Coexistence of hepatitis B virus quasispecies enhances viral replication and the ability to induce host antibody and cellular immune responses

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Hepatitis B virus (HBV) quasispecies contain a large number of variants that serve as a reservoir for viral selection under antiviral treatment and the immune response, leading to the acute exacerbation and subsequent development of liver failure. However, there is no clear experimental evidence for a significant role of HBV quasispecies in viral pathogenesis. In the present study, HBV sequences were amplified from a patient with severe liver disease and constructed into HBV replication-competent plasmids. Western blot, Enzyme-linked immunosorbent assay (ELISA), and immunofluorescence staining were performed to analyze the expression, secretion and subcellular localization of viral proteins in vitro. Viral replication intermediates were detected by Southern blot. HBV gene expression and replication and the induction of specific immune responses in an HBV hydrodynamic injection (HI) mouse model were investigated. The results demonstrated that two naturally occurring HBV variants, SH and SH-DPS, were identified. The variant SH-DPS expressed only a non-exportable hepatitis B surface antigen (HBsAg) with abnormal intracellular accumulation. The coexistence of the HBV variants at a ratio of 1 to 4 (SH to SH-DPS) increased HBV replication. Significantly stronger intrahepatic cytotoxic T lymphocyte (CTL) responses and antibody responses specific to HBsAg were induced in mice by the HBV variants when co-applied by HI. These findings uncovered an unexpected aspect of HBV quasispecies: that the coexistence of different variants can significantly modulate specific host immune responses, representing a novel mechanism for the immunopathogenesis of HBV infection.
Hepatitis B virus (HBV) is an important human pathogen. HBV quasispecies with genetically heterogenous variants are thought to play a role in the progression of HBV-associated liver diseases. So far, direct evidence is only available in few cases to confirm the proposed role of HBV variants in the pathogenesis. We report here that the coexistence of two naturally occurring HBV variants at a ratio of 1 to 4 increased HBV replication and induced significantly stronger intrahepatic cytotoxic T lymphocyte responses and antibody responses specific to HBsAg in mice. Our discovery uncovered an unexpected aspect of HBV quasispecies that the coexistence of different variants can significantly modulate specific host immune responses and may enhance immune-mediated liver damage under some circumstances, representing a novel mechanism for the immunopathogenesis of HBV infection.

KEYWORDS: Hepatitis B virus, acute liver failure, quasispecies, immune response
INTRODUCTION

Hepatitis B virus (HBV) infection causes a wide spectrum of clinical manifestations ranging from an asymptomatic carrier state to acute or chronic hepatitis, with progression to liver cirrhosis, hepatocellular carcinoma, and other severe liver diseases (1-4).

The HBV population in the host consists of genetically heterologous variants and exists in the form of quasispecies. It is proposed that quasispecies may contribute to viral persistence and pathogenesis because quasispecies contain a large number of mutated genes that serve as a reservoir for viral selection under antiviral treatment and the immune response (5). It has been previously reported that co-infection with human immunodeficiency virus (HIV) and HBV results in low quasispecies complexity (4). In patients with HBV infection-related liver failure (HB-LF), the quasispecies showed distinct characteristics with higher complexity and diversity within the HBV precore (preC)/core gene (6). However, there is no clear experimental evidence for a significant role of HBV quasispecies in the pathogenesis of HB-LF.

A number of publications have suggested that the emergence of HBV variants leads to acute exacerbation and subsequently contributes to the development of liver failure (LF). Some mutations in HBV genomes, such as the A1762T/G1764A double mutation in the basal core promoter (BCP) and the G1896A mutation in the preC region, are thought to play a role in the progression of liver diseases (1, 6-8). These mutations were found to enhance HBV replication in vitro and to abrogate expression of hepatitis B e antigen (HBeAg), which is suspected to interrupt immune tolerance in the host and to contribute to the development of LF (1, 7, 8). However, no direct
evidence is available to confirm the proposed role of HBV mutations in the pathogenesis of HB-LF so far. In addition, other studies have reported contradictory findings, indicating that there is no obvious link between HBV BCP/preC mutations and the development of LF (9, 10). It also remains mechanistically unclear how HBV BCP/preC mutations affect the development of HB-LF.

In general, HBV variants may cause liver damage by a direct cytopathic effect or by indirectly promoting immunopathology. There are a few examples of exacerbation of liver diseases associated with cytopathic HBV variants (11-15). However, it is currently unknown whether the appearance of HBV variants has any influence on host immune responses which would in turn cause liver damage.

In the present study, we characterized HBV isolates from a patient with severe liver disease and identified two major HBV variants, HBV-SH (SH) and HBV-SH-DPS (SH-DPS), which harbored a number of mutations including two deletions within the preS regions and hepatitis B surface antigen (HBsAg) sequences. The variant SH-DPS expressed only a non-exportable SHBsAg with abnormal intracellular accumulation. Both SH and SH-DPS coexisted at a ratio of 1 to 4. These two isolates were phenotypically characterized alone or together in different ratios by transient transfection. The results demonstrated that the coexistence of SH and SH-DPS at a ratio of 1 to 4 increased HBV replication and led to a predominant nuclear localization of HBV core antigen (HBcAg). Using an HBV hydrodynamic injection (HI) mouse model, we found that mice mounted significantly stronger antibody and cytotoxic T lymphocyte (CTL) responses to HBsAg only if SH and SH-DPS were co-applied. Thus, the coexistence of different variants may...
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significantly modulate specific host immune responses and may enhance immune-mediated liver damage under some circumstances, representing a novel mechanism for the immunopathogenesis of HBV infection.
MATERIALS AND METHODS

Patient. A 38-year-old male patient from China had a history of chronic hepatitis B infection for over 30 years. He was positive for HBsAg and the antibody to the hepatitis B e antigen (anti-HBe) and was negative for HBeAg and the antibody to HBsAg (anti-HBs).

The patient was diagnosed with HB-LF manifesting as a rise in alanine aminotransferase (ALT) to 283 U/L along with HBV DNA levels > 10^6 copies/ml, jaundice (bilirubin, 7.9 mg/dL) and coagulopathy (grade II), complicated within 4 weeks by ascites and encephalopathy. The patient received artificial liver support 3 times as well as other treatments, but the illness worsened precipitously, complicated by hepatic encephalopathy, infection and hepatorenal syndrome (Fig. 1).

The patient gave signed, informed consent. Sample collection, processing, and storage conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the institution's human research committee.

Characterization of HBV isolates from patient serum samples and cloning.

Isolation of HBV viral DNA from patient serum samples was performed as described previously with minor modifications (16, 17). A polymerase chain reaction (PCR) was performed to amplify 2.1 kb fragment (1821-699 bp) and 1.2 kb fragment (669-1825 bp) with the primer pairs P1/P3 and P2/P4, respectively: P1, 5’-CCGGC
GTCGACGAGCTCTTCTTTTTCACCTCTGCCTAATCA-3' (nt1821-1841); P2, 5'-
CCGGCGTCGACGAGCTCTTCAAAAAGTTGCATGGTGCTGG-3' (nt1825-1806); P3, 5'-
CACTGAAACAAATGGCACTAGTAAACTGAGCC-3' (nt699-669); P4, 5'-
GCTCAGTTTACTAGTGCCATTTGTCAGT3-3' (nt669-699). To reduce the
possibility of error-prone amplification, an enzyme with excellent high PCR fidelity
and efficiency, KOD-PLUS (Toyobo) was used in PCR. The two PCR products were
cloned into the pGEM®-T Easy vector (Promega, Madison, Wisconsin, USA) for
sequence analysis (ten clones of each fragment). The sequences of the 1.2 kb
fragments were consistent, while the 2.1 kb fragment had two types with either
entirely HBsAg gene (2 clones) or 2 deletions within HBsAg gene (8 clones) (Fig.
2A). To get full-length HBV genomes, the 1.2 kb  SacI-SpeI fragment and a 2.1 kb
SacI-SpeI fragment were released from the pGEM®-T Easy vector and cloned into the
cloning vector pUC19 pre-digested with  SacI and  SacI. The two head-to-tail fragments
were sub-cloned into the pUC19 vector and resulted in pUC19-HBV1-SH and
pUC19-HBV1-SH-DPS harboring a complete HBV genome and a type of mutant
genome with two deletions within the HBV preS region (GenBank accession number:
SH: KC492739.1 and SH-DPS KC492740.1) (Fig. 2A).

Two plasmids with replication-competent, 1.3-fold HBV genomes, pUC19-
HBV1.3-SH (pSH) and pUC19-HBV1.3-SH-DPS (pSH-DPS) (18), and a positive
control (PC) pUC19-HBV1.3-B were constructed as described previously (19). A
previously described replication deficient HBV clone pHBV1.3-rtG244Y (20) was
used to serve as a negative control (NC) in Southern blot experiment. Additionally, the 1.3-fold over-length HBV genomes were sub-cloned into the pAAV vector to produce pAAV-SH, pAAV-SH-DPS and pAAV-PC for HI in mice.

**Cells and mice.** The human hepatoma cell line Huh7 (provided by American Type Culture Collection, Manassas, VA) was maintained and transfected as described previously (20). Male C57BL/6 (H-2b) mice (6 to 8 weeks of age) were kept under specific-pathogen-free (SPF) conditions in the Central Animal Laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences and treated by following the guidelines of the institutional animal ethical standard.

**Western blot analysis.** Western blot analysis was performed as described previously (21, 22). The following antibodies were used: anti-HBs (Abcam, Cambridge, UK), anti-HBc (Santa Cruz, Santa Cruz, CA) and anti-beta-actin (Santa Cruz, Santa Cruz, CA). Relative band intensities for viral proteins were quantified using NIH ImageJ software.

**Enzyme-linked immunosorbent assay (ELISA).** HBsAg, HBeAg, antibodies to HBsAg in mouse sera, culture supernatants, and cell lysates of transfected cells were detected as described previously (23-25).

**Immunofluorescence (IF) staining and confocal laser scanning microscopy.**
Indirect IF staining of transfected cells was performed as described previously (21, 23, 24). Anti-HBc (Dako, Carpinteria, CA) and anti-HBs (S1, kindly provided by Yan Bin) were used as primary antibodies and Alexa Fluor 488-conjugated and Alexa Fluor 568-conjugated antibodies (Life Technologies, Carlsbad, CA) were used for secondary detection.

**Southern Blot Analysis.** Huh7 cells were transiently transfected with 2.5 μg of pHBV-B, pSH or pSH-DPS alone or co-transfected with 2.5 μg of mixtures of pSH and pSH-DPS, or pHBV-B and pSH-DPS at the indicated ratios. Encapsidated HBV replication intermediates were extracted and subjected to Southern blot analysis as described previously (20). The hybridization signals were quantified with ImageJ software (National Institutes of Health).

**HBV challenge by hydrodynamic injection.** Mice in each group were challenged by hydrodynamic injection (HI) as described previously with minor modifications (17). In brief, 10 μg of HBV plasmid DNA was incubated with 20 μl Lipofectamine 2000 (Life Technologies, Carlsbad, CA) for 20 min at room temperature to form the DNA–Lipofectamine 2000 complex. Then, the complexes were injected into the tail veins of mice in a volume of PBS equivalent to 8% of the mouse body weight within 5 seconds.

**Detection of serum HBV DNA and intrahepatic core-associated HBV DNA.**
Purification of core-associated HBV DNA in liver tissue and quantification of HBV DNA were performed as described previously (23, 26). The real-time PCR primers P5/P6 and the TaqMan probe are used: P5, 5’-AAATCTCCAGTCACTCACCAACC-3’ (nt321-343); P6, 5’-CATAGCAGCAGG ATGCAGAGG-3’ (nt423-403); TaqMan probe, 5’-FAM-TCCTCCAATTTGTCTGGTTATCGCT-MGB-3’ (nt349-374). The plasmid pHBV-B was used in 10-fold serial dilutions ranging from 10^1 to 10^9 copies per reaction as a standard curve.

**Immunohistochemistry (IHC).** Liver tissues were collected from mice sacrificed at 5 days post-hydrodynamic injection (dpi) and subjected to IHC staining as described previously (20). Intrahepatic HBcAg and HBsAg were detected by IHC staining of formalin-fixed, paraffin-embedded liver tissue sections using anti-HBc antibody (Dako, Carpinteria, CA) and anti-HBs antibody (Thermo Scientific Pierce, Rockford, IL) with an appropriate HRP-conjugated secondary antibody and visualized by the Envision System.

**Intracellular cytokines staining and flow cytometry.** Splenocytes and intrahepatic lymphocytes were isolated from mice at 14 and 28 days and subjected to flow cytometry analysis (27). Lymphocytes were supplemented with CD28 (1:1000, ebioscience, San Diego, CA) and Brefeldin A (1:1000, ebioscience, San Diego, CA), stimulated with or without peptide derived from HBcAg and HBsAg corresponding to S protein CD8+ T cells epitope (Kb/S190-197, VWLSVIWM), core protein CD8+ T...
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cells epitope (Kᵇ/C93–100, VWLSVIWM), S protein CD4⁺ T cells epitope (S₁₈₂–1₉₆, FFLLTFTULITFQSLD) and core protein CD4⁺ T cells epitope (C₁₂₈–₁₄₀, TPPAYRPPNAPIL), respectively, and then stained for surface marker CD4 and CD8 (BD Biosciences, San Jose, CA). Lymphocytes were fixed and permeabilized in Cytofix/Cytoperm solution (Cytofix/Cytoperm™ kit, BD Biosciences, San Jose, CA) followed by intracellular staining for IFN-γ (BD Biosciences, San Jose, CA). Dead cells were excluded by staining with 7-aminoactinomycin D (7AAD, Biolegend, San Diego, CA).

Statistical analysis. The statistical analysis was carried out using GraphPadPrism 5.0 software (GraphPad Software). Two tailed t-test was used to determine the differences in multiple comparisons. A P value < 0.05 was considered statistically significant. Results were presented as mean ± standard deviation (SD).
RESULTS

Sequence analysis of HBV genomes. First, HBV DNA was isolated from the serum of the patient and subjected to sequence analysis. A comparison of the cloned HBV DNA sequences with published HBV sequences in Genbank indicated that the isolates belong to the genotype B and serotype adw2 and harbor the A1762T/G1764A double mutation in the BCP region and the G1896A mutation in the preC region (Fig. 2A). The A1762T/G1764A double mutation has been reported to reduce the production of HBeAg and to enhance the HBV replication level in vitro (28, 29), whereas the G1896A stop codon mutation abolishes HBeAg expression (30). In 8 of 10 sequenced HBV genomes, 2 deletions in the preS1 region (nt 2976-3102) and in the preS2 promoter and preS2 open reading frame (ORF) region (nt 3203-3215, nt 1-31) were found (Fig. 2A), resulting in a stop codon mutation in the ORF of large HBsAg (LHBsAg) and the deletion of the start codon in the ORF of middle HBsAg (MHBsAg), respectively. In addition, two typical point mutations, N146S and P120S, were present. The mutation N146S prevents the glycosylation of HBV envelope proteins (31). The mutation P120S may be associated with immune escape (Fig. 2A) (32). Thus, two major HBV variants coexisted in this patient; the one with complete HBsAg was designated as SH, and the other one with deletion mutations in HBsAg ORFs was designated as SH-DPS. The ratio of SH to SH-DPS was 1 to 4 based on the numbers of the sequenced clones.

Phenotypic characterization of SH and SH-DPS in vitro. The plasmids pSH
and pSH-DPS containing the respective 1.3-fold over-length HBV genomes were transfected into Huh7 cells. PSH produced much less intracellular and supernatant HBsAg (52% and 30%, respectively) than PC (32%) (Fig. 2B). PSH-DPS did not produce detectable HBsAg in supernatant but produced a low level of intracellular HBsAg. These results suggested that HBsAg with deletions and mutations encoded by SH-DPS was secretion deficient. No HBeAg was detected in culture supernatants, consistent with the presence of the G1896A mutation in the preC region that abolishes HBeAg expression (Fig. 2C) (1, 2). WB with anti-HBs antibodies indicated that glycosylated and un-glycosylated bands corresponding to L, M and SHBsAg were detected for both SH and PC as reported previously (33), while only the non-glycosylated form of SHBsAg could be detected for SH-DPS, consistent with the sequence information that included the termination of L and MHBsAg and the loss of the glycosylation site by N146S substitution (Fig. 2D). Based on immunofluorescence (IF) staining with anti-HBs antibodies, HBsAg was evenly distributed in the cytoplasm of cells transfected with pSH or PC (Fig. 2E) (23). In contrast, a dot-like distribution of HBsAg was observed in the cytoplasm of cells transfected with pSH-DPS (Fig. 1E). Thus, the mutations in HBsAg expressed from pSH-DPS caused abnormal localization of HBsAg and prevented its secretion.

The coexistence of SH and SH-DPS at a ratio of 1 to 4 increased HBV replication. We co-transfected pSH and pSH-DPS at different ratios and analyzed HBV gene expression and replication by enzyme linked immunosorbent assay.
(ELISA), Western blot, Southern blot, and IF staining (Fig. 3). Increasing amounts of pSH-DPS led to a decrease of HBsAg production in supernatant (Fig. 3A), as well as a decrease of HBcAg expression level in cell lysates (Fig. 3B). Both the pSH and pSH-DPS clones were replication competent. However, pSH-DPS produced much more core-associated DNA than pSH (Fig. 3B and C). Interestingly, when pSH and pSH-DPS were co-transfected at different ratios, the highest amount of core-associated HBV replication intermediates was detected at a ratio of 1 to 4, which yielded a 15% increase compared to pSH-DPS alone (Fig. 3B and C). This demonstrated a synergistic effect of HBV quasispecies on genomic replication.

The coexistence of SH and SH-DPS led to a predominant nuclear localization of HBcAg. In Fig. 3D, HBcAg evenly distributed in both the cytoplasm and nucleus in cells transfected with pSH-DPS but only in the cytoplasm in cells transfected with pSH and PC. When the ratio of pSH and pSH-DPS was 4 to 1, both HBsAg and HBcAg were evenly distributed in the cytoplasm, similar to pSH transfection. At a ratio of 1 to 4, HBsAg aggregated in the cytoplasm and HBcAg was located both in the cytoplasm and nucleus similar to the distribution with pSH-DPS transfection.

Phenotypic characterization of SH and SH-DPS in vivo. We further characterized these two HBV variants using an HBV HI mouse model (23, 25). pAAV-SH and pAAV-SH-DPS were generated by cloning the 1.3-fold over-length
HBV genomes into the pAAV plasmid. After HI of HBV constructs into C57BL/6 mice, serum HBsAg levels were monitored from day 1 on and up to day 63 post-HI. A high serum HBsAg level was detected in all mice receiving pAAV-PC and pAAV-SH at 1 dpi to 7 dpi, then declined to an undetectable level at 28 dpi (Fig. 4A). HBsAg was not detected in mice receiving pAAV-SH-DPS, consistent with the secretion deficiency of mutated HBsAg (Fig. 4A). For the pAAV-SH and pAAV-SH-DPS groups co-injected at a ratio of 4 to 1, HBsAg was detected at 1 dpi and seroconversion occurred at 21 dpi (Fig. 4A). However, when pAAV-SH and pAAV-SH-DPS were co-injected at a ratio of 1 to 4, no HBsAg was detected (Fig. 4A).

Further, we detected serum and hepatic HBV DNA in mice by real-time PCR at the indicated time points after HI. In pAAV-PC-injected mice, the serum HBV DNA level was $1.4 \times 10^6$ copies/ml at 3 dpi and increased to $2.4 \times 10^7$ copies/ml at 5 dpi (Fig. 4B). HI with pAAV-SH in mice resulted in significantly lower serum HBV DNA levels, at approximately 6% of the levels in the control with pAAV-HBV-B. The serum HBV DNA levels were below the detection limit in other mice receiving pAAV-SH-DPS and pAAV-SH/pAAV-SH-DPS co-injection (Fig. 4B). The intrahepatic HBV DNA levels at 5 dpi were positive in all mice injected with pAAV-SH, pAAV-SH-DPS, and pAAV-SH/pAAV-SH-DPS but were less than 10% of that of the PC group (Fig. 4C), indicating that both SH and SH-DPS were replication competent in vivo but at a lower level. However, there were no significant differences
among these groups. Southern blot hybridization detected HBV replication intermediates from the PC group but significantly lower levels of intermediates from the liver samples from the other groups (Fig. 4C).

Immunohistochemistry (IHC) staining for HBsAg and HBcAg with liver tissue sections collected at 5 dpi showed a cytoplasmic distribution of HBsAg in mice injected with pAAV-PC and pAAV-SH and a dot-like distribution in pAAV-SH-DPS-injected mice (Fig. 5). The staining of HBcAg showed both cytoplasmic and nuclear distribution (Fig. 5B). When SH and SH-DPS were co-injected, both diffused distribution and dot-like distribution of HBsAg in the cytoplasm were observed (Fig. 5A). Additionally, the percentage of HBsAg- or HBcAg-positive hepatocytes was calculated and positively related to the intrahepatic HBV DNA levels of each group (Figs. 5A and B).

HBV-specific antibody- and cell-mediated immune responses. The levels of HBsAg in the sera of all mice receiving pAAV-PC and pAAV-SH declined to an undetectable level at 28 dpi. Inversely, anti-HBs antibodies were initially undetectable but became positive at 21 dpi and then were maintained at a high level through the experiment period in mice of both groups (Fig. 6A). However, like HBsAg, anti-HBs antibodies were not detected in mice receiving pAAV-SH-DPS, consistent with the secretion deficiency of mutated HBsAg (Fig. 4A). Strikingly, when pAAV-SH and pAAV-SH-DPS were co-injected at a ratio of 1 to 4, a significantly higher anti-HBs
antibody response was induced in the mice of this group, although we could not detect HBsAg previously (Figs. 4A, 6A and B). When mice were co-injected with pAAV-SH and pAAV-SH-DPS at a ratio of 4 to 1, the HBsAg levels decreased before 21 dpi and anti-HBs antibody levels increased after 21 dpi (Figs. 4A and 6A). Therefore, the coexistence of SH and SH-DPS variants enhanced the HBsAg-specific immune responses.

The IgG1/IgG2a antibody responses could serve as an indication for the Th bias of specific immune responses. Thus, we determined the subtypes of HBsAg-specific IgGs. The IgG1 response to HBsAg was comparable in all mice receiving SH, SH/SH-DPS at the ratio of 1 to 4 and PC. Interestingly, HBsAg-specific IgG2a antibodies were only detected in mice receiving SH/SH-DPS (Figs. 6C and D), suggesting that the coexistence of SH and SH-DPS induced a significantly stronger Th1 response.

Next, we further examined the induction of cell-mediated, specific immune responses to HBsAg and HBeAg. Splenocytes and intrahepatic lymphocytes were isolated at 14 and 28 dpi, respectively, and stimulated with two H-2Kb-restricted CTL epitope peptides derived from HBsAg and HBeAg, as well as a CD4+ T cell epitope peptide. Flow cytometric analysis was performed for the detection of CD4+ and CD8+ IFN-γ-producing cells. CD8+ IFN-γ-producing cell populations specific to HBsAg were detected in mice from all groups except the mock group at 14 dpi and 28
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dpi. Notably, at 28 dpi, the frequencies of CD8+ IFN-γ-producing cells were the
highest if pAAV-SH and pAAV-SH-DPS were co-injected at a ratio of 1:4 (Figs. 7A
and B), consistent with the production of anti-HBs antibody. However, the HBsAg-
and HBcAg-specific CD4+ T cell response and the HBcAg-specific CD8+ T cell
response in all mice were not detected in both splenocytes and intrahepatic
lymphocytes (Figs. 7C, D, E, F, G and H).

**DISCUSSION**

HBV mutants are thought to play a role in the progression of HBV-associated
liver diseases (1, 6-8). The HBV population in the host exists in the form of
quasispecies and contains a great spectrum of mutants (5). The role of HBV
quasispecies in the pathogenesis of HBV infection is currently not understood. In the
present study, we found that the coexistence of two naturally occurring HBV variants
at a ratio of 1 to 4 increased HBV replication and led to a predominant nuclear
localization of HBcAg. More importantly, significantly stronger intrahepatic CTL
responses and antibody responses specific to HBsAg were induced in mice by these
HBV variants when co-applied by HI. These findings revealed the potential role of
HBV quasispecies and their mutations in the pathogenesis of HBV infection.

The cell-mediated immune response is considered to contribute to both viral
clearance and liver injury in HBV infection (34). In mice receiving HI with HBV
genomes, a higher frequency of HBsAg-specific CD8+ T cells was detected in the
liver than in the spleen. Strikingly, the coexistence of SH and SH-DPS at a ratio of 1
to 4 induced a stronger CTL response than each HBV vector alone (Figs. 7A and B). At the same time, the magnitude and the IgG subtype distribution of anti-HBs antibody responses were significantly changed by the coexistence of the HBV quasispecies (Figs. 6B, C and D). Both higher CTL frequencies and the appearance of the IgG2a subtype of anti-HBs antibodies indicated a shift of HBV-specific immune responses toward the Th1 type. The enhancement of HBV-specific immune response may be due to secretion defect of HBsAg. In addition, increased HBV replication activity when both HBV clones were co-injected and changed subcellular localization of HBsAg may also contribute to the induction of stronger specific immune responses.

Though our study does not prove a direct link between the HBV quasispecies and HBV-associated severe liver diseases, it hints at a possible explanation for the nearly obligatory presence of HBV variants in patients suffering from acute LF. It also gives an idea for why the characterization of HBV variants in cell culture systems seldom delivers a clear answer about their pathogenic potential. We could hypothesize that only the combination of different WT and mutant proteins effectively trigger specific host immune responses and leads to observed immunopathology in LF patients. This hypothesis implies that HBV variants are not innocent bystanders selected by decreased liver functions but are the major causes for LF, and thus may need attention in clinical monitoring.

Previously, the A1762T/G1764A and G1896A mutations in BCP/preC were
found to affect HBV replication and to abrogate expression of HBeAg. It was suspected that these mutations may contribute to the development of LF (1, 6-8). However, most of these results were based on in vitro transfection of single HBV replication-competent plasmids with such mutations. Here, co-transfection of two HBV replication-competent plasmids increased viral replication in the ratio of 1 to 4, suggesting that the HBV quasispecies should be paid more attention in clinical monitoring.

HBcAg tended to localize in the cytoplasm in the presence of the A1762T/G1764A double mutation (35, 36). However, the localization of HBcAg from a fulminant HBV strain was changed to the nucleus when both G1862T and G1896A were also present (3). Early studies on clinical samples indicated that the subcellular localization of HBcAg was closely correlated with hepatitis activity in HBeAg-positive patients (37, 38). However, the relationship between intracellular distribution of HBcAg and hepatitis activity is unknown in HBeAg-negative or anti-HBe-positive patients. Here, we observed a predominant nuclear localization of HBcAg when SH DNA and SH-DPS DNA were transfected into cells at a ratio of 1 to 4 (Fig. 3C), suggesting that the coexistence of SH and SH-DPS changed the preferential localization of HBcAg from the cytoplasmic to nuclear compartment. To clarify the mechanism, we performed Western blot and found that increasing amounts of pSH-DPS led to a decrease of HBcAg expression level (Fig.3B). Low level of HBcAg could not drive capsid assembly and virion formation in cytoplasm for
budding (39) but could freely enter the nucleus by nuclear pore complex (40).
Moreover, mature nucleocapsids could be targeted back to the nucleus and amplify the pool of cccDNA, or be targeted to the Endoplasmic reticulum to bud and exit the cell.
Envelope proteins play a regulatory role in the process (41). However, SH-DPS produced a low level of intracellular HBsAg (Fig.2). Therefore the difference in core protein localization between SH, SH-DPS and the co-existence of SH and SH-DPS was observed. However, the mechanism determining the shuttle of HBcAg between the nucleus and cytoplasm is not completely understood. Nuclear localization/export signals and some host factors may play a role in this process (42). The presence of different HBV proteins including mutated HBsAg may change the expression or function of such viral and host factors. Future investigation is required to identify the factors involved in the regulation of HBcAg localization.

To our knowledge, the present study demonstrated for the first time that the interaction of HBV quasispecies may enhance HBV replication and induce stronger host immune responses, thereby potentially contributing to acute exacerbation of liver diseases and the development of liver failure.

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FIGURE LEGENDS

**Fig. 1. Clinical course of the patient with HB-LF.** (A) The level of serum transaminase (ALT and AST), total bilirubin (TBIL) and direct bilirubin (DBIL) level. (B) The level of PT (prothrombin time), PTA (prothrombin time activity percentage), APTT (activated partial thromboplastin time) and FIB (fibriogen).

**Fig. 2. Expression, secretion and subcellular localization of viral proteins.** (A) Schematic representation of SH and SH-DPS. Stop codon in the preS1 ORF is indicated by an asterisk. The mutations and deletions are marked by dark arrows. (B, C, D and E) Huh7 cells were transfected with PC, pSH or pSH-DPS. At 72 hours (h) post transfection, HBsAg (B) and HBeAg (C) in culture supernatants and cell lysates were detected by ELISA. Surface proteins (D) were detected by Western blot. (E) Cells were fixed 48 h after transfection and stained for HBsAg. The nuclei were stained with Hoechst 33258. Higher-magnification images of the selected area are also shown. PC, Huh7 cells transfected with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. Mock, Huh7 cells transfected with a mock plasmid.

**Fig. 3. SH and SH-DPS synergistically enhance viral replication and change subcellular localization of the core protein.** Huh7 cells were transfected with pSH,
pSH-DPS or pSH and pSH-DPS at the indicated ratios. (A) HBsAg in culture supernatants was detected by ELISA. (B) HBV replication intermediates were detected by Southern blot. The positions of relaxed circular (RC), double stranded linear (DL), and single stranded (SS) DNA are indicated (upper panel). HBcAg were detected by Western blot. The last lane was mock (Middle panel). The levels of β-actin served as a loading control (lower panel). Relative band intensities for HBV replication intermediates or HBcAg were quantified using NIH ImageJ software and presented as the percentage of DNA or protein in positive control (PC). (C) Viral DNA levels of three independent Southern blot experiments were quantified and plotted as relative level (mean±SD) of PC samples. (D) Cells were fixed and stained for HBsAg and HBcAg. The nuclei were stained with Hoechst 33258. Higher-magnification images of the selected area are also shown. PC, Huh7 cells transfected with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. NC, Huh7 cells transfected with a HBV replication-deficient plasmid pHBV1.3-rtG244Y as a negative control. Mock, Huh7 cells transfected with a mock plasmid.

**Fig. 4. Phenotypic characterization of SH and SH-DPS in vivo.** Hydrodynamic injection (HI) was performed with HBV constructs at the indicated ratios in C57BL/6 mice (n=5). (A) The levels of HBsAg in the serum were detected by ELISA. Results were presented as the OD 450 value. HBV DNA expression in mouse sera (B) or liver samples (C) collected at 5 days post HI (dpi) was determined by real-time PCR and
Southern blot. PC, mice HI with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. Mock, mice HI with PBS as a negative control. Two tailed t-test was used to determine the differences in multiple comparisons (*, P<0.05, ***, P<0.01, compared to the PC).

**Fig. 5. Immunohistochemical (IHC) staining of HBsAg and HBeAg.** C57BL/6 mice were challenged by HI with 10 μg of HBV-DNA as indicated. Hydrodynamic injection (HI) was performed with HBV constructs at the indicated ratios in C57BL/6 mice (n=5). The liver tissues were collected at 5 dpi for IHC staining for HBsAg (A) and HBeAg (B). HBsAg- and HBeAg-positive hepatocytes were counted and positive rate was calculated as the percentage of total hepatocytes in one field of microscope. The average values from positive rate of 9 fields of microscope were calculated and given as the final positive rate and presented at the upper left. Magnification is at 400×. PC, mice HI with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. Mock, mice HI with PBS as a negative control.

**Fig. 6. Analysis of HBV-specific antibody responses.** C57BL/6 mice were challenged by HI with 10 μg of HBV-DNA as indicated. The dynamic (A) and level (B) of anti-HBs antibodies were monitored by ELISA. Results were presented as the OD 450 value. Two tailed t-test was used to determine the differences in multiple comparisons (**, P<0.01, ***, P<0.001, compared to the pAAV-SH and pAAV-SH-DPS co-injected group at a ratio of 1 to 4). The levels (C) and dynamic (D)
of subtypes of HBsAg-specific IgGs were determined. Filled circles represent the level of IgG1/IgG2a antibody in individual mice. Solid lines represent geometric mean values. The dotted line represents the cutoff, which was assumed to be 2.1-fold the mean value of the negative samples. PC, mice HI with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. Mock, mice HI with PBS as a negative control.

**Fig. 7. Analysis of HBV-specific antibody- and cell-mediated immune responses.**

C57BL/6 mice were challenged by HI with 10 μg of HBV-DNA as indicated. Splenocytes and intrahepatic lymphocytes isolated from mice (n=4 for 14 dpi, n=7 for 28 dpi) were stimulated for 5 h with 2 μg/ml of peptides derived from HBeAg and HBsAg corresponding to S protein CD8+ T cells epitope (Kb/S190–197, VWLSVIWM), core protein CD8+ T cells epitope (Kb/C93–100, VWLSVIWM), S protein CD4+ T cells epitope (S 182-196, FFLLTFULTIFQSLD) and core protein CD4+ T cells epitope (C128-140, TPPAYRPPNAPIL), respectively. HBV-specific IFN-γ secreting cells were analyzed by FACS. Dead cells were excluded by staining with 7-aminoactinomycin D (7AAD). PC, mice HI with a HBV replication-competent plasmid pHBV-B originated from genotype B as a positive control. Mock, mice HI with PBS as a negative control. Two tailed t-test was used to determine the differences in multiple comparisons (*, P<0.05, **, P <0.01 and ***, P<0.005).
**Fig. 1.**

(A) The level of serum transaminase (ALT and AST), total bilirubin (TBIL) and direct bilirubin (DBIL) level. (B) The level of PT (prothrombin time), PTA (prothrombin time activity percentage), APTT (activated partial thromboplastin time) and FIB (fibriogen).
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