Thus, investigating the molecular reasons for the slow/incomplete virological response to entecavir is of clinical importance. To examine this, studies with 2 important properties are required: (1) a large number of subjects for testing, because only 20%–30% of patients are expected to have suboptimal responses, and (2) a sensitive and robust viral sequence revelation of the whole genomic constitution of the HBV rt region. Our present study is specifically designed to solve these issues.

In the present study, HBV rt sequence analysis of 64 partial responders and 241 optimal responders showed that 4 variations (rt53N, rt118N, rt124N, and rt332S) were found to be present in a higher proportion in the partial responders. Detailed analyses showed that these 4 rt variants were also associated with other baseline parameters, such as HBV genotype and cirrhosis. In silico analysis showed that they were mostly associated with HBV genotype B, which was found to be more prevalent in the partial responders in the present study. However, logistic regression analysis showed that HBV genotype B was not an independent factor for partial response to entecavir. This is in accordance with the findings observed in other studies that the difference in the entecavir response has not been observed with different HBV genotypes in different studies [5, 6]. The prevalence of the 4 rt variants was also lower in patients with cirrhosis, which was also found to be associated with a better entecavir response in this present study. This is in accordance with a recent study showing that cirrhosis predicts early HBV DNA clearance upon entecavir therapy [30]. However, it should be noted that the patients with cirrhosis were generally HBeAg negative, had a lower HBV DNA level, and were older ($P = .013$, .002, and < .0001, respectively; data not shown), all of which were associated with a better entecavir response. Thus, a multivariate analysis was performed to identify the independent factors associated with a partial entecavir response.

Multivariate analysis showed that high baseline HBV DNA level, HBeAg positivity, and the rt variant rt124N were

associated with a partial entecavir response. This agrees with a previous study from our center, which found that patients who tested positive for HBeAg and had an HBV DNA level of ≥8 logs copies/mL (7.3 logs IU/mL) had a lower rate of HBV DNA undetectability at years 1–3 [25]. One novel finding of the present study is that the rt124N variant was significantly associated with a partial entecavir response at year 1. Furthermore, rt124N may also be associated with a higher chance of persistently detectable HBV DNA at year 3. Although the percentage of subjects with the rt124N variant was comparable between patients with detectable and those with undetectable HBV DNA at year 3 (64% vs 44%, respectively), it may be due to the limited number of subjects being tested. In particular, among the HBeAg-positive patients with a high baseline HBV DNA level, rt124N was significantly more frequently found in the patients who still had detectable HBV DNA at year 3. These data suggested that rt124N, although not the sole factor, is associated with a slower long-term response to entecavir, particularly when other coexisting adverse factors are present.

At the molecular level, the effect of rt124N on entecavir response was further studied by adopting our molecular docking simulation model. HBV rt124 is located in the rt fingers domain, but it is not inside the catalytic pocket within the palm domain of rt [17, 31]. Thus, it is less likely that rt124 variants will cause a remarkable conformational change to the entecavir binding site. Nevertheless, our molecular docking simulation model indicated that rt124N caused a slight interference with entecavir binding, suggesting that the rt124N may be slightly less susceptible to entecavir, without completely abolishing the binding. This may partly explain the slower response to entecavir in some patients who still achieve undetectable HBV DNA upon continuation of treatment beyond the first year. However, this modeling is based on the assumption that HBV rt forms a dimerized structure like that of HIV-1 rt. Should the crystal structure of HBV rt be available, the steric effect of HBV rt124N to entecavir binding will have to be confirmed with an HBV-based model. It should also be noted that the direct effect of rt124N should be best studied by an in vitro phenotypic assay. Nevertheless, this present study served as an initial identification of the HBV variants. An in vitro phenotypic study is required as a subsequent confirmation.

Another interesting finding from the clonal sequencing data is that the optimal responders had a higher quasispecies complexity and diversity than the partial responders. Studies of quasispecies complexity and diversity in NA-treated patients with chronic hepatitis B are rare. Our present finding differs from that in a recent study by Liu et al, which involved 31 entecavir recipients and showed that the baseline quasispecies complexity and diversity are comparable between the optimal and partial responders [21]. However, our study had a greater power because of the greater number of patients (n = 97) being studied.

The reason for the higher baseline quasispecies complexity and diversity in optimal responders in this present study is unclear. From the classical view of viral genetics, high quasispecies diversity would imply that the population has an increased possibility to harbor drug-resistant variants. However, according to the quasispecies theory, all variants within a viral population form an interacting network and react as a whole unit in response to stimulants such as antiviral therapy [19]. Under this quasispecies theory, a high quasispecies diversity may imply that the viral population reaches a critical status, termed self-organized criticality, in which the quasispecies network is maintained at an optimal capability [32]. At such a self-organized criticality status, the viral quasispecies population would be extremely sensitive to external perturbation [19, 33]. Therefore, a viral population with a high quasispecies diversity will be more prone to the external pressure exerted by entecavir. In accordance with the same line of thought, it has been demonstrated that higher baseline HCV quasispecies diversity and complexity are associated with an early virological response to interferon and ribavirin therapy [33]. It is also possible that, in the optimal responders with higher baseline complexity and diversity, the viral quasispecies were "less-fit" subpopulations coexisting in the total viral population with no single "good-fit" and replicatively competent dominant strain. Consequently, they are more susceptible to entecavir.

In conclusion, apart from the known factors of high baseline HBV DNA level and HBeAg positivity, a novel single nucleotide polymorphism, rt124N, was found to be a significant factor associated with partial entecavir response. In addition, lower baseline quasispecies complexity and diversity were also found to be associated with partial response to entecavir. These 4 factors may exert additive or synergistic influences on the suboptimal responsiveness to entecavir treatment.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Financial support. This work was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (grant 08070852).

Potential conflicts of interest. M.-F. Y. and C.-L. L. have received speakers bureau and/ or research grants from Bristol-Myers-Squibb, Gilead Sciences, LG Life Sciences, and Novartis. Y. T. has received speakers bureau and/ or research grants from Bristol-Myers-Squibb. J. F. has been an invited speaker for Bristol-Myers-Squibb. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Hepatology Research

Hepatology Research 2015; 45: 179–189 doi: 10.1111/hepr.12439

Review Article

Immunobiology of hepatitis B virus infection

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The adaptive immune response, particularly the virus-specific CD8⁺ T-cell response, is largely responsible for viral clearance and disease pathogenesis during hepatitis B virus (HBV) infection. The HBV-specific CD8⁺ T-cell response is vigorous, polyclonal and multispecific in acutely infected patients who successfully clear the virus and relatively weak and narrowly focused in chronically infected patients. The immunological basis for this dichotomy is unclear. A recent study using HBV transgenic mice and HBV-specific T-cell receptor transgenic mice suggests that intrahepatic antigen presentation by HBV positive hepatocytes suppresses HBV-specific CD8⁺ T-cell responses through a co-inhibitory molecule, programmed cell death 1 (PD-1). In contrast, antigen presentation by activated professional antigen-presenting cells induces functional differentiation of HBV-specific CD8⁺ T cells. These findings suggest that the outcome of T-cell priming is largely dependent on the nature of antigen-presenting cells. Another study suggests that the timing of HBV-specific CD4⁺ T-cell priming regulates the magnitude of the HBV-specific CD8⁺ T-cell response. Other factors that could regulate HBV-specific cellular immune responses are high viral loads, mutational epitope inactivation, T-cell receptor antagonism and infection of immunologically privileged tissues. However, these pathways become apparent only in the setting of an ineffective cellular immune response, which is therefore the fundamental underlying cause. Understanding the cellular and molecular mechanisms by which HBV evades host immune responses will eventually help develop new immunotherapeutic strategies designed to terminate chronic HBV infection.

Key words: hepatitis B virus, immune response, immunological priming, immunotherapy, T cells

INTRODUCTION

HEPATITIS B VIRUS (HBV) is a partially double-stranded DNA virus that causes necroinflammatory liver disease of variable severity.[1] More than 240 million people worldwide are persistently infected with HBV. Persistent infection by HBV is often associated with chronic liver disease that can lead to the development of cirrhosis and hepatocellular carcinoma (HCC). HBV is thought to be largely non-cytopathic for the infected hepatocyte under normal circumstances,[2,3] but could be cytopathic in immunodeficient chimeric mice carrying human hepatocytes,[4] suggesting that unmitigated HBV replication and gene expression trigger cellular stresses and cell death. The immune response mediates the clearance of HBV and disease pathogenesis.[2] Interestingly, HBV appears to be a poor inducer of innate immune responses, acting as a stealth virus in this regard by evading recognition by innate immune sensor molecules.[5] Despite the weak innate immune responses, acute HBV infection of adults is usually self-limited and cleared by functional HBV-specific CD8⁺ T-cell responses. The induction of functional HBV-specific CD8⁺ T-cell responses is dependent on early CD4⁺ T-cell priming prior to HBV spread,[6] which might explain why patients infected with HIV become persistently infected following horizontal transmission of HBV.[7,8] Once chronic HBV infection is established, HBV-specific CD8⁺ T cells are subjected to powerful immunoregulatory mechanisms in the liver. Ample evidence suggests that HBV actively suppresses adoptive immune responses, particularly HBV-specific CD8⁺ T-cell responses. Reduction of viral antigen expression by antiviral treatment

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Received 8 August 2014; revision 13 October 2014; accepted 14 October 2014.

can overcome CD8+ T-cell hyporesponsiveness in patients with chronic HBV infection, suggesting that T cells are present in these subjects but suppressed by high viral loads.[9] In addition, recent evidence suggests that antigen recognition in the liver suppresses HBV-specific CD8+ T-cell effector functions by signaling through a co-inhibitory molecule, programmed cell death 1 (PD-1).[10,11] Furthermore, several viral proteins have been shown to regulate the adaptive immune response to HBV,[12–14] which may explain the varying chronicity rate between genotypes that express different levels of viral antigens.[15,16] We herein review our current knowledge of the host immune responses to HBV as well as the cellular and molecular mechanisms by which HBV evades these immune responses. We also discuss how the accumulating knowledge of HBV immunobiology will help us to develop more inventive therapeutic strategies that are designed to reinvigorate HBV-specific immune responses, thereby terminating chronic infection with this deadly virus.

INNATE IMMUNE RESPONSE

Absence of an innate response by infected cells during acute HBV infection

VIRUS INFECTION USUALLY triggers a robust innate immune response that is heralded by rapid induction of interferon (IFN)-α/β by the infected cell.[17] Production of IFN-α/β induces the transcriptional expression of a large number of IFN-inducible genes (ISG), which in turn exert a variety of intracellular antiviral mechanisms.[17,18] Surprisingly, intrahepatic gene expression profiling in acutely HBV-infected chimpanzees revealed that HBV acts like a stealth virus early after infection because it does not induce any cellular gene expression, including ISG, as it spreads through the liver.[5] This contrasts strikingly with the induction of many ISG during the spread of hepatitis C virus that is highly visible to the innate immune system.[19] The relative invisibility of HBV to the innate sensing machinery of the cells probably reflects its replication strategy. HBV retains its transcriptional template, namely cccDNA, in the nucleus[20] and the HBV mRNA is capped and polyadenylated, resembling normal cellular transcripts.[21,22] In addition, HBV replicates within viral capsid particles in the cytoplasm[20] and is therefore shielded from innate sensing machinery. Thus, the typical widespread expansion of HBV in the liver may reflect the absence of IFN-α/β production to which the virus is exquisitely sensitive, as shown in HBV transgenic mice.[23,24]

Natural killer and natural killer T-cell responses during HBV infection

The role of natural killer (NK) cells in acute HBV infection remains largely unknown. Studies that analyzed NK-cell functions during acute HBV infection have provided conflicting evidence. A recent study demonstrated that the activation and cytokine-producing ability of NK cells were impaired in acute HBV patients,[25] while others showed enhanced IFN-γ production by, and cytotoxicity of, NK cells.[26,27] The cause of this discrepancy is unclear. On the other hand, NK cells in chronic HBV patients seem generally cytolytic but their cytokine-producing ability is selectively impaired.[28,29] A recent report demonstrated a strong correlation between liver disease, IFN-α production and NK-cell expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) during chronic HBV infection,[30] suggesting a pathogenic role of NK cells (Fig. 1, grey area). Interestingly, NK cells seem highly immunoregulatory as well, because they can apparently delete HBV-specific, but not cytomegalovirus-specific, CD8+ T cells through TRAIL–TRAIL receptor 2 interaction.[31] Taken together, NK cells appear to exert a detrimental effect on the host during chronic HBV infection. The precise role of NK cells during chronic HBV infection must be fully elucidated to develop new NK-cell-based therapeutic approaches against chronic HBV infections. The role of natural killer T (NKT) cells during natural HBV infection is even less clear. The therapeutic potential of NKT-cell activation against HBV infection was initially indicated in a HBV transgenic mice model,[32] and subsequent studies with HBV and woodchuck hepatitis virus suggested that NKT cells were activated soon after infection.[33,34] However, the degree of NKT-cell activation was relatively modest in both cases, and the physiological importance of such activation has not been addressed in these studies. Studies with several mouse models provided more convincing evidence that CD1d-restricted NKT cells are important for the control of HBV replication and gene expression.[35,36] It is well known, however, that hepatic NKT-cell repertoires are phenotypically and functionally very different between humans and mice.[37] Further studies are required to confirm the role of NKT cells during HBV infections.

ADOPTIVE IMMUNE RESPONSE

Antibody response

ANTIBODIES TO THE hepatitis B surface antigen (HBsAg) neutralize HBV infection presumably by preventing virus attachment to hepatocytes. They also

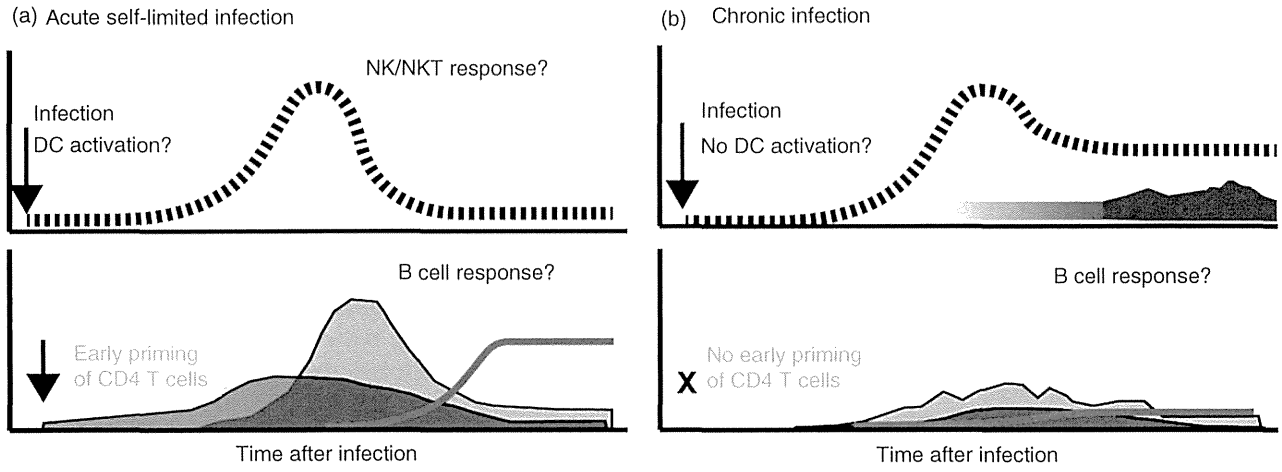

Figure 1 Schematic representation of immune responses during acute and chronic hepatitis B virus (HBV) infections. (a,b) Schematic representation of the time course of serum HBV titer and immune responses during acute and chronic HBV infections. The upper panel in each figure represents the timing of HBV infection as an arrow, the serum HBV DNA as a dashed black line, and the natural killer (NK) cell response as gray area. The lower panel in each figure represents the timing of CD4 T-cell priming to HBV as an arrow. the CD4 T-cell response as red area, the CD8 T-cell response as green area, the blue line as the antibody response. ▮▮▮▮, HBV titer; ▭, NK response; ▭, CD4 T-cell response; ▭, CD8 T-cell response; ——, anti-HBs. DC, dendritic cells; NKT, natural killer T cell.

facilitate viral clearance by complexing with free viral particles and removing them from the circulation. The induction of anti-hepatitis B surface (HBs) by immunization is a T-cell-dependent process and requires $CD4^+$ T-cell help.[38] While anti-HBs neutralizing antibodies are essential for providing protection after vaccination, their importance in resolving natural HBV infection is somewhat underappreciated, mostly because the appearance of anti-HBs occurs relatively late after HBV exposure (Fig. 1, blue lines).[39] It is generally believed that anti-HBs neutralizing antibodies prevent viral spread from the rare cells that remain infected, even after resolution of HBV infection. Supporting this notion, HBV is frequently reactivated long after resolution of infection in patients who receive conventional chemotherapy or a newly developed monoclonal antibody (rituximab) therapy that targets the CD20 antigen expressed on the surface of normal and malignant B lymphocytes.[40] More detailed analysis of HBV-specific B-cell responses may reveal a heretofore unappreciated role of humoral responses in the control of HBV infection.

$CD4^+$ T-cell response

Ample evidence suggests a clear relationship between the $CD4^+$ T-cell response to HBV and the outcome of HBV infection. For example, the peripheral blood $CD4^+$ T-cell response to HBV is vigorous and multispecific in patients with acute hepatitis who ultimately clear the virus, while it is relatively weak in chronic HBV patients (Fig. 1, red areas).[39,41,42] Despite the association between a strong $CD4^+$ T-cell response and viral clearance, $CD4^+$ T-cell depletion at the peak of HBV infection had no effect on viral replication and liver disease in infected chimpanzees.[43] In contrast, $CD4^+$ T-cell depletion before HBV infection resulted in quantitatively and qualitatively reduced HBV-specific $CD8^+$ T-cell responses, leading to persistent HBV infection.[6] These results suggest that $CD4^+$ T cells probably contribute indirectly to the control of HBV infection by facilitating the induction and maintenance of the virus-specific $CD8^+$ T-cell responses. It is likely that $CD4^+$ T cells help induce functional HBV-specific $CD8^+$ T-cell responses by producing interleukin (IL)-2 that is essential for T-cell proliferation and by activating professional antigen-presenting cells (pAPC) that are capable of providing co-stimulation to T cells.[44] In contrast, in the absence of early $CD4^+$ T-cell responses, $CD8^+$ T-cell priming could occur in the liver, resulting in T-cell inactivation, tolerance or apoptosis,[11,45,46] as summarized later in this review. This requirement of $CD4^+$ T cells for the induction of HBV-specific $CD8^+$ T cells is one of the reasons patients infected with HIV (low CD4 count) become persistently infected after horizontal transmission of HBV[7,8] and why genetic variants in the human leukocyte antigen DP locus are associated with the risk of developing chronic HBV infection.[47] Interestingly, early $CD4^+$

T-cell priming does not occur when the size of inoculum is small (i.e. 1–10 copies) and the appearance of HBV DNA is delayed after infection,[6] suggesting that slow spread of HBV may facilitate chronicity, as often observed with genotype A infections.[15,16] Interestingly, recent studies indicated that the frequency of IL-17-producing CD4 T cells (T-helper [Th]17 cells) is increased in chronic HBV patients,[48] and IL-17 and another Th17-related cytokine IL-22 are shown to exacerbate liver disease.[49] These results suggest that CD4 T cells not only help induce antiviral cellular immune responses during acute HBV infections, but also mediate inflammatory responses during chronic HBV infection.

CD8⁺ T-cell response

The HBV-specific CD8⁺ T-cell response plays a fundamental role in viral clearance and liver disease. A vigorous polyclonal CD8⁺ T-cell response is readily detectable in the peripheral blood of patients with acute hepatitis B who ultimately clear the virus. In contrast, the peripheral blood T-cell response in chronically infected patients is weak and narrowly focused (Fig. 1, green areas).[2,50] The livers of these patients contain virus-specific T cells that likely contribute to disease pathogenesis but, for functional and/or quantitative reasons, are unable to clear the infection. Interestingly, a recent study that examined the relationship between the number of intrahepatic HBV-specific CD8⁺ T cells, extent of liver disease and levels of HBV replication in chronically infected patients indicated that inhibition of virus replication could be independent of liver damage and that the functionality of HBV-specific CD8⁺ T cells was more important than the number of T cells in controlling HBV replication.[51] Experiments in chimpanzees have shown that the viral clearance and the onset of liver disease coincide with the accumulation of virus-specific CD8⁺ T cells and the induction of IFN-γ and ISG in the liver.[52] Importantly, depletion of CD8⁺ T cells at the peak of viremia delays viral clearance and the onset of viral hepatitis until the T cells return, providing the most definitive evidence that viral clearance and liver disease are mediated by virus-specific CD8⁺ T cells.[43]

NON-CYTOLYTIC AND CYTOLYTIC T-CELL EFFECTOR FUNCTIONS EMERGE ALTERNATELY DURING HBV CLEARANCE

IT IS WIDELY believed that virus-specific CD8⁺ T cells clear the viral infection by killing infected cells. However, CD8⁺ T-cell killing is an inefficient process, requiring direct physical contact between the CD8⁺ T cells and the infected cells. Therefore, it may not be possible for HBV-specific CD8⁺ T cells to kill all the HBV infected cells if the CD8⁺ T cells are greatly outnumbered, as occurs during HBV infection in which as many as 10^{11} hepatocytes can be infected.[53] Hence, although the liver disease in HBV infection is clearly due to the cytopathic activity of the CD8⁺ T cells, viral clearance may require more efficient CD8⁺ T-cell functions than killing. Important insights into the pathogenic and non-cytopathic antiviral functions of the CD8⁺ T-cell response have come from studies of HBV transgenic mice that develop an acute necroinflammatory liver disease after adoptive transfer of HBV-specific effector or effector memory CD8⁺ T cells that were generated by immunizing non-transgenic mice.[10,54] In that model, the HBV-specific CD8⁺ T cells rapidly enter the liver and recognize viral antigen and secrete IFN-γ, which non-cytopathically inhibits HBV replication in the remaining hepatocytes[54] by preventing the assembly of HBV RNA-containing capsids in the cytoplasm[23,55] in a proteasome-[56] and kinase-dependent[57] process. During this remarkable process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes, yet the hepatocytes remain perfectly healthy.[54] Antibody blocking and knockout experiments in the HBV transgenic mouse model further demonstrated that the cytopathic and antiviral functions of CD8⁺ T cells are completely independent of each other.[58,59] Following the secretion of IFN-γ and the suppression of HBV replication, HBV-specific CD8⁺ T cells expand vigorously *in situ*. Surprisingly, however, the ability of HBV-specific CD8⁺ T cells to produce IFN-γ is quickly downregulated as they expand. Despite the suppression of IFN-γ secretion, the cytotoxic capabilities of the HBV-specific CD8⁺ T cells increase over time, causing necroinflammatory liver disease and complete inhibition of HBV mRNA expression.[10] Taken together, these results suggest that, in the context of a vigorous immune response during acute HBV infection, the sequential and altering patterns of non-cytolytic and cytolytic effector function, coupled with T-cell expansion and contraction, cooperate to minimize the extent of tissue injury required for viral clearance. Conversely, we suggest that diminution of the CD8⁺ T-cell response or poor coordination of these responses could lead to viral persistence and varying degrees of chronic liver disease.

WHERE, HOW AND WHAT KIND OF HBV-SPECIFIC CD8⁺ T CELLS ARE GENERATED?

THE RESULTS DESCRIBED above were generated by adoptively transferring splenocytes from

HBV-immunized mice to HBV transgenic mice. While these results suggest that intrahepatic antigen recognition has a profound impact on the distribution, expansion and effector functions of primed effector-memory $CD8^+$ T cells, they may not reflect the events that occur when a naïve individual becomes infected with HBV, because immunologically naïve T cells are known to behave very differently than memory T cells.[60–62] Accordingly, we generated transgenic mice whose $CD8^+$ T cells express T-cell receptors (TCR) specific for HBV, to study where and how immunologically naïve T cells are primed in response to intrahepatic HBV.

When HBV-specific naïve $CD8^+$ T cells were adoptively transferred into HBV transgenic mice, they were predominantly activated rapidly in the liver, independent of T-cell homing to lymphoid organs, suggesting that HBV-specific native T cells are primed in the liver.[11] While this intrahepatic priming of HBV-specific $CD8^+$ T cells challenges the current T-cell priming model postulating that naïve T cells are primed by activated pAPC in lymphoid organs,[63,64] several studies previously indicated that the liver is an exception to this rule.[45,46,65] This rather unusual ability of the liver to prime naïve T cells presumably reflects the unique architecture of the hepatic sinusoid, which is characterized by a discontinuous endothelium, the absence of a basement membrane and a very slow flow rate,[66] allowing circulating T cells to make prolonged direct contact with resident liver cells, including hepatocytes. Furthermore, the liver is replete with diverse and unique antigen-presenting cell populations, including liver sinusoidal endothelial cells (LSEC), hepatic stellate cells, Kupffer cells, and conventional and plasmacytoid dendritic cells (DC), all of which are capable of priming and/or tolerizing naïve T cells.[67] In our model, HBV-specific $CD8^+$ T cells are presumably primed by HBV-expressing hepatocytes, because HBV-specific $CD8^+$ T cells were activated when co-cultured with hepatocytes isolated from HBV transgenic mice but not with LSEC, liver residential DC or Kupffer cells. Importantly, the intrahepatically primed HBV-specific $CD8^+$ T cells expand vigorously in the liver but are functionally impaired because they do not express IFN-γ or the cytolytic granule granzyme B, suggesting that intrahepatic priming by HBV-expressing hepatocytes expands functionally defective $CD8^+$ T cells. Consequently, such intrahepatically primed T cells are incapable of suppressing HBV gene expression or inducing liver disease.[11] Interestingly, functionally defective HBV-specific $CD8^+$ T cells expressed a co-inhibitory molecule, PD-1, after antigen recognition in the liver.[11] Furthermore, a significant fraction of PD-1-deficient HBV-specific naïve T cells expanded more vigorously than PD-1 positive controls, and differentiated into effector T cells capable of producing IFN-γ, as well as granzyme B, which in turn suppressed HBV gene expression and caused liver disease.[11] Collectively, these results suggest that PD-1 signaling impairs the effector functions of HBV-specific $CD8^+$ T cells. Importantly, PD-1 expression was also observed on HBV-specific $CD8^+$ T cells in chronic HBV patients,[68] indicating that PD-1-mediated functional suppression is active during natural HBV infection. Taken together, these results suggest that intrahepatic priming induces T-cell tolerance and such tolerance is at least partially mediated by PD-1 signaling. This PD-1-meditated intrahepatic suppression may be the underlying cause of the weak T-cell responsiveness during chronic HBV infection.

OTHER MECHANISMS OF HBV PERSISTENCE

THE POTENTIAL MECHANISMS by which HBV evades the immune responses are listed in Table 1. Neonatal tolerance to HBV is probably responsible for viral persistence following mother–infant transmission.[69,70] The underlying cause of adult-onset chronic HBV infection is probably multifaceted. Potential contributing factors include mutational escape leading to inactivation of B- and T-cell epitopes and specific inhibition of the adaptive immune response by viral proteins.[71] For example, hepatitis B e-antigen (HBeAg) has

Table 1 Causes of HBV persistence

Potential mechanisms of HBV persistence
• Neonatal tolerance
• Peripheral deletion/exhaustion (due to high viral load and negative signaling)
• Anergy
• Ignorance
• Weak CD4 T-cell response (associated with low viral titer and HIV infection)
• Reduced dendritic cell numbers and functions
• Negative signaling (PD-1/CTLA-4/Tim-3)
• Immunosuppressive microenvironment (IL-10, TGF-β)
• Regulatory T cells/myeloid-derived suppressor cells
• Escape mutations in T- and B-cell epitopes
• Inhibit antigen processing or presentation
• Inhibit cytokine signaling

CTLA-4, cytotoxic T-lymphocyte antigen-4; HBV, hepatitis B virus; IL, interleukin; PD-1, programmed cell death 1; Tim-3, T-cell immunoglobulin and mucin 3; TGF, transforming growth factor.

been shown to suppress the antibody and T-cell response to hepatitis B core antigen in T-cell receptor transgenic mice.[12,13] The immunosuppressive potential of HBeAg may explain the clinical observation that viral mutations precluding the production of HBeAg are often associated with exacerbations of liver disease and, sometimes, even with viral clearance from chronically infected patients.[72,73] HBsAg may also prevent immune elimination of infected cells by functioning as a high-dose tolerogen, because extremely high serum HBsAg titers in the mg/mL range are often seen in chronically infected patients[74,75] and chronically infected patients have an absence or subnormal levels of HBsAg-specific $CD8^+$ T cells.[75] In addition, HBV X protein, a transcriptional transactivator that is required for the initiation of infection,[76,77] can inhibit cellular proteasome activity when it is overexpressed[78] and may interfere with antigen processing and presentation. Finally, genotypic variation clearly influences the outcome of HBV infection. Up to 10% of adult-onset infections with genotype A become persistent, while less than 5% of infections with other genotypes do so.[15] The precise mechanism for the difference is unclear, but the high levels of HBsAg expression and low replication activity of genotype A infections may facilitate immune evasion.[16]

In both neonate and adult onset of chronic HBV infections, weak CD4 and CD8 T-cell responses are clearly responsible for the viral persistence. The mechanism that suppresses HBV-specific immune responses during chronic HBV infection is likely multifactorial. In addition to PD-1, other negative signaling molecules, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and T-cell immunoglobulin and mucin 3 (Tim-3), are expressed on functionally compromised HBV-specific CD8 T cells in chronic HBV patients.[79] For example, a fraction of CD8 T cells in patients with chronic hepatitis B express the co-inhibitory molecule CTLA-4, and its expression level is correlated with serum HBV DNA titer and pro-apoptotic protein Bim expression. Blockade of CTLA-4 increases the expansion of IFN-γ producing HBV-specific CD8 T cells *in vitro*. Similarly, Tim-3 is also highly expressed on functionally impaired HBV-specific CD8 T cells in chronic HBV patients, and the blockade of Tim-3 *in vitro* restored effector functions of the CD8 T cells,[79] suggesting that multiple layers of negative co-regulation contribute to T-cell exhaustion in chronic HBV infection. Interestingly, Tim-3-expressing CD8 T cells appear to have a immunosuppressive function similar to regulatory T cells (Treg), limiting proliferation of antigen-specific effector T cells *in vitro* and *in vivo*.[80] Extrinsic factors could also contribute to the reduced T-cell responses during chronic HBV infection. The livers of chronic HBV patients have been shown to express elevated levels of IL-10,[81] transforming growth factor-β[82] and arginase,[83] all of which are known to suppress cellular immune responses. These immunosuppressive soluble factors may be produced by regulatory cells, as the frequency of Treg is increased in chronic HBV patients.[84] Interestingly, recently identified myeloid-derived suppressor cells have been shown to induce T-cell tolerances in mouse models of HBV infection.[85,86] In addition, several studies suggest that the number and functionality of DC are reduced in chronic HBV patients compared with healthy donors, and the reduction of HBV DNA titer by a nucleotide analog adefovir dipivoxil significantly restored the number and functionality of DC. Caution should be exercised, however, to interpret the physiological relevance of the antigen non-specific immunosuppression during chronic HBV infections, because chronic HBV patients are no more susceptible than healthy people to other infectious agents such as influenza virus.

NEW IMMUNOTHERAPEUTIC STRATEGIES TO CURE CHRONIC HBV INFECTIONS

THE MANAGEMENT OF chronic HBV has improved significantly in the last decade, mainly because of the development of very effective and safe nucleoside analogs (NA) that primarily inhibit reverse transcription and DNA replication.[87] Although NA strongly inhibit HBV replication and have much fewer side-effects than IFN-based therapies, they usually do not achieve sustained viral suppression. Failure to sustain viral suppression by NA presumably reflects the persistence of cccDNA, which is untouched by reverse transcriptase inhibitors. Because the elimination of cccDNA usually requires the turnover of infected hepatocytes, the induction of functional HBV-specific $CD8^+$ T cells that can specifically kill virus-infected hepatocytes has been deemed the most promising approach to cure chronic HBV infections. Immunization of chronically infected patients is the most straightforward approach to boost HBV-specific $CD8^+$ T-cell responses. Several strategies have been tested in clinical trials with disappointing results,[88] indicating that more inventive approaches are required to induce functional HBV-specific $CD8^+$ T-cell responses. There are three approaches that could effectively reinvigorate HBV-specific $CD8^+$ T-cell responses (Table 2).

The first approach is to block signaling pathways that negatively regulate HBV-specific $CD8^+$ T-cell responses.

Table 2 Immunotherapeutic strategies to terminate chronic HBV infection

Strategies to reinvigorate T-cell responses
1. Block negative signaling pathways (e.g. PD-1, CTLA-4, Tim-3)
2. Stimulate pAPC (e.g. anti-CD40, TLR) and cytokine signaling
3. Redirect peripheral T cells with high avidity TCR

CTLA-4, cytotoxic T-lymphocyte antigen-4; HBV, hepatitis B virus; pAPC, professional antigen-presenting cells; PD-1, programmed cell death 1; TCR, T-cell receptor; Tim-3, T-cell immunoglobulin and mucin 3; TLR, Toll-like receptor.

As described in "Where, How and What Kind of HBV-Specific CD8+ T Cells are Generated", we showed that blockade of PD-1 signaling induced functional differentiation of intrahepatically primed, HBV-specific CD8 T cells in a HBV transgenic mouse model, indicating that PD-1 could be a new therapeutic target to treat chronic HBV infection. HBV-specific CD8 T-cell responses may be restored by blocking other negative signaling molecules, such as CTLA-4, Tim-3 and arginase, as well as by depleting Treg. More evidence is needed to support this approach.

The second approach is to provide extra stimulus to override negative signaling that suppresses effector functions of HBV-specific CD8 T cells. One of the promising approaches is to activate pAPC, particularly DC. Activated DC are capable of providing the second and third signals necessary for the functional differentiation of HBV-specific $CD8^+$ T cells. Indeed, we showed that activation of myeloid DC (mDC) with an agonistic anti-CD40 antibody (αCD40) restored HBV-specific $CD8^+$ T-cell responses that are otherwise suppressed by PD-1 signaling.[11] Interestingly, PD-1 expression on the functional HBV-specific $CD8^+$ T cells is strongly downregulated in αCD40-treated HBV transgenic mice. These results suggest that activation of mDC directly or indirectly suppresses PD-1 expression, thereby rescuing HBV-specific $CD8^+$ T cells from PD-1-mediated functional suppression. The data also illustrate the therapeutic potential of mDC activation by αCD40 for the treatment of chronic HBV infection. Other signaling pathways that stimulate pAPC, such as Toll-like receptor signaling pathways, should be tested for their ability to restore HBV-specific $CD8^+$ T-cell responses. In addition, cytokines required for T-cell expansion and differentiation, particularly common γ-chain cytokines such as IL-2, IL-4, IL-7, IL-15 and IL-21, could serve as effective adjuvants for a therapeutic vaccine against chronic HBV infections.

The third approach is to genetically engineer peripheral blood T cells from chronic HBV patients to express TCR specific for HBV-derived epitopes. The first and second strategies described above require the presence of HBV-specific $CD8^+$ T cells that can efficiently recognize HBV-derived T-cell epitopes presented on infected hepatocytes. However, such T cells are often deleted in chronic HBV patients after years of exposure to HBV antigens. To circumvent this problem, genes encoding TCR that efficiently recognize HBV-derived epitopes can be introduced into the patient's peripheral blood T cells using retroviral or lentiviral vectors. These TCR redirected autologous T cells could be subsequently transferred into the chronic HBV patient. Such approaches are employed in treating cancer patients[89,90] and the results so far have been very encouraging. In addition, T cells with redirected specificity toward the HBV envelope protein have been shown to recognize and lyse HCC cell lines with natural HBV DNA integration in *in vitro* and animal models.[91] Safety concerns, cost-effectiveness, and ethical issues should be fully addressed before this promising approach is adapted to chronic HBV patients.

SUMMARY AND CONCLUSIONS

IN SUMMARY, HBV acts like a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response several weeks later. The relative invisibility of HBV to the innate sensing machinery of the cells probably reflects its replication strategy, with the replicating viral genome being sheltered within viral capsid particles in the cytoplasm. On the other hand, HBV can be controlled when properly activated HBV-specific $CD8^+$ T cells enter the liver, recognize antigens, kill infected cells and secrete IFN-γ, which triggers a broad-based cascade that amplifies the inflammatory process and has non-cytopathic antiviral activity against HBV. However, HBV-specific $CD8^+$ T-cell responses are subjected to a powerful immunoregulatory mechanism in the liver and the induction of an effective HBV-specific $CD8^+$ T-cell response is dependent on activation of pAPC. Failure to activate pAPC induces functionally impaired $CD8^+$ T-cell responses due to intrahepatic priming by HBV-expressing hepatocytes, leading to persistent infection (Fig. 2). The precise mechanism by which pAPC are activated during HBV infection remains to be elucidated but HBV-specific $CD4^+$ T cells probably play a critical role. The functional impairment is mediated, at least partially, by PD-1 signaling but other co-inhibitory molecules are likely to be

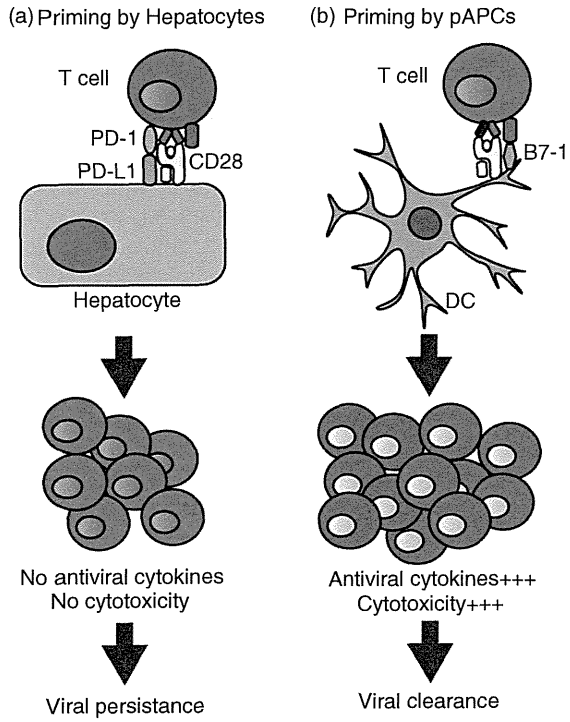

Figure 2 Outcome of T-cell priming is determined by the antigen-presenting cell population. (a) Hepatitis B virus (HBV)-specific naive CD8 T cells that are primed by HBV positive hepatocytes receive co-inhibitory signals rather than co-stimulatory signals, resulting in the expansion of functionally impaired CD8 T cells. (b) T-cell priming by professional antigen-presenting cells (pAPC), such as dendritic cells (DC), that can deliver co-stimulatory signals is presumably required to induce functional HBV-specific CD8 T-cell responses.

involved as well. Thus, activating pAPC and suppressing PD-1 and other negative signaling pathways may have therapeutic potential to treat chronic HBV patients. Adoptive transfer of genetically engineered T cells with redirected specificity towards HBV may represent a powerful immune strategy to cure chronic HBV infection, but safety, cost and ethical issues should be fully addressed before clinical trials.

ACKNOWLEDGMENTS

THIS WORK WAS supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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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).

Received 3 October 2014 Returned for modification 3 November 2014
Accepted 8 December 2014
Accepted manuscript posted online 15 December 2014
Citation Watanabe T, Hamada-Tsutsumi S, Yokomaku Y, Imamura J, Sugiura W, Tanaka Y. 2015. Postexposure prophylactic effect of hepatitis B virus (HBV)-active antiretroviral therapy against HBV infection. Antimicrob Agents Chemother 59:1292–1298. doi:10.1128/AAC.04459-14.
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doi:10.1128/AAC.04459-14

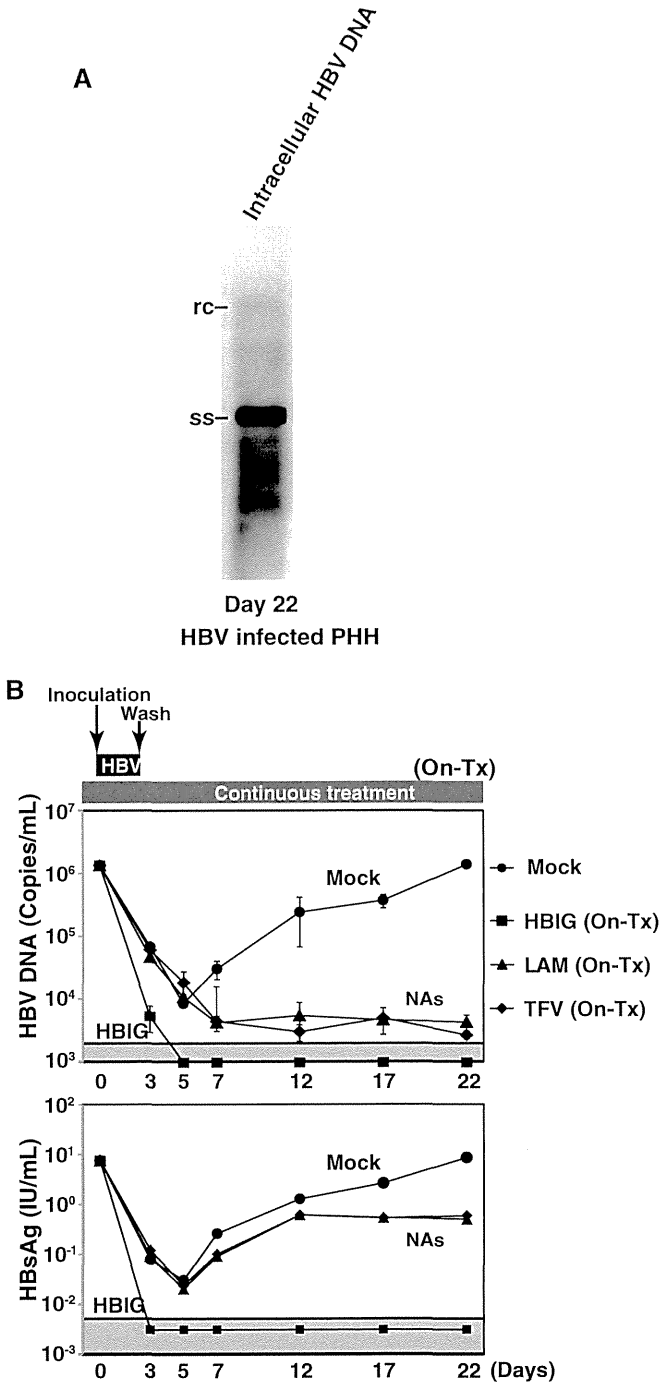

FIG 1 Incomplete prevention of primary HBV infection by NA. (A) Freshly isolated PHH susceptible to HBV infection. Single-stranded HBV DNA (ssDNA), a replicative intermediate in PHH on day 22 postinfection, was visualized by Southern blotting. rcDNA, relaxed-circular, partially double-stranded HBV DNA. 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

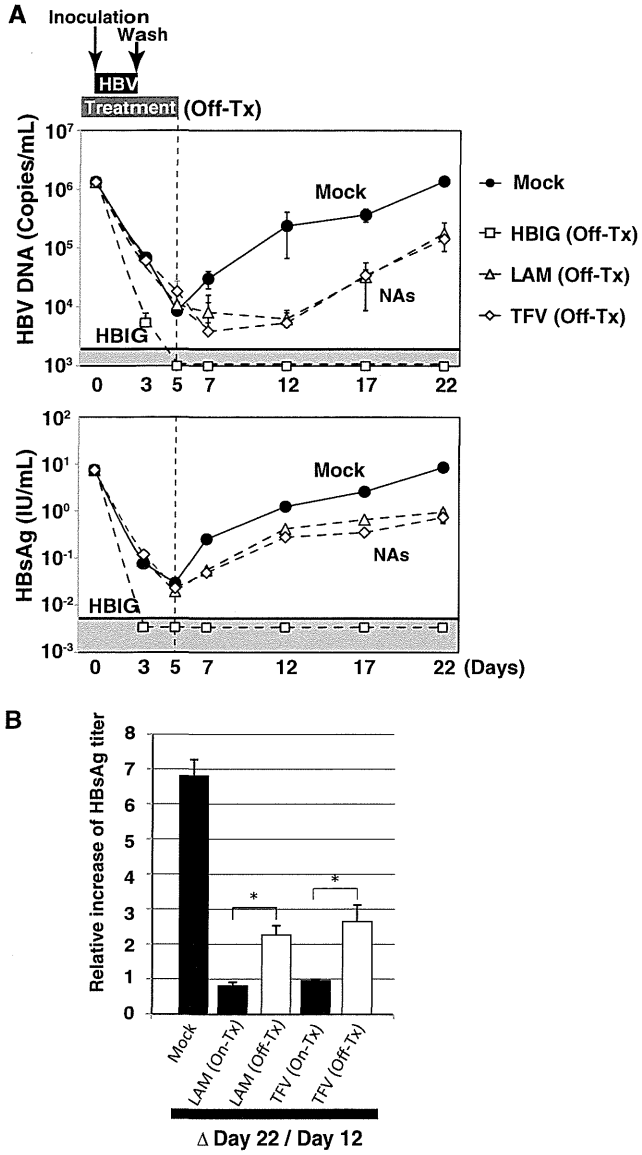

FIG 2 Preexposure NA treatment fails to prevent HBV infection. (A) PHH were inoculated with HBV for 48 h at 37°C and washed several times, and sampling of the supernatants was performed (as described in Fig. 1B). The concentration of HBV DNA increased gradually after day 12 when the LAM or TFV treatment 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. *, $P < 0.05$.

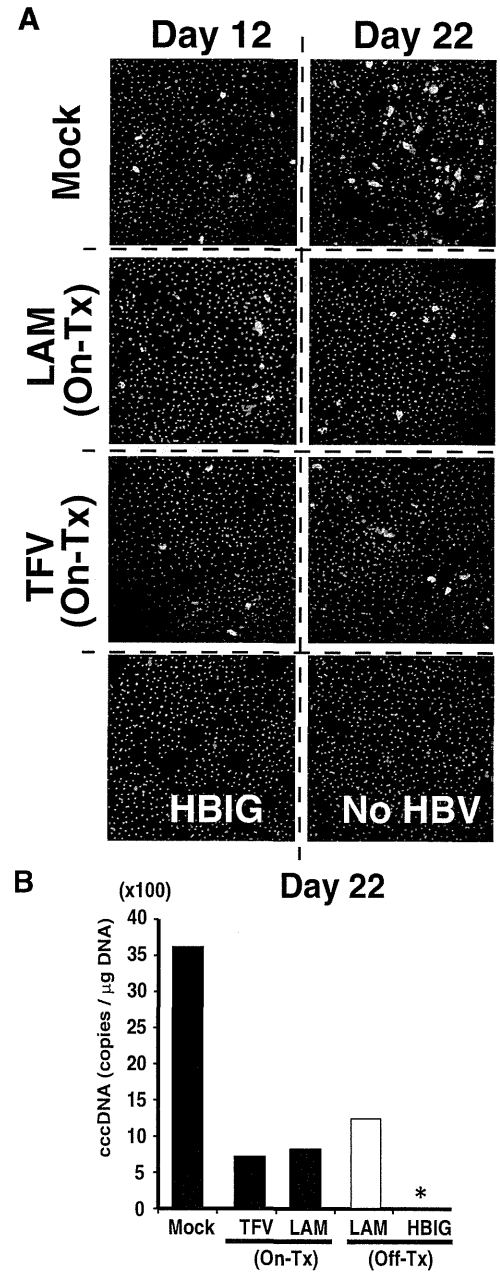

FIG 3 HBV-active NAs can inhibit viral spread after acquisition of HBV infection in freshly isolated PHH. (A) Immunohistochemical staining of HBV-infected PHH with various treatments. Viral spread occurred over 10 days from day 12 to day 22 in the nontreatment experiment (Mock); however, the numbers 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-

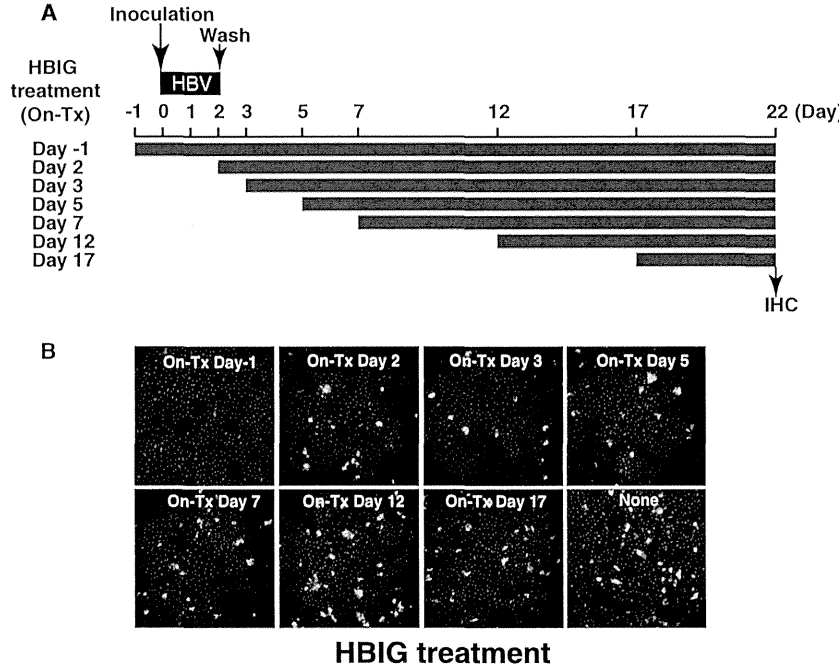

FIG 4 HBIG treatment in freshly isolated PHH post-HBV inoculation inhibits viral spread, while delaying HBIG addition allows viral spread. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with hepatitis B immune globulin (HBIG) after HBV inoculation. IHC, immunohistochemical 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

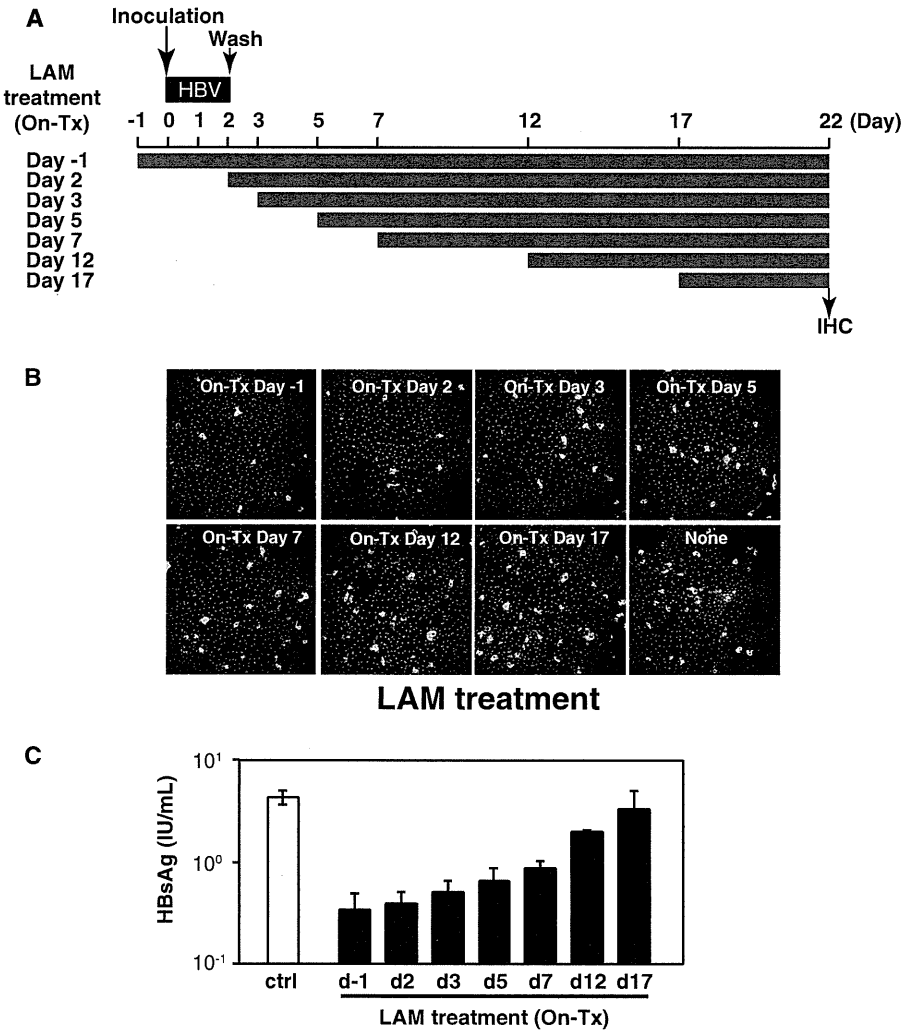

FIG 5 Effects of LAM on post-HBV inoculation *in vitro*. (A) Schematic representation of the schedule for continuous treatment (On-Tx) with lamivudine (LAM) after HBV inoculation. IHC, immunohistochemical staining for intracellular HBsAg. (B) IHC of PHH on day 22 with LAM treatment started at 2, 3, 5, 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.

ACKNOWLEDGMENTS

We thank Mayumi Hojo and Kyoko Ito for excellent technical assistance.

This study was supported, in part, by a grant for Practical Research on Hepatitis B (009) from the Ministry of Health, Labor, and Welfare of Japan and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

We have no conflicts of interest to declare.

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