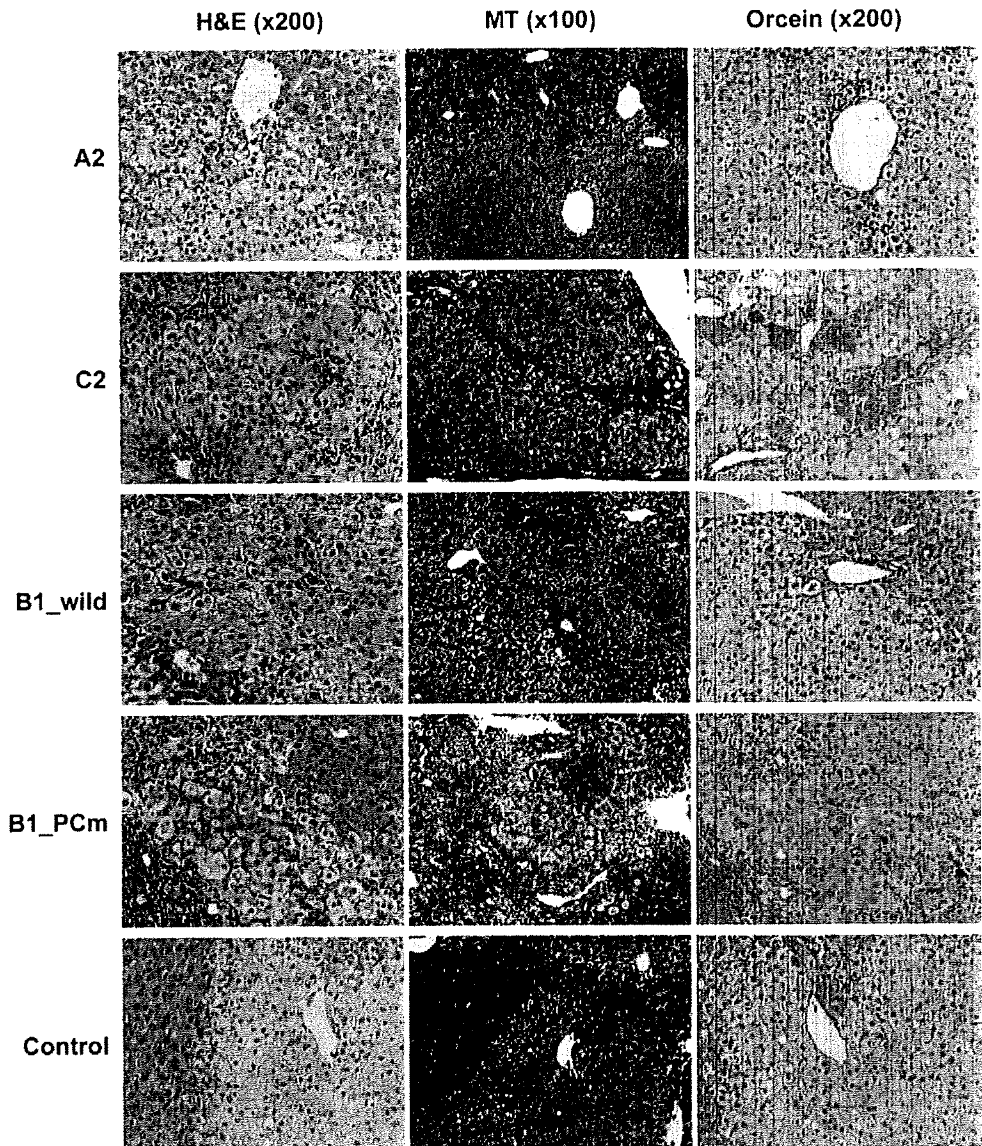

Figure 3. Immunohistochemical analysis of liver tissue. Comparison of liver histology in mice long-term (25 weeks) infected with HBV/A2, C2, B1_wild, B1_PCm, and noninfected control. Liver sections stained with H&E, MT, or orcein are shown. After deparaffinization, tissue slides were stained according to each method. Representative staining of C2 and B1_PCm showed a ground-glass appearance, fibrosis, and cytoplasmic positivity of human hepatocytes by orcein staining (brown), whereas these were absent in A2, B1_wild, and control mice. Original magnifications: H&E and orcein, 200×; MT, 100×.

expression of HBV DNA and antigens. This has allowed for an assessment of the direct cytopathic potential of different HBV genotypes (ie, particular subgenotypes) to be investigated without the host-related bias, under conditions of the absence of immune pressure. In addition, this may represent a novel mouse model for human liver fibrosis associated with ROS production leading to the activity of TGF-β by viral infection but not chemical trigger. The study thereby has shown that infection with HBV/C2 in contrast to HBV/A2 or B1_wild has induced an abundant ground-glass appearance of the human hepatocytes along with an increased fibrosis in the humanized liver of the chimeric mice in an immunosuppressive condition. A strong staining of α-SMA observed around areas of fibrosis indicated activation of HSCs in cases of HBV/C2 and B1_PCm, but not in A2 and B1_wild. In the chimeric mice, therefore, ROS production could play a critical role in HSC activation. In connection with this study, we have evaluated the liver damages in chimeric mice killed at 3 months postinfection (early phase dynamics). The viral dynamics and ROS production of HBV/C2 or B1_PCm evaluated in the early phase indicated levels of alterations similar to those observed after long-term infection (Supplementary Figure 2; see Supplementary material online at www.gastrojournal.org). Fibrosis stage and orcein staining levels (ground-glass appearance), however, were expressed in lesser levels than in the long-term infected mice, suggesting that the liver damage can be detected even in the early stage of the infection, but its level correlates with the duration of exposure to oxidative stress.

Our previous report showed that the intracellular virion retention and endoplasmic reticulum stress were the highest for HBV/C2.[21] Our data obtained in vitro and

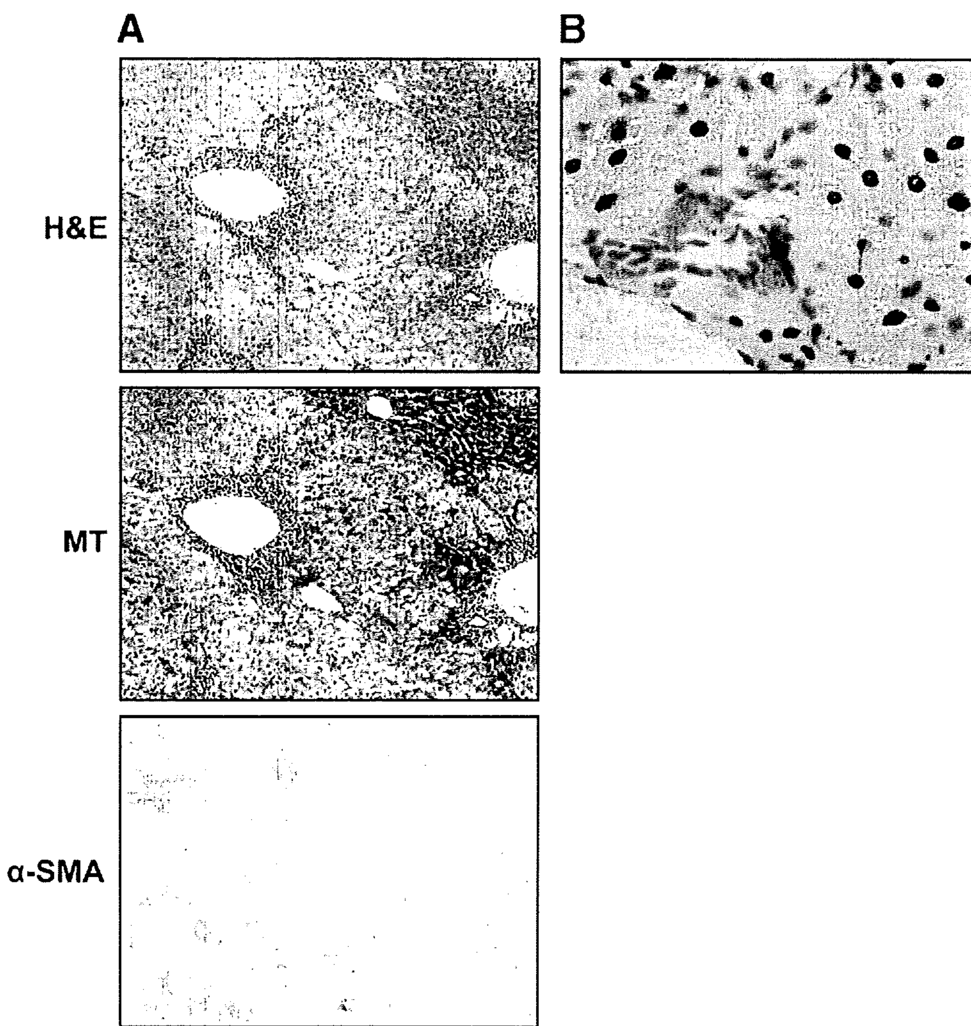

Figure 4. Confirmation of liver fibrosis by immunostaining using anti–α-SMA antibody. (*A*) Liver sections stained with H&E, MT, or immunostaining using anti–α-SMA antibody (as described in the Materials and Methods section). (*B*) Nuclei stained *brown* with the antibodies indicate human origin, and α-SMA is stained in *red*, located in the cytoplasm without a stained nucleus. Shown are representative staining of images expressing fibrosis. Original magnification, 200×.

in vivo may explain in part previous results accumulated from clinical studies indicating that HCC more often was associated with HBV/C and the mean age of patients with HCC is younger in the HBV/C-infected group compared with the HBV/B1-infected group.[28,29] On the other hand, the low replicative capacity and hepatic injury of HBV/A2 may contribute to the ability of the subgenotype to evade the immune response and chronically persist in up to 10% of acutely infected adults (which is exceptionally rarely observed with HBV/C or HBV/B).[11,30-32] High levels of HBsAg secretion for HBV/A2 are in contrast with its low replicative activity, and this may be an important mechanism for the immune escape. However, some cautions must be exercised when extrapolating the results of in vivo models to patients because immune responses are not taken into account.

The hepatic injury during acute and chronic HBV infection genuinely is considered to be caused by the host's immune response against the infected hepatocytes.[33] However, in some immunosuppressed chronic HBV patients, high viremia and liver fibrosis may occur.[34,35] Previous reports have shown that HBV genotypes E or G cause intracellular changes and hepatocellular damage in human hepatocytes in severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator.[2,3] We showed here that activation of oxidative stress led to TGF-β1 production in chimeric mice as reported in previous studies.[26] Accumulation of oxidative damage, 8-OHdG, might enhance the possibility of carcinogenesis as observed in HCC patients. These findings suggest that hepatic injuries could arise in the absence of a mature immune system and the difference of genotype would affect the cytopathic potential of the virus.

Chimeric mice were infected with HBV recovered from serum or culture medium containing virion from Huh7 cells transfected with HBV construct.[2,20,21,36,37] In our previous study, by using a single clone corresponding to HBV/A or C, we showed 2 logs difference during weeks 4-7 in the serum levels of HBV DNA between the cohort of mice inoculated with HBV/C and HBV/A.[21] In the present study, we extended the examination of the geno-

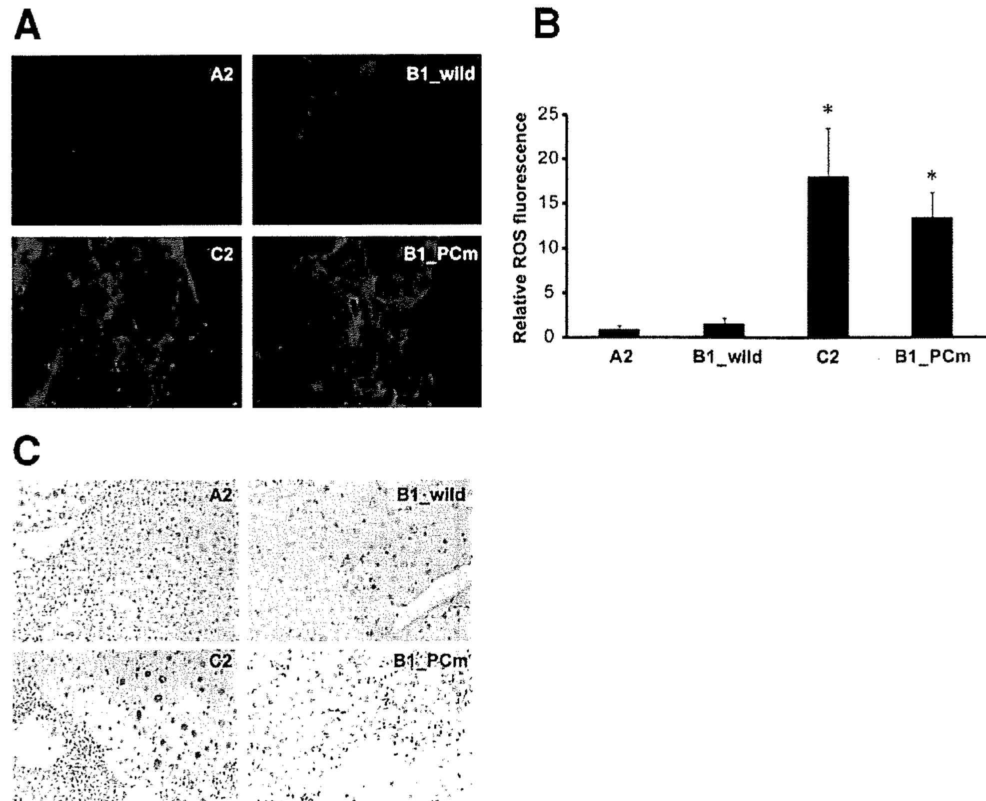

Figure 5. Differences in production of oxidative damage among HBV genotypes. (*A*) Frozen liver sections of mice inoculated with different HBV genotypes were stained by dihydroethidium. Fluorescence was detected with a laser scanning microscope. (*B*) Fluorescence intensities in randomly selected areas of digital images were quantified by National Institutes of Health image analysis software. $^{*}P < .01$: A2 or B1_wild vs C2 or B1_PCm. (*C*) Oxidative damages in liver tissue were evaluated by staining of 8-OHdG–positive nuclei. Original magnifications, 200×.

type differences by using 3 clones, representative of each genotype. The results of the present study in concordance with our previous study showed that the replication efficiency of HBV/C is significantly higher than that of HBV/A, as was indicated by 2 logs difference during weeks 6–8 in the levels of HBV DNA detected in murine sera ($P < .05$). The ability of HBV/A to express more HBsAg, and that of HBV/C to produce more HBcrAg revealed in our previous in vitro study,[21] were both thereby confirmed by the present in vivo replication model using the chimeric mice.

Previous clinical observations on HBV/B1[11,28] prompted a deeper investigation on the impact of the PC mutation on the virologic characteristics of the genotype. The unique characteristic of HBV/B1_wild stood out among genotypes harboring no major mutations. The HBV/B1_wild group revealed low replication efficiency with window periods and low antigen expression. The lower replicative activity and hepatic injuries of HBV/A2 and B1_wild may partially explain why carriers with either HBV/A2 or HBV/B1 often are asymptomatic in contrast to those with HBV/C infection.[28,38,39] In our study, the PC mutation was the only difference between HBV/B1_PCm and HBV/B1_wild clone, and the former showed higher replication efficiency and severe damage in liver tissue. The antigen levels of the HBV/B1_PCm increased rapidly and decreased earlier than those of the HBV/A2 or C2 clone, whereas HBV/B1_wild showed that the concentrations of HBV antigens remained low for several months postinfection. These particular characters were observed for the HBV/B1_PCm group inoculated with sera from both preinfected mice and patients with fulminant hepatitis. The majority of patients with fulminant hepatitis and fatal acute exacerbation have been found to have the G1896A mutation.[11,40,41] A greater incidence of fulminant hepatitis might be associated with the high replication and protein production in the early phase, as was shown on the HBV/B1_PCm clone in this study. The defect of immunologic tolerance as a result of the absence of HBeAg may play an important role in the fulminant course of precore mutation in HBV infection.[42] This would concur with a previous report by Bocharov et al which proposed that enhanced HBV replication would efficiently stimulate immune responses, represented by the cytotoxic T-lymphocyte response,[43] suggesting that enhanced replication by HBV/B1 with G1896A mutation might lead to an extremely high cytotoxic T-lymphocyte response, resulting in fulminant hepatitis. But in this study, HBV/B1_PCm showed similar responses to HBV/C2 infection because chimeric mice did not have an immune system that was strong enough to invite strong cytotoxic T-lymphocyte response against viral infection. To uncover these unique characteristics of PC mutant, further study would be needed by using the infection model but not gene transfer.

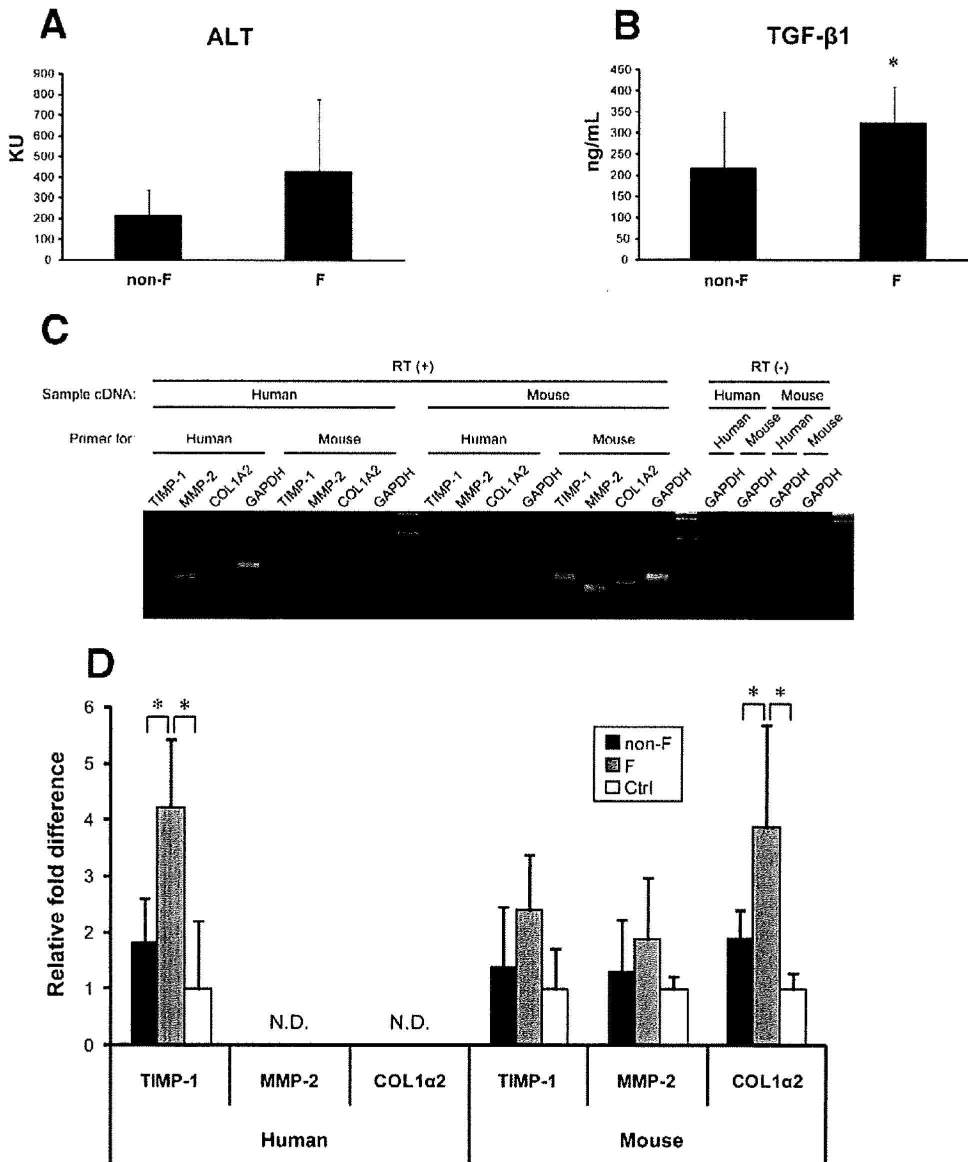

Figure 6. Differences in the expression levels of fibrosis-related genes among HBV genotypes. Quantification of (*A*) ALT and (*B*) TGF-β1 levels in mouse sera with enzyme-linked immunosorbent assay (see Supplementary Materials and Methods section. non-F, no fibrosis group (A2 and B1_wild); F, fibrosis group (C2 and B1_PCm). $^*P < .01$: non-F vs F. (*C*) The specificity of each PCR using species-specific primer sets. The species-specific primer sets were established to determine whether mRNA of fibrosis-related genes were of human or mouse origin. Liver tissue of a HCC patient or a mouse without transplantation of human hepatocytes was used to check the primer sets for real-time detection PCR. The PCR products were run on 2% agarose gels to confirm the molecular sizes as well as species-specific amplifications. (*D*) Quantification of mRNA expression on fibrosis-related genes in each group by real-time reverse-transcription PCR. non-F group, n = 15; F group, n = 22; control, n = 8; ND, not detected; $^*P < .001$.

Finally, the discrepancy between in vitro[21] and in vivo (present study) observations on HBV/B1_wild might have been caused by differences in the cells used for transfection (Huh7 cells) and infection (human hepatocytes from Caucasoid donors), respectively. Nonrecombinant type HBV/B strains (B1 and B6) have been detected in limited areas including Japan[44] and Alaska,[45] which were settled mainly by Mongoloid people. The existence of a window period on HBV/B1 might indicate a possibility that a receptor or coreceptor used by HBV/B1 is not equal to one adopted by other genotypes as shown in the human herpes virus.[46] Further studies using human hepatocytes from Mongoloid people would be required.

In conclusion, using an in vivo experimental system, we show that different HBV genotypes and even particular mutations are associated with different virologic and histopathologic characteristics. Infection with HBV/C2 as well as PC mutant of the HBV/B1 in immunosuppressive conditions can induce a direct cytopathic effect in the humanized part of the murine liver. This mouse model appears to be useful in the evaluation and prediction of pathogenic effects of various genotypes of HBV and certain HBV mutations.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.10.048.

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Received April 20, 2008. Accepted October 23, 2008.

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The authors disclose the following: Supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, and a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan, the Toyoaki Foundation.

The authors thank Drs C. Tateno, H. Yokomichi, K. Kuramoto, and T. Nakamura of PhoenixBio Co, Ltd for providing chimeric mice with a high replacement for hepatocytes; Dr T. Wakita of the National Institute of Infectious Diseases, Tokyo, Japan for quantifying the alanine aminotransferase level; Dr Ikehara of the National Institute of Advanced Industrial Science and Technology for the differential diagnosis of neutrophil/monocyte in liver tissue; Dr S. Nishina of Yamaguchi University Graduate School of Medicine for assistance with histological reactive oxygen species evaluation; Ms K. Tatematsu of Nagoya City University Graduate School of Medical Sciences for performing sequencing; and Mr S. Sato and Ms Y. Tanizaki of Nagoya City University Hospital for slicing liver tissues of chimeric mice.

The nucleotide sequences of HBV-DNA isolates used in this study have been deposited in the international DNA database under the following accession numbers: AB246337, AB246338, AB246341, AB246342, AB246344, AB246345, and AB362931–362933.

Supplementary Data

Materials and Methods

Plasmid Constructs of HBV DNA and Sequencing

The 1.24-fold HBV genomic constructs used in the present study were prepared as described previously.[1] The constructs were designed to transcribe oversized pregenome and precore mRNA. Table 1 shows the list of 12 plasmids used in this study. Nine wild-type clones were used including 3 HBV/A (Ae/A2), 3 HBV/B (Bj/B1), and 3 HBV/C (Ce/C2). An additional 3 HBV/B plasmids identical to the earlier-mentioned HBV/B clone were constructed with precore stop-codon (PC) mutation (G1896A), which abolishes HBeAg expression. Briefly, for site-directed mutagenesis, the wild-type clone was digested by *Hind*III and *Eco065I* and ligated with the fragment carrying the PC mutation (G1896A). Cloned HBV-DNA sequences were confirmed with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer. Furthermore, the HBV DNA spanning the complete genome were amplified from murine sera and cloned into the pGEM-T Easy Vector (Applied Biosystems) with followed sequencing.

Cell Culture and Transfection

Huh7 cells were transfected with plasmids equivalent to 5 μg of HBV-DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by cotransfecting 0.5 μg of reporter plasmids expressing secreted alkaline phosphatase in the culture media.

Determination of HBV Markers

HBsAg and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial kits (Fujirebio Inc, Tokyo, Japan). HBcrAgs, which included both HBeAg and HBcAg, were measured in serum using the chemiluminescent enzyme immunoassay as described previously.[2,3] HBcAg was measured by enzyme-linked immunosorbent assay as previously reported.[2]

Detection and Quantification of Serum HBV DNA

HBV-DNA sequences spanning the S gene were amplified by real-time detection PCR by the method of Abe et al.[4] The detection threshold of the method is 100 copies/mL (equivalent to 20 IU/mL). However, because of the small volume of the serum available from each mouse for the HBV-DNA quantification, 10-fold template dilution was used, which resulted in a higher detection threshold of the method in this study: 1000 copies/mL (200 IU/mL). Quantification standards used in the assay were prepared based on World Health Organization standard serum containing HBV genotype A (kindly provided by Dr Hiroshi Yoshizawa of Hiroshima University). The amplification and detection were performed in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to the protocol.

Detection of 8-OHdG in Liver Tissue

The slides obtained from frozen tissues for 8-OHdG determination were placed in Bouin's fixative overnight at room temperature, and washed in water for 20 minutes. Tissues were incubated with 0.3% H_2O_2 in methanol for 30 minutes and rinsed in phosphate-buffered saline (PBS) buffer. The slides were placed in 0.05 N NaOH in 40% ethanol for 12 minutes, rinsed in PBS, and incubated with 250 μg/mL ribonuclease for 1 hour. An avidin/biotin block (Vector Laboratories) was applied for 20 minutes, and super block and mouse-to-mouse blocking reagent (ScyTek Laboratories, Logan, UT) were used to eliminate background staining caused by endogenous mouse immunoglobulin (Ig)G. The primary 8-OHdG antibody (Japan Institute for the Control of Aging, Shizuoka, Japan) then was applied to the slides overnight at 4°C (20 μg/mL, 1:100). To detect positive cells binding primary antibody, these slides were treated with Vectastain Elite ABC kit (Vector Laboratories).

Quantification of TGF-β1 and ALT Levels in Sera

Serum TGF-β1 and ALT levels were determined by using commercially available enzyme-linked immunoassay kits (Bender MedSystems GmbH, Vienna, Austria; and Nissui Pharmaceutical Co, LTD, Tokyo, Japan) according to the manufacturer's instructions, respectively.

Quantification of Gene Expression Levels of Fibrosis Markers

Fresh liver tissues (n = 45) from killed mice were used for quantification of fibrosis markers. Total RNAs were isolated using the RNeasy Mini Kit, and DNA contamination of samples was eliminated using the RNase-free DNase Set (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized in reaction mixtures with SuperScript II RNase H^- Reverse Transcriptase kit (Invitrogen), adding 0.5 μg oligo$(dT)_{12-18}$ primer at 70°C for 10 minutes. Reaction mixtures were incubated sequentially at 42°C for 60 minutes, at 95°C for 5 minutes, and at 60°C for 5 minutes. To check DNA contamination of samples, PCR was performed using isolated samples without reverse transcriptase. Primer sets to detect species-specific cDNA were designed using Primer Express software (Applied Biosystems) and are shown in Supplementary Table 1. Equal aliquots (1 μL) of cDNA were amplified by real-time detection PCR according to the manufacturer's Power SYBR Green PCR Master Mix instructions (Applied Biosystems) using the ABI Prism 7700 Sequence Detection System (Applied

Biosystems) in triplicate. The PCR conditions were as follows: (1) stage 1, 50°C for 2 minutes; (2) stage 2, 95°C for 10 minutes; and (3) stage 3, 95°C for 15 seconds followed by amplification at 60°C for 1 minute. Stage 3 was repeated for 40 cycles. Specificity of the amplification products was confirmed by examination of dissociation reaction plots, and a distinct single peak indicated a single DNA sequence amplified by the real-time detection PCR. The PCR products were run on 2% agarose gels to confirm the molecular sizes as well as species-specific amplifications (Figure 6C). Data were analyzed by the 2 [-Delta Delta C(t)] method using Sequence Detector version 1.7 software (Applied Biosystems),[5] and were normalized using human or mouse-specific glyceraldehyde-3-phosphate dehydrogenase. A standard curve was prepared by serial 10-fold dilutions of human or mouse cDNA. The curve was linear over 7 logs with a 0.998 correlation coefficient.

Immunofluorescence Immunofluorescence was performed as previously reported.[1] Briefly, fresh-frozen specimens were cut at 5–6 μm by cryostat, and fixed in acetone at room temperature for 10 minutes. Liver sections were blocked with Antibody Diluent (Dako, Glostrup, Denmark), incubated with rabbit anti-HBc antibody (Dako) at room temperature for 1 hour, and then incubated with goat anti-rabbit IgG antibody conjugated with Cy3 (Chemicon) or goat anti-human albumin antibody labeled with FITC (Bethyl Laboratories Inc, Montgomery, TX). Sections were observed in a fluorescent microscopy (Eclipse E800M; Nikon, Tokyo, Japan).

Statistical Analysis

Group means were compared by an independent Student *t* test or 1-way analysis of variance.

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Supplementary Table 1. Sequence of Species-Specific Primers on Fibrosis-Related Genes

Primer	Sequence
hTIMP1/F1	5′-ATGGCCCCCTTTGAGCCC-3′
hTIMP1/R1	5′-GTCTGGTTGACTTCTGGTGTC-3′
mTIMP1/F1	5′-ATGGCCCCCTTTGCATCT-3′
mTIMP1/R1	5′-GTCTCGTTGATTTCTGGGGAA-3′
hMMP2/F1	5′-CCTTCTTGTTCAATGGCAA-3′
hMMP2/R1	5′-GGACAGAAGCCGTACTTGC-3′
mMMP2/F1	5′-CCTTCCTGTTCAACGGTCG-3′
mMMP2/R1	5′-GGGCAGAAGCCATACTTGC-3′
hCOL1α2/F1	5′-AGGAAATGGCTACCCAACTT-3′
hCOL1α2/R1	5′-TTAGAGCCCTGTAGAATG-3′
mCOL1α2/F1	5′-AGGAAATGGCAACTCAGCTC-3′
mCOL1α2/R1	5′-TTGGAACCCTGCAGAAGC-3′
hGAPDH/F2	5′-CACCAGGGCTGCTTTTAACTC-3′
hGAPDH/R2	5′-AGATGGTGATGGGATTTCCA-3′
mGAPDH/F2	5′-CACCAGGGCTGCCATTTGCAG-3′
mGAPDH/R2	5′-AGATGGTGATGGGCTTCCCG-3′

COL1α2, collagen type 1α2; F, sense primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human specific; m, mouse specific; MMP2, matrix metalloproteinase 2; R, antisense primer; TIMP1, tissue inhibitor of metalloproteinase 1.

Virology 376 (2008) 408–415

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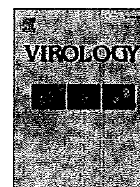

Characteristics of hepatitis B virus genotype G coinfected with genotype H in chimeric mice carrying human hepatocytes ☆

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ARTICLE INFO

Article history:
Received 14 February 2008
Returned to author for revision 18 March 2008
Accepted 1 April 2008
Available onlin 13 May 2008

Keywords:
HBV genotype G
HBV genotype H
Chimeric mice
MSM
Replication
Fibrosis

ABSTRACT

Accumulated evidence indicated that hepatitis B virus genotype G (HBV/G) is present exclusively in coinfection with other HBV genotypes. In Mexico, HBV/G from 6 men who had sex with men were coinfected with HBV/H. Phylogenetically complete genomes of the 6 Mexican HBV/G strains were closely related to previous ones from the US/Europe. Using uPA/SCID mice with human hepatocytes, monoinfection with HBV/G did not result in detectable HBV DNA in serum, whereas superinfection with HBV/G at week 10 inoculated HBV/H when HBV/H DNA was elevated to $>10^7$ copies/mL has enhanced the replication of HBV/G. The HBV/G was enhanced in another 3 inoculated with a serum passage containing HBV/G with a trace of HBV/H. Coinfection of mice with HBV/G and H induced fibrosis in the liver. In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions.

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Introduction

On the basis of the nucleotide sequence divergence exceeding 8% throughout the complete genome sequence, hepatitis B virus (HBV) has been classified into eight genotypes designated by capital letters A (HBV/A) through H (HBV/H) (Arauz-Ruiz et al., 2002; Norder et al., 1994; Okamoto et al., 1988; Stuyver et al., 2000). The genotypes have different geographical distributions, virological characteristics and clinical manifestations (Magnius and Norder, 1995; Miyakawa and Mizokami, 2003).

One of the less studied genotypes is the HBV/G. It was first described in 2000, among inhabitants of France and Georgia, USA (Stuyver et al., 2000). The isolated strains had 36 base-pairs' insertion in the core gene and two stop codons in the precore region depriving ability of the virus to translate HBeAg. Nevertheless, some of the carriers were HBeAg positive (Stuyver et al., 2000) that was shortly after explained by the coexistence of the "HBeAg-potent" HBV/A strains in coinfection (Kato et al., 2002a,b). Further studies reported circulation of the genotype in Thailand (Suwannakarn et al., 2005), Japan (Ozasa et al., 2006) and Mexico (Sanchez et al., 2007) indicating global distribution and association of the infection with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM). The studies also demonstrated that throughout the world HBV/G strains possess unprecedented genetic homology and are mainly presented in coinfection with another endemic genotype. However, little is known about peculiarities of interaction of the HBV/G with various genotypes as well as about virological and clinical concerns of the coinfection.

Produced by genetic engineering, a mouse with severe combined immunodeficiency, carrying urokinase-type plasminogen activator transgenes controlled by albumin promoter (uPA/SCID) with transplanted human hepatocytes (Heckel et al., 1990; Rhim et al., 1994) was recently shown as an appropriate animal model for studying HBV (Dandri et al., 2001; Tsuge et al., 2005). Using this model it was demonstrated that during monoinfection, HBV/G might be able to replicate in hepatocytes at low level; but its replication was

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcrAG, antigens related to HBV core; uPA/SCID, severe combined immunodeficiency transgenic with urokinase-type plasminogen activator.

☆ Supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, and a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan.

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doi:10.1016/j.virol.2008.04.001

significantly enhanced by coinfection with HBV/A or C (Sugiyama et al., 2007).

Our purpose is to determine the complete sequences of HBV/G coinfected with HBV/H in sera obtained from MSM in Mexico and to elucidate the characteristics of HBV/G in coinfection with HBV/H using recently developed *in vivo* model.

Results

Phylogenetic relatedness of HBV complete genome sequences in Mexico

Six HBV/G strains in coinfection with HBV/H among MSM in Mexico were described in our previous study (Sanchez et al., 2007). In the present study, the complete genome sequences of not only 6 HBV/G strains but also 6 HBV/H strains from the same patients were determined by each specific PCR. The phylogenetic analyses indicated that the 6 HBV/G strains were close to those previously reported from the United States (US), France and Germany, and the 6 HBV/H strains were related to the previously reported ones in the US (Fig. 1). All 6 coinfected patients were positive for HBeAg, and asymptomatic carriers of chronic HBV infection at the time of sample collection (the details unknown).

Characteristics of HBV/G strains in Mexico

Sequence analyses revealed unique insertion of 36 nt in the core gene, two stop codons in the precore region and double mutation in the core promoter (CP) in all Mexican HBV/G strains, whereas no related mutations were found in the corresponding HBV/H strains (Fig. 2a and b). These data suggest that the HBeAg detected in serum of those patients had been produced by HBV/H. Additionally, several mutations, which might have affected the replication of the virus genome and amino acid substitutions of HBx, were found in the first half of the CP region, including the above double mutation (nt 1701–1765) (Fig. 2a).

Examining the genetic diversity and recombination of HBV/G

Comparing the complete sequences, both overall genetic distance among the HBV/G strains (0.0037±0.0005 per site) and percent nucleotide homology (0.30±0.24%) were much lower than those among the other intra-genotype groups. In consideration of previously reported recombination between HBV/A and HBV/G strains (i.e. AB056516) (Kato et al., 2002a), we have examined the Mexican HBV/G strains for possible event of intergenotypic recombination. Complete sequences of 3 to 5 clones isolated from each of the 6 HBV/G carriers revealed; no evidence of recombination by similarity and bootstrap scan (data not shown).

Intracellular expression of HBV DNA and antigens

Huh7 cells were transfected with a pUC19 vector carrying 1.24-fold the HBV genome. Three days post-transfection, they were harvested, lysed with NP-40 and tested for HBV DNA and antigens. The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. The expression of HBV DNA was higher for HBV/H than G, indicating that HBV/G had very low replication *in vitro* (Fig. 3a). As well, HBsAg, HBeAg and HBcrAg levels were much higher in HBV/H (Fig. 3b).

Superinfection with HBV/G on mice infected with HBV/H

Chimeric mice were infected with HBV/G and H particles propagated in Huh7 cells in order to confirm the infective efficiency. Monoinfection with HBV/G from the Huh7 cells culture medium did not result in detectable HBV DNA in mice serum (data not shown).

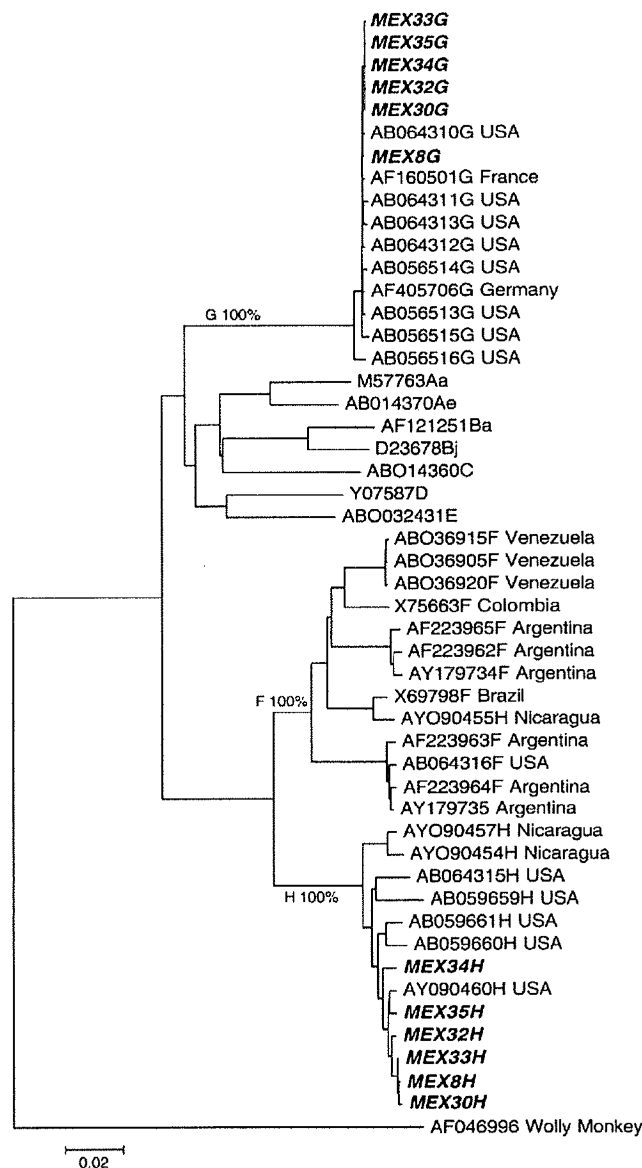

Fig. 1. A phylogenetic tree constructed using the complete nucleotide sequences of 50 HBV strains. The 6 HBV/G strains coinfected with HBV/H in Mexico (MEX), shown in bold italic, were compared with reference sequences recruited from GenBank/DDBJ/EMBL databases; 10 HBV/G, 7 HBV/H, 13 HBV/F, 7 representing genotypes A–E and 1 outgroup (Wolly Monkey). Aa and Ae are subgenotypes of HBV/A (Sugauchi et al., 2004). Ba and Bj are subgenotypes of HBV/B (Sugauchi et al., 2002). The country of origin is indicated after the accession number for each HBV/F, HBV/H and HBV/G strain. Bootstrap values are shown at the nodes of the main branches.

Then, according to our previous method (Sugiyama et al., 2007), the dynamics of HBV DNA, HBsAg and HBeAg assessed in 3 chimeric mice (ChiM_H1–H3) with HBV/G on H superinfection, are shown in Fig. 4 (a–c). Initially each of the mice received inoculation of around 10^5 copies of HBV/H recovered from the Huh7 cells culture supernatants, and the dynamics of HBV/H DNA indicated approximately 2 logs elevation within the following 5 weeks. At week 10 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, the chimeric mice were superinfected by inoculation of HBV/G. The HBV/G DNA level increased within 5 weeks after the superinfection and plateaued around 10^7 copies/mL. Two HBV antigens (HBsAg and HBeAg) waxed and waned in profiles similar to that of HBV DNA.

(a)

Core promoter region

```
                                                                              1753      1762/64
AB014370Ae   1700 GGCCTACTTCAAAGACTGTGTGTTTAAGGACTGGGAGGAGCTGGGGGAGGAGATTAGGTTAATGATCTTTGT
M57763Aa     1700 ...........................A............T.....................A.G.......
AF121251Ba   1700 ...A......................CT..G.........T...........T.........A.G.......
D23678Bj     1700 ...A.......................T..G...............................A.G.......
AB014360C    1700 ...A...............T....................T............C..............A...
Y07587D      1700 ...A...............T....C..A............T.....................A.G.......
AB0032431E   1700 ...A...............T.......A............T................A....A.G........
AF223962F    1700 A.AA...A.............A.....A............T.............C.......A.G.......
AB064316F    1700 A.AA...A.............A..................................................
H AB064315H  1700 ..AA...A.............A.......G..........TC..........T.G...........T.A...
H AB059659H  1700 ..AA...A....G........A..................TC..........T.G.......A.G.......
H MEX30H     1700 ..AA...A.............A..................TC..........T.G.......A.G.......
H MEX32H     1700 ..AA...A.............A..................TC..........T.G.......A.G.......
H MEX33H     1700 ..AA...A.............A..................TC..........T.G.......A.G.......
H MEX34H     1700 ..AA...A.............A..................TC..........T.G.......A.G.......
H MEX35H     1700 ..AA...A.............A..................TC..........T.G.......A.G.......
H MEX8H      1700 ..AA...A.............A..................TC..........T.G.......A.G.......
G AF405706   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AF160501   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB056513   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB056514   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB056515   1700 .AAA........G............GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB056516   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB064310   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB064311   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB064312   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G AB064313   1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX30G     1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX32G     1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX33G     1700 .AAA.....G..G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX34G     1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX35G     1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
G MEX8G      1700 .AAA........G........T...GCT..G.....A..AT.A..CA.T...TCC..........C......
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(b)

Precore region

Encapsidation Signal

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                                                                            1858                                  1896
AB014370Ae   1800 TGCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATCTCTTGTACATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGG
M57763Aa     1800 .........TT.T.................................................T.........................A...........
AF121251Ba   1800 ..TT..........................................A...T.......T.....................................A...
D23678Bj     1800 ..TT..................................G...........T.......T.....................................A...
AB014360C    1800 ..TT..............................................T.......T........................................A
Y07587D      1800 .............................................T....T.......T.........................................
AB0032431E   1800 ..................................................T.......T.........................................
AF223962F    1800 ..TT.........................................T....T.......T.........................................
AB064316F    1800 ..TT.........................................T....T.......T.....................................A...
H AB064315H  1800 ..TT.........................................T....T.................................................
H AB059659H  1800 ..TT.........................................T....T................................................A
H MEX30H     1800 ..TT.........................................T....T.................................................
H MEX32H     1800 ..TT.........................................T....T.................................................
H MEX33H     1800 ..TT.........................................T....T.................................................
H MEX34H     1800 ..TT.........................................T....T.................................................
H MEX35H     1800 ..TT.........................................T....T.................................................
H MEX8H      1800 ..TT.........................................T....T.................................................
G AF405706   1800 .................T................................T.......T.....................................A...
G AF160501   1800 .................T................................T.......T.....................................A...
G AB056513   1800 .................T................................T.......T.....................................A...
G AB056514   1800 .................T................................T.......T.....................................A...
G AB056515   1800 .................T................................T.......T.....................................A...
G AB056516   1800 .................T................................T.......T.....................................A...
G AB064310   1800 .................T................................T.......T.....................................A...
G AB064311   1800 .................T................................T.......T.....................................A...
G AB064312   1800 .................T................................T.......T.....................................A...
G AB064313   1800 .................T................................T.......T.....................................A...
G MEX30G     1800 .................T................................T.......T.....................................A...
G MEX32G     1800 .................T................................T.......T.....................................A...
G MEX33G     1800 .................T................................T.......T.....................................A...
G MEX34G     1800 .................T................................T.......T.....................................A...
G MEX35G     1800 .................T................................T.......T.....................................A...
G MEX8G      1800 .................T................................T.......T.....................................A...
                                   stop codon                                                                   stop codon
```

Fig. 2. Nucleotide sequences spanning (a) core promoter and (b) precore gene in HBV genotypes A–H. The reference sequence is shown for the AB014370Ae. The precore region includes pregenome encapsidation signal. Positions of the double mutation (A1762T and G1764A), T1753C, and C1858T making a pair with G1896A are indicated by inverted triangles. Two stop codons in the precore region are shaded.

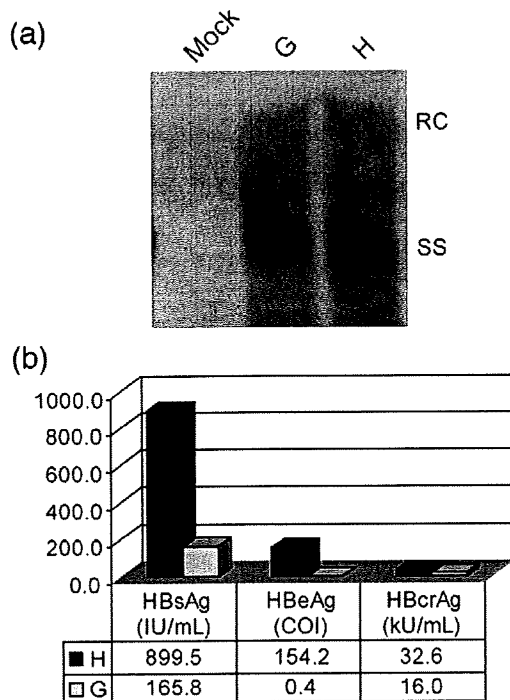

Fig. 3. Intracellular expression of HBV DNA and antigens. (a) The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. RC; relaxed circular double stranded DNA forms. (b) HBsAg, HBeAg and HBcrAg levels were also compared.

Coinfection of mice with HBV/H and G by inoculation with a mouse passage of G-on-H superinfection

Another 3 chimeric mice (ChiM202-17, ChiM212-22 and ChiM314-12) received serum from sacrificed ChiM_H2 with G-on-H superinfection taken at week 34 when the HBV/G and H DNA was around 5×10^6, 10^8 copies/mL, respectively (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^6 copies of HBV DNA, were similar among the 3 chimeric mice. Despite receiving the inoculation with a mouse passage supposedly containing HBV/G strain, the HBV/G DNA was not detectable until week 4 after the passage. At the week 4 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, HBV/G started to increase and plateaued around 10^8 copies/mL at week 16 (Fig. 4d).

Cloning and sequencing HBV DNA in chimeric mice coinfected with HBV/H and G

HBV DNA clones from sera of the ChiM_H1 and ChiM_H2 sampled at 26 and 34 weeks, respectively (Fig. 4a and b) included those of HBV/H and G invariably. At least 5 clones were propagated and completely sequenced in each serum; but no mutation was observed when the clones were compared to the original inoculum of either genotype. No evidence of recombinations was detected between HBV/H and G on the basis of complete genome analyses.

Pathology in the liver of chimeric mouse infected with HBV/G and H

Fig. 5a shows histology of liver of a chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1). The mouse coinfected with HBV/G and H revealed fibrosis of stage 1 (F1) and inflammation of grade 2 (A2) with Hematoxlin–Eosin and Masson's trichrome stain (Fig. 5a), whereas the mouse monoinfected with HBV/H had no fibrosis (Fig. 5b). ChiM_H2 also had F1A1 at week 34, but ChiM_H3 was not available for histological examination due to sudden death. Interestingly, a chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24 (Fig. 5c). It might be difficult to evaluate its statistical significance due to small number.

Discussion

The HBV/G was initially reported in the US, Canada and Europe in coinfection with HBV/A (Kato et al., 2002a,b; Osiowy and Giles, 2003; Stuyver et al., 2000), which is the one of the prevalent genotypes in both of the regions. Recently, coinfection of HBV/G with H as well as G/C recombinant were reported in Mexico and Thailand, respectively (Sanchez et al., 2002, 2007; Suwannakarn et al., 2005), and accumulated data indicated trend to preferential occurrence of the HBV/G coinfection in population of MSM and IDU, possibly via particular infectious routes of transmission. A phylogenetic analysis of the complete genome of HBV/G strains isolated in the present study indicated their close relationship with those previously reported, despite the fact that they were all isolated from HBV carriers infected with different genotypes that were prevalent in the respective area. These suggest that the HBV/G is genetically homologous and has no specific preference for HBV genotype to be coinfected. Thus, the HBV/G may be found among HBV carriers in any part of the world regardless of the prevalent genotype.

Invariably coinfected with other genotypes, HBV/G was once deemed a defective virus that cannot replicate by itself. Recently, however, Chudy and his associates reported plasmapheresis and platelet donor who was infected with HBV/G alone and had transmitted it to two recipients in look-back studies (Chudy et al., 2006). Since the donor and two recipients were not coinfected with HBV of the other genotypes by the reversed hybridization assay (Hussain et al., 2003), capable of detecting HBV infection of any genotype in low titers, the authors concluded that HBV/G can monoinfect human beings without help from coinfecting HBV of other genotypes (Chudy et al., 2006).

The uPA/SCID mouse with the liver replaced for human hepatocytes (Heckel et al., 1990) is a very useful model for studies on hepatitis virus infection and replication *in vivo* (Dandri et al., 2001; Tsuge et al., 2005). Using this model we have previously demonstrated that despite of apparent dependence on other genotype strain (HBV/A or HBV/C) in replication, the viral load of the HBV/G eventually outgrows and takes over the coinfecting strain in the chimeric mice (Sugiyama et al., 2007).

In the present study, the chimeric mice had undetectable HBV DNA in the serum when monoinfected with HBV/G, whereas superinfection on HBV/H enhanced replication of the HBV/G. This observation is concordant with previous experimental study which indicated a rapid takeover in mice initially infected with HBV/A or C and superinfected with HBV/G (Sugiyama et al., 2007). The takeover was significantly enhanced in mice infected with HBV/G on HBV/A when compared to HBV/G on HBV/C; with the mean time required for a 10-fold increase (log time) in the HBV/DNA level 1.6 vs. 3.3 weeks, respectively (Sugiyama et al., 2007). Interestingly, the present study demonstrates that pattern of the genotypic interaction between HBV/G and H was similar to that described for HBV/G on C; with the HBV DNA log time 3.0 weeks, suggesting that HBV/G is better sustained in human population by its association with HBV/A than with other HBV genotypes. These experimental results were also supported by clinical observations of the takeover which was demonstrated in a patient coinfected with HBV/G and A by cloning and sequencing (Kato et al., 2002a). However, such a heavy dependence of HBV/G on other genotype strain does not seem to require intergenotypic recombination between them, as no recombination event was observed in chimeric mice coinfected with HBV/G and any other genotype strain. The mechanism how HBV/G depends on other genotypes for replication had been pursued in cotransfection experiments in Huh7 cells; our previous *in vitro* study showed that trans-complementation with core protein of HBV/A would be required for HBV/G to replicate

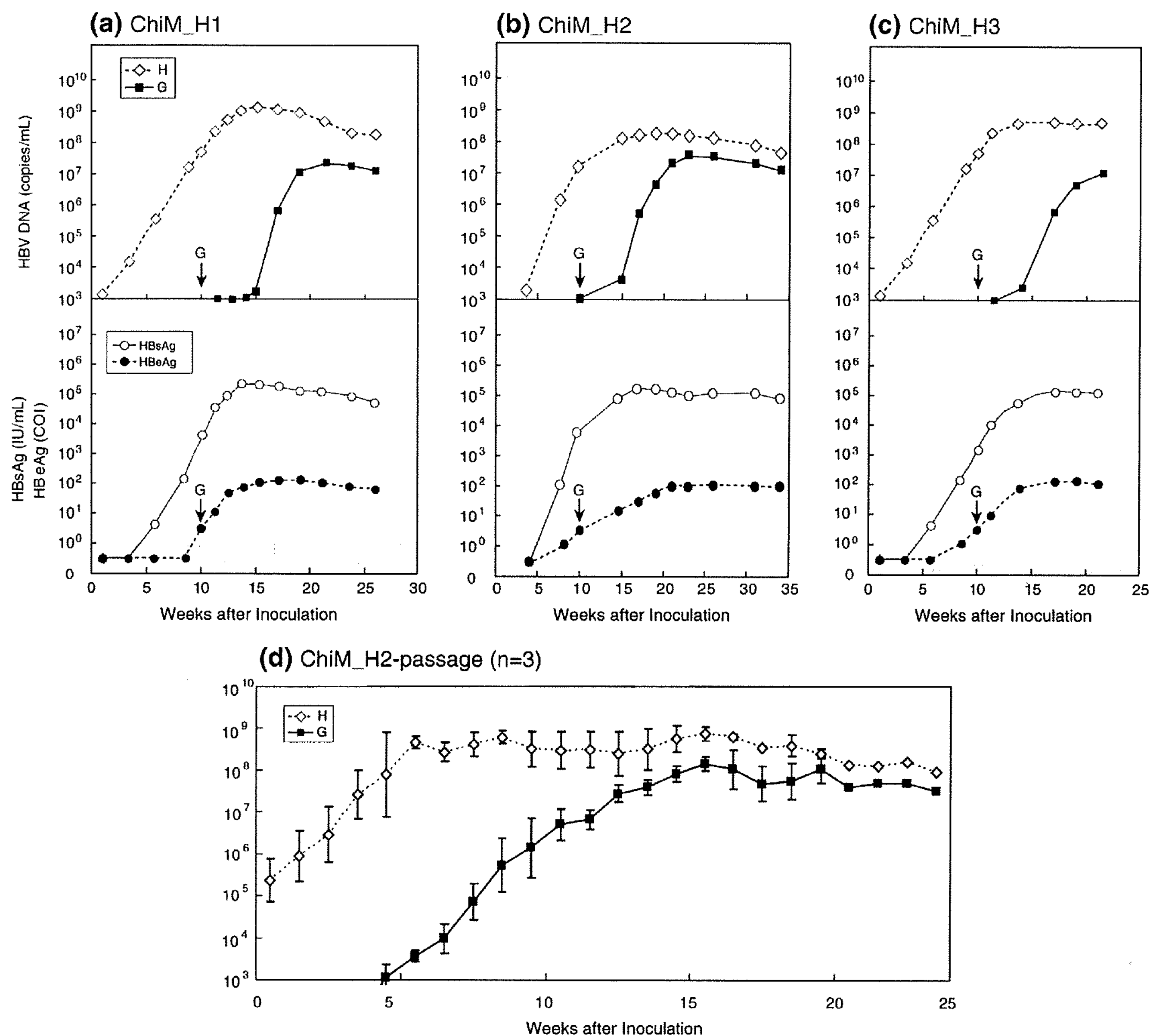

Fig. 4. Superinfection with HBV/G on chimeric mice infected with HBV/H [(a) ChiM_H1, (b) ChiM_H2, (c) ChiM_H3]. Inoculation with genotype G is indicated by large arrows. The dynamics of HBV DNA, HBsAg and HBeAg assessed superinfection of 3 chimeric mice (ChiM_H1-H3) are shown in panels a–c. Another 3 chimeric mice received serum from ChiM_H2 with G-on-H superinfection taken at week 34 when HBV/G DNA plateaued around 10^7 copies/mL (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^6 copies of HBV DNA, were similar among the 3 chimeric mice. Mean HBV DNA and standard deviation (bar) were shown in panel d.

actively (Sugiyama et al., 2007), suggesting that the replication of HBV/G might be also enhanced by core protein of HBV/H. Two other functional analyses of HBV/G had been already reported. Kremsdorf et al. have proposed the involvement of polymerase encoded by HBV/G in active replication (Kremsdorf et al., 1996) and Li et al. showed that lack of HBeAg expression rather than a replication defect could be the primary determinant for the rare occurrence of HBV/G monoinfection (Li et al., 2007). Hence, possibility remains for other viral elements beyond core protein from coinfecting genotypes to enhance the replication of HBV/G. Further studies are needed to elucidate the mechanism of HBV/G replication by cotransfection of other proteins beyond core protein of HBV/H or construction of domain-switch experiments between the genome of HBV/G and H.

Considering that coinfection with HBV/G may be associated with pathological manifestations, liver histology was investigated in tissue obtained from the mice used in this study. The chimeric mouse co-infected with HBV/G and H had developed fibrosis and inflammation (F1–2, A1–2) in the liver that was not observed in mice with HBV/G or H monoinfection. This might be supported by clinical data in Mexico; Mexican patients infected with the most prevalent HBV/H have milder liver damage when it is in monoinfection rather than in coinfection with other genotypes (personal communication). Our recent study also showed fibrosis of F1–F2 stage in the majority of the mice superinfected with HBV/G on A or C (Sugiyama et al., 2007). Clinically, Lacombe and her colleagues reported more severe fibrosis in human immunodeficiency type-1 (HIV)-positive French patients who were infected with HBV/G than the others (Lacombe et al., 2006). Taken together, clinical and experimental observations indicate that in immunodefficient conditions HBV/G possesses stronger disease-inducing capacity when it is coinfected with other genotype. Unfortunately, studied patients did not have exact diagnosis due to neither histopathological examination nor abdominal ultrasonography, although they were asymptomatic.

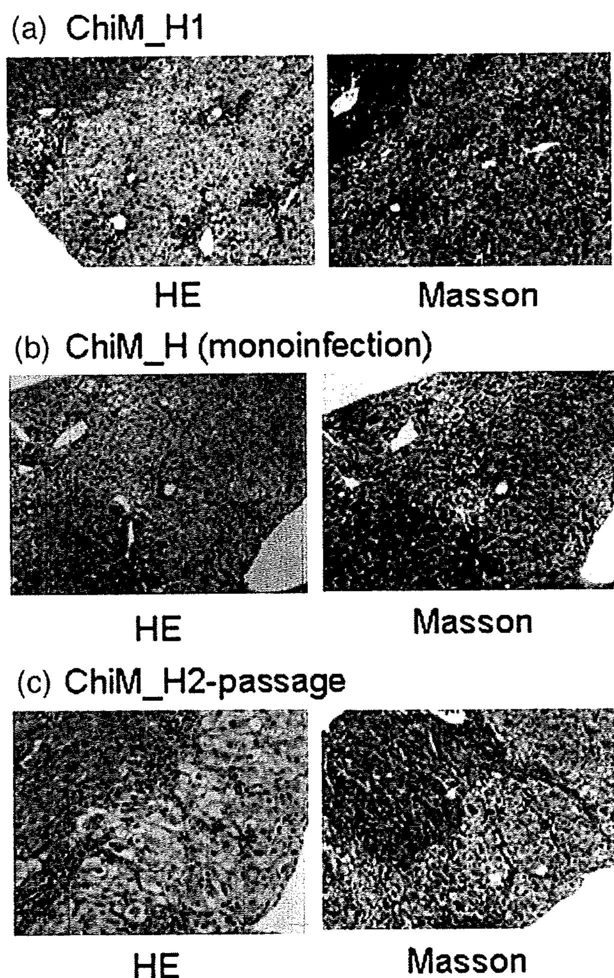

Fig. 5. Liver histology in chimeric mice superinfected with HBV/G on H. (a) A chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1) revealed F1A2 with Hematoxlin–Eosin (HE) and Masson's trichrome stain. (b) A chimeric mouse 24 weeks after monoinfection with HBV/H had no fibrosis. The peak of HBV DNA was around 10^8 copies/mL. (c) Another chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24.

Further prospected studies are required to investigate whether HBV DNA levels (viral replication) and/or other factors would affect liver fibrosis in immunosuppressive conditions.

Two remarkable viral genomic characteristics of the HBV/G have been established by previous reports; one of them is the unique insertion of 36 bp in the core gene, which is shared by all HBV/G strains studied herein and previously (Fig. 2). Although screening of the entire DNA Genome Bank (BLAST search) did not reveal any homologous to the insertion parts within other sequences including those of human, we took the advantage of these peculiar insertion sequences for designing the type-specific primers to be used for screening (Kato et al., 2001) and quantification of the genotype (Sugiyama et al., 2007). In respect to the function of this insertion, it may induce modifications of both the encapsidation signal sequence and the core protein structure, as previously proposed (Junker-Niepmann et al., 1990). Furthermore, HBV/G strains include two stop codons in the precore region at positions 2 and 28, both of which prohibit the translation of the HBeAg precursor (Carman et al., 1989; Okamoto et al., 1990), assumably resulting in HBeAg-negative phenotype of all HBV/G strains. In this study all patients infected with HBV/G, however, were positive for HBeAg. The coinfection with HBV/H would explain the presence of HBeAg in individuals infected with HBV/G, which would be consistent with a previous report (Kato et al., 2002a).

In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H prevalent in Mexico. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions. Further epidemiological, clinical and *in vitro* studies are required to confirm the clinical manifestation of the HBV/G coinfection with various genotypes and evaluate its genotypic peculiarities.

Methods

Serum samples

Nineteen HBsAg-positive sera were obtained from previously described MSM cohort (age range 22–30 years) in Mexico (Sanchez et al., 2007). HBeAg was detected with chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan).

Determination of HBV genotypes and the complete sequences of HBV

Initially, HBV genotypes were determined by EIA with monoclonal antibodies directed to the preS2 epitopes (Usuda et al., 1999, 2000), with use of commercial kits (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd., Tokyo, Japan), allowing the determination of HBV/A–F and HBV/H. HBV/G was confirmed by the G-specific PCR with one of the primers deduced from the sequence of 36 nt. insertion in the core gene (Kato et al., 2001). The complete genomes were further determined. In brief, two partially overlapping fragments were amplified by nested PCR using two sets of primers with LA Taq (TaKaRa Bio Inc., Tokyo, Japan) (Table 1). Similarly, the complete genomes of HBV/H strains were also amplified as two overlapping fragments using different set of primers shown in the Table 2. Thereafter, the PCR products were cloned in a plasmid pGEM-T easy vector (Promega Corp., Madison, WI, US) and sequenced using sequencing primers (Tables 1 and 2) with Prism Big Dye (Applied Biosystems, Foster City, CA, US) on the ABI 3100 DNA automated sequencer. Reference sequences for comparative analyses were retrieved from the DDBJ/EMBL/GenBank database.

Plasmid constructs of HBV DNA

HBV DNA from a Mexican patient (MEX33) was extracted from 100 μL of serum using the QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Two overlapping fragments, fragment A and fragment B approximately 1700 bp long, covering the entire genome

Table 1
HBV DNA oligonucleotide primers for complete genomes of genotype G

Primer	Nucleotide sequence (5′–3′)	Position*	Polarity
1) Fragment A			
HBVG3157F	CCTCCTGCCTCCACCAATCG	3157–3176	Sense
HBVG1917R	AGCCAAAAAGGCCATATGGCA	1937–1917	Antisense
HBVG3187F	AGGCAGCCTACTCCCATCTC	3187–3206	Sense
HBVG1797R	CATGGTGCTGGTGCGCAGAC	1816–1797	Antisense
2) Fragment B			
HBVG1601F	ACGTTACATGGAAACCGCCA	1601–1620	Sense
HBVG103R	GATTGACGAGATGTGAGAGGCA	124–103	Antisense
HBVG1630F	CTCATCATCTGCCAAGGCAGT	1630–1650	Sense
HBVG56R	GAACTGGAGCCACCAGCAGG	75–56	Antisense
3) Sequencing primers			
HBSF2	CTTCATCCTGCTGCTATGCCT	407–426	Sense
HBVG894F	AAGTTGGGGTACTTTGCCAC	894–913	Sense
HBVG1013R	TGGGTAAAAGGAGCAGCGAAAC	1034–1013	Antisense
HBVG2052F	GGGAATCCTTAGAGTCCTCTG	2052–2072	Sense
HBVG2491F	TTCCTTGGACTCACAAGGTG2	2491–2510	Sense

* Nucleotide position of reference sequence (AB056513).

Table 2
HBV DNA oligonucleotide primers for complete genomes of genotype H

Primer	Nucleotide sequence (5′–3′)	Position	Polarity
1) Fragment A			
HBVH55F	TCCTGCTGGTGGCTCC	55–70	Sense
HBVH1801R	GTTGCATGGTGCTGGTGAAC	1820–1801	Antisense
HB6R	AACAGACCAATTTATGCCTA	1803–1784	Antisense
2) Fragment B			
HBVH1611F	GAGACCACCGTGAACGCCC	1611–1629	Sense
HBVH285R	GCCAGGACACCCGGGTGGTA	304–285	Antisense
HBVH229R	CGAGTCTAGACTCTGTGGTATTGTGAGG	256–229	Antisense
3) Sequencing primers			
HB2F	TGCTGCTATGCCTCATCTTC	414–433	Sense
HBVH760F	GCCAAATCTGTGCAGCATCTTGAG	760–783	Sense
HB5F	CTCTGCCGATCCATACTGCGGAA	1256–1278	Sense
HBVH1859F	ACTGTTCAAGCCTCCAAGCTGT	1859–1880	Sense
HBVH2415F	GTCGCAGAAGATCTCAATCTC	2415–2435	Sense
HBVH2814F	GGGTCACCATATTCCTGGGAA	2814–2834	Sense

*Nucleotide position of reference sequence (AB059659).

of HBV, were amplified by nested PCR (primers sequences shown in Table 2). Primers used for fragment A were HBVH55F and HBVH1801R for 1st PCR and HBVH55F and HB6R for 2nd PCR. Primers used for fragment B were HBVH1611F and HBVH285R for 1st PCR and HBVH1611F and HBVH229R for 2nd PCR. Then these fragments were ligated into pGEM-T vector (Promega, Madison, WI) and cloned in DH5α cells. Ten clones each (pGEM-fragA-1 to 10, pGEM-fragB-1 to 10) were obtained and the nucleotide sequences were determined. As reported previously (Fujiwara et al., 2005; Sugiyama et al., 2006), these fragments were constructed into the pUC19 vector deprived of promoters (Invitrogen Corp., Carlsbad, CA) by digestion with HindIII and EcoRI, resulting in 1.24-fold the HBV genome, just enough to transcribe over-sized pregenome and precore mRNA. Cloned HBV DNA sequences were determined with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer. Based on our previous report (Sugiyama et al., 2007), a plasmid of HBV/G with 1.24-fold the HBV genome was also constructed in this study.

Cell culture and transfection

Huh7 cells were transfected with plasmids equivalent to 5 μg of HBV DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by enzymatic activity of secreted alkaline phosphatase (SEAP) in the supernatant of culture with addition of 0.5 μg of reporter plasmids expressing the SEAP.

Determination of HBV markers

HBsAg and HBeAg were determined by chemiluminescent enzyme immunoassay (CLEIA) with commercial kits in a fully automated Lumipulse CLEIA analyzer (Fujirebio Inc., Tokyo, Japan). HB core-related antigen (HBcrAg) was measured in serum using the CLEIA described previously (Kimura et al., 2002). Briefly, 150 μL of serum was incubated with 150 μL pretreatment solution containing 15% sodium dodecylsulfate at 60 °C for 30 min. The pretreated serum was added to a well coated with three monoclonal antibodies against denatured HBcAg and HBeAg. After washing, two other alkaline phosphatase-labelled monoclonal antibodies against denatured HBcAg and HBeAg were added as secondary antibodies. 200 μL substrate (AMPPD:(3-(2′-spiroadamantan)-4-methoxy-4- (3′-phosph oryloxy) phenyl-1, 2-dioxetane disodium salt) (Applied Biosystems, Bedford, MA, USA) solution was added and the assay tube was incubated for 5 min at 37 °C. HBcrAg assay with the relative chemiluminescence intensity was measured with chemiluminescent enzyme immunoassay (CLEIA) system for fully automated Lumipulse f CLEIA analyzer (Fujirebio Inc., Tokyo, Japan), and the HBcrAg concentration was estimated by comparison to a standard curve generated using recombinant HBeAg. In the present study, the cutoff value was tentatively set at 3.0 log U/mlmL. Sera containing over 7.0 log U/ml mL of HBcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Detection and quantification of serum HBV DNA

For HBV/G-specific real-time detection PCR (RTD-PCR), previously reported set of primers was used, where one of the primers contained sequence of the HBV/G unique 36-bp insertion (Sugiyama et al., 2007). For HBV/H DNA quantification, following primers were applied; HBVH29F: 5′-GTT CCA CCA AGC ACT GTT GG-′3, HBV229R: 5′-CGA GTC TAG ACT CTG TGG TAT TGT GAG G-′3. Amplification and detection were performed using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The quantification standard was prepared by serial dilution of a known amount of the cloned plasmid of HBV/G or H. The specificity of these primers was confirmed in every PCR run by dissociation curve analysis (ABI Prism 7700 dissociation curve software; Applied Biosystems). In assays for HBV DNA in mouse sera, in which only 10 μL of sample is used, the sensitivity of the assay allowed detection of 1000 copies/mL of HBV/G or HBV/H DNA.

Molecular evolutionary analysis

Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the 6-parameter method (Gojobori et al., 1982) in the Hepatitis Virus Database (Robertson et al., 1998). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). To confirm the reliability of the phylogenetic tree, bootstrap resampling test was performed 1,000 times (Felsenstein, 1985).

Examination for recombination

Intergenotypic recombination was searched for using the method of Robertson et al. (Robertson et al., 1995) with use of the SimPlot program and bootscanning analysis (Lole et al., 1999). The mean genetic distances were calculated with a window size of 200 bp and a step size of 50 bp in this study.

Southern blot hybridizations

Southern blot hybridizations were performed with a full-length probe of HBV/G or H by previous methods (Fujiwara et al., 2005). No significant differences were observed in the detection between internal control HBV DNA and each probe.

Inoculation of chimeric mice with the liver repopulated for human hepatocytes

$uPA^{+/+}/SCID^{+/+}$ mice with the liver repopulated for human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The human hepatocytes were obtained from a single donor (female, 6 years, African American). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co.Ltd, Tokyo, Japan). They were inoculated with HBV recovered from culture supernatants of Huh7 cells transfected with plasmids containing 1.24-fold HBV genome constructs of the HBV/G or H (Sugiyama et al., 2006, 2007).

Histopathological examination

Liver tissues were fixed in formaldehyde, embedded in paraffin and stained with hematoxylin–eosin (H–E) or Masson's trichrome. The fibrosis stage and inflammation grade were evaluated by independent expert pathologists.

Acknowledgments

We thank Mr. T. Nakamura and T Shimada (PhoenixBio Co., Ltd., Higashi-Hiroshima, Japan) for providing chimeric mice with a high replacement for hepatocytes, and Ms. Y. Yotani in our laboratory for constructing HBV clones.

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JOURNAL OF VIROLOGY, Aug. 2008, p. 8241–8242 Vol. 82, No. 16
0022-538X/08/$08.00+0 doi:10.1128/JVI.00793-08

When Should "I" Consider a New Hepatitis B Virus Genotype?

Recently, Huy et al. described a new hepatitis B virus (HBV) strain isolated in Vietnam (3) and claimed it to be a "new genotype," "HBV genotype I," with a complex recombination involving genotypes C, A, and G. We refute both claims.

Using complete genome sequence analysis of their single isolate, VH24 (AB231908), the authors documented an over 98% similarity with three other Vietnamese strains (2). Earlier, Hannoun et al. provided comprehensive information regarding those strains, showing recombination between genotype C and an unknown genotype in the pre-S/S region (2). Mean genetic divergence from genotype C of <8% in the entire genome and evidence of recombination had prevented the authors from assigning the strains to a new genotype. The same conclusion for the strains was reached by a later study using a new methodological approach (10). By providing neither additional information nor a new analytical approach, Huy et al. (3) surprisingly conclude that their strain, with those previously reported, represent a new genotype.

First, phylogenetic analysis of the complete genome of the four Vietnamese HBV isolates shows them to cluster with subgenotypes of C (C1 to C5) and to differ from genotype C by a mean nucleotide distance of only 7.0% ± 0.4%, which falls within the range of intragenotype and not intergenotype divergence (4). Furthermore, their conclusion of a "complex A/G/C recombination" arose from the use of Simplot software that has methodological limitations, which can be overcome by using GroupScanning (10). Reanalyzing AB231908 by using GroupScanning provides no strong evidence for recombination with known human or ape HBV genotypes in the pre-S/S regions (apart from two restricted regions, with association values of >0.5), in contrast to its consistent penetration into the genotype C clade from position 1600 (Fig. 1). In the pre-S/S regions, AB231908 formed variable, inconsistent outgroup associations with a range of genotypes, including A and G (originally identified as recombination partners by Huy et al. [3], using SimPlot) and with chimpanzee variants (Fig. 1, gray line; not included in the original analysis), a recombination partner even more improbable geographically than genotype A or G.

Finally, Huy et al. (3) "justified" assigning the four Vietnamese strains into a new genotype on the basis of seven "unique" conserved amino acids: His[56], Ala[60], Asn[87], Val[90], Val[91], Ile[136], and Lys[198]. From the databases, it is evident that His[56] is present in subgenotype B1 and genotype C; Ala[60] is the consensus for genotype D and present in subgenotypes C2 to C4; Val[90], found in only three of the four Vietnamese sequences, is present in subgenotype C2; Val[91] is common in genotype A; and Lys[198] is found in subgenotypes B1 to B4, C3, F1, and F2 and genotypes E and H. Ile[136] and Asn[87] are therefore the only amino acids unique to the four Vietnamese strains, a far-from-recognized criterion of HBV genotyping.

Since 1988, when nucleotide diversity of >8% in the entire genome was first proposed for genotyping (9), eight genotypes have been described and named A to H (1, 7, 8, 11), and their geographical distribution and clinical relevance have been extensively reported (5, 6). In addition to the eight currently recognized genotypes, intergenotype recombination generates novel HBV variants, with over 24 phylogenetically independent recombinant variants described (10, 13). These recombinants can spread in humans and develop specific distributions and epidemiology as shown for the B/C recombinant, which accounts for the majority of genotype B strains in mainland Asia (12). Since sequencing and phylogenetic analyses are widely available, numerous further reports on HBV variation can be expected. If every new recombinant is assigned to a new genotype, we would soon be running out of alphabet letters. Principles of HBV classification must be established and accepted by the international community of experts in the field in order to ensure that genotyping is consistent, relevant, and significant.

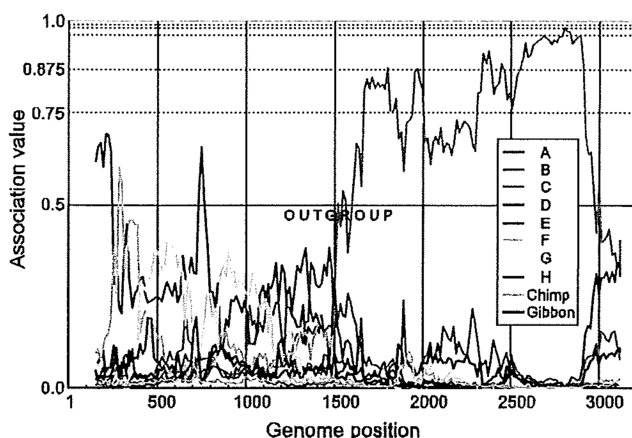

FIG. 1. GroupScanning analysis (10) of VH24 against reference groups of nonrecombinant HBV sequences of human genotypes A to H and nonhuman ape-derived variants (chimpanzee/gibbon) (n = 288), incorporating all HBV sequences used for recombination detection in the Huy et al. study (3). Association values of approximately 0.5 or lower indicate an outgroup position or no phylogenetic clustering with a reference group. Analysis of previously described Vietnamese variants (AF241407 to AF241409) produced almost identical results (data not shown).

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Ed. Note: *The author of the published article declined to reply.*

Early Dynamics of Hepatitis B Virus in Chimeric Mice Carrying Human Hepatocytes Monoinfected or Coinfected with Genotype G

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Of the 8 genotypes of HBV (genotypes A-H), genotype G is unique in that it has an insertion in the core gene and two stop codons in the precore region preventing the synthesis of hepatitis B e antigen. Most individuals with genotype G are coinfected with other genotypes, typically genotype A. Mice with severe combined immunodeficiency disease carrying human hepatocytes were infected with HBV particles propagated in Huh7 cells in culture. Mice monoinfected with genotype G did not raise detectable HBV DNA in serum, although products of the core gene emerged 4 to 8 weeks after inoculation. When they were superinfected with genotype A at week 10, however, HBV DNA of genotype A developed, which was replaced almost completely by that of genotype G within 10 weeks. Such a rapid takeover was also observed in mice initially infected with genotype A or C and superinfected with genotype G. Similar viral dynamics occurred in mice simultaneously coinfected with genotypes G and A. Takeover was markedly enhanced in mice inoculated with a serum passage containing genotype G with a trace of genotype A. Coinfection of mice with genotypes G and A induced abundant cellular steatosis along with increased fibrosis in the liver, which was not detected in mice monoinfected with genotype A or G. *Conclusion:* Genotype G can monoinfect chimeric mice at very low levels, and its replication increases maredly when coinfected with other genotypes. Coinfection with genotype G could enhance fibrosis under immunocompromised states. (HEPATOLOGY 2007;45:929-937.)

HBV infects an estimated 350 million people worldwide and causes 1 million deaths annually.[1] Eight genotypes of HBV have been classified by the sequence divergence in the entire genome exceeding 8% and have been assigned the names A through H in order of discovery.[2-6] HBV genotypes have distinct geographic distributions and can influence the severity of liver disease and response to antiviral therapies.[7-10] HBV genotypes are further divided into subgenotypes, such as A1/Aa and A2/Ae, B1/Bj and B2/Ba, and C1/Ce and C2/Cs.[11-13] These genotypes may influence clinical outcomes of HBV infection.[14,15]

HBV genotype G (HBV/G) was first described in 2000 among inhabitants of France and the state of Georgia.[5] It has an insertion of 36 base pairs in the core gene and two stop codons in the precore region.[5,16] Despite the inability in encoding hepatitis B e antigen (HBeAg), carriers of HBV/G possess it in serum.[5] They are usually coinfected with HBV of other genotypes, most frequently HBV/A, which is responsible for serum HBeAg.[17] Coinfection with HBV/C, F, and H has also been reported.[18-20] In spite of heavy dependence on other genotypes for replication, HBV/G outgrows them and eventually takes over the great majority of HBV DNA in the circulation.[16,17]

Recently, HBV/G DNA in low levels was reported in a German donor of plasmapheresis who had transmitted it to 2 recipients in look-back studies.[21] Hence, HBV/G would be able to infect recipients by itself. Furthermore,

Abbreviations: ChiM, chimeric mice; HBcrAg, HBV core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; RFLP, restriction fragment length polymorphism; SCID, severe combined immunodeficiency disease.

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Received August 12, 2006; accepted December 1, 2006

Supported in part by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan (H16-kanen-3), Scientific Research from the Ministry of Education (18590741), and Toyoaki Foundation.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21584

Potential conflict of interest: Nothing to report.

Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).