Hepatology Research 2007 doi: 10.1111/j.1872-034X.2007.00292.x

Original Article

Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine

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Aim: Lamivudine (LAM) has been widely used to treat chronic hepatitis B (CHB) patients, but the emergence of a LAM-resistant virus greatly limits its therapeutic efficacy. In this study, we tried to identify factors affecting the emergence of a LAM-resistant virus in CHB patients treated with LAM.

Methods: The subjects were 190 CHB patients in continuous LAM therapy (139 males, mean age 50 years, 87 HBeAg-positive). The mean duration of follow-up was 39 months (range 12–104). The initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) at 6 months.

Results: IVR was positive in 86% of the patients. The cumulative emergence rates of LAM-resistant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years. In univariate analysis, factors contributing to the emergence of LAM-resistant virus were baseline HBV DNA > 6.5 logcopies/mL ($P = 0.0044$), HBeAg-positivity ($P = 0.0062$), IBR ($P = 0.01$) and IVR ($P < 0.0001$). The cumulative emergence rates of LAM-resistant virus in IVR-positive and -negative patients were 4% and 41% at 1 year, and 41% and 79% at 3 years. In multivariate analysis, only IVR was an independent factor affecting the emergence of LAM-resistant virus ($P < 0.0001$).

Conclusion: IVR is a useful factor for predicting the emergence of LAM-resistant virus in CHB patients treated with LAM. For IVR-negative patients, therapeutic options other than LAM monotherapy should be used because of the high incidence of the emergence of LAM-resistant virus.

Key words: chronic hepatitis B, initial viral response, lamivudine monotherapy, lamivudine-resistant virus

INTRODUCTION

MORE THAN 350 million people are chronically infected with hepatitis B virus (HBV) worldwide.[1] Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death. Nucleos(t)ide analogs are widely used to suppress HBV replication and the progression of HBV-related liver diseases. Lamivudine (LAM), the first approved nucleoside analog for chronic HBV infection, has been shown to suppress viral replication and disease activity.[2] In addition, LAM therapy has recently been reported to reduce the incidence of HCC, the risk of major complications and to improve survival.[3,4] However, the relatively high incidence of LAM resistance is a serious problem in the case of LAM therapy for chronic HBV infection. The emergence of LAM-resistant HBV is linked to the reappearance of active viral replication, followed by the worsening of liver disease.

LAM-resistant HBV is based on point mutation within the YMDD motif of the reverse transcriptase domain of

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Received 24 August 2007; revision 9 October 2007; accepted 14 October 2007.

HBV (YMDD mutation).[5,6] The emergence rates of the mutant virus have been reported to be 24% at 1 year and 70% at 4 years from the start of treatment.[7]

Recent work has shown that newly developed nucleos(t)ide analogs, such as adefovir dipivoxil (ADV) and entecavir (ETV), are also useful agents for controlling patients with chronic HBV infection.[8-11] In particular, the drug-resistant mutant virus has been reported to appear less frequently in cases of treatment with ADV and ETV than with LAM.[12,13] For this reason, LAM has been replaced by ADV and ETV for the treatment of chronic hepatitis B. However, there are still a considerable number of patients with chronic HBV infection who are already on continuous LAM therapy. Thus, further clarification is needed of what factors influence the emergence of the LAM-resistant HBV in LAM treatment for chronic HBV infection.

For a more precise evaluation, we investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus in patients with chronic hepatitis B treated with LAM.

METHODS

Patients and treatment

THIS STUDY WAS conducted at nine institutions in the Osaka area of Japan (Osaka Police Hospital, Osaka Minami Medical Center, Osaka Kouseinenkin Hospital, Osaka Rousai Hospital, Kinki Central Hospital, Ikeda City Hospital, Osaka National Hospital, Otemae Hospital and Osaka University Hospital). The subjects were 190 consecutive patients with chronic hepatitis B who underwent continuous LAM therapy for more than 12 months. All patients tested positive for hepatitis B surface antigen (HBsAg) or had detectable levels of HBV DNA in their sera by the polymerase chain reaction (PCR)-based method (for 100 patients)[14] or the transcription-mediated amplification (TMA) method (for 90 patients).[15] Exclusion criteria were patients with antihepatitis C antibody, antihuman immunodeficiency virus antibody and other forms of liver diseases (alcoholic liver disease, drug-induced liver disease and autoimmune hepatitis). Forty-one (22%) patients had previously received interferon (IFN)-α therapy for 24 weeks.

All patients were treated with 100 mg of LAM daily. After the beginning of the therapy, liver function tests and HBV DNA were measured every other month for the first 6 months and every two months thereafter. HBeAg and anti-HBe were tested every 6 months. In 33 patients (18%), combination therapy with IFN was carried out for the initial 6 months. Three or six megaunits of natural IFN-á were administered daily for the first 2 weeks and three times a week thereafter, followed by LAM monotherapy. The mean follow-up period of the 190 patients was 39 (range 12–104) months. The LAM-resistant YMDD mutant virus was detected by the PCR-enzyme-linked minisequence (ELMA) assay[16] when the virological or biochemical breakthrough was observed. The YMDD mutant virus was found in 86 (45%) patients during follow-up. Fifty-eight of these patients underwent ADV therapy in addition to ongoing LAM treatment and were excluded from the follow-up when ADV administration began. In this study, the initial viral response (IVR) was defined as HBV DNA < 4.0 logcopies/mL, and the initial biochemical response (IBR) as normalization of alanine aminotransferase (ALT) (<40 IU/L) after 6 months of therapy.

Table 1 Patient characteristics

Gender (male/female)	139/51
Age (years)	50 ± 11
Chronic hepatitis/liver cirrhosis	113/77
Hepatocellular carcinoma	14 (7%)
AST (IU/L)	122 ± 157
AST (IU/L)	177 ± 236
ALT (≤1/1–2/2–5/>5 × ULN)	22/53/65/50
Platelet (10^4/mm^3)	12.6 ± 5.1
Prothrombin time (%)	71.5 ± 16.6
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)
HBeAg (positive/negative)	87/103
Combination with interferon	33 (17%)
Duration of treatment (months)	38.9 ± 17.5

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ULM, upper limit normal.

The patients' clinical characteristics are shown in Table 1. There were 139 males and 51 females, ranging in age from 25 to 75 (mean 50) years. Of them, 113 (59%) patients were diagnosed as having chronic hepatitis and the remaining 77 patients (41%) as having cirrhosis according to liver histology and/or the imaging procedure. HCC was developed in 14 (7%) patients. The aspartate aminotransferase (AST) at baseline was 122 ± 157 IU/l, and the ALT at baseline was 177 ± 236 IU/L. Abnormal ALT was observed in 168 (88%) patients. Eighty-seven patients (46%) tested positive for HBeAg. The median HBV DNA at baseline was 6.5 (range 3.0 to 7.6<) logcopies/mL.

HBV testing

HBsAg, hepatitis B e antigen (HBeAg) and antihepatitis B e antibody (anti-HBe) were examined by chemiluminescent immunoassay or enzyme immunoassay.

The HBV DNA level was measured by the PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan)[14] or the TMA method (TMA-HPA; Fujirebio, Tokyo, Japan),[15] which have lower detection limits of 2.6 and 3.7 logcopies/mL, respectively. The LAM-resistant YMDD mutant virus was examined by the PCR-ELMA method.[16]

Statistical analysis

Comparisons of categorical and continuous variables between groups were done by the χ^2-test, Student's *t*-test and Mann–Whitney's *U*-test. The cumulative emergence rates of LAM-resistant virus were evaluated with the Kaplan–Meier's curve and the differences between groups were analyzed by the log–rank test. For multivariate analysis to investigate factors affecting the cumulative emergence rate of LAM-resistant virus, Cox proportional hazard regression analysis was carried out. A *P*-value of less than 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

Therapeutic efficacy and the emergence of LAM-resistant mutant virus

AMONG THE 190 patients with chronic hepatitis B who underwent continuous LAM therapy, reduction of HBV DNA to less than 4 logcopies/mL was observed in 86% (163/190) at 6 months, 89% (151/170) at 1 year, 88% (83/94) at 2 years and 89% (48/54) at 3 years of the treatment. Normalization of ALT was achieved by 77% (146/190) at 6 months, 83% (141/170) at 1 year, 81% (76/94) at 2 years and 83% (45/54) at 3 years. Among the 87 HBeAg-positive patients, HBeAg was cleared in 22% (19/86) at 6 months, 26% (21/80) at 1 year, 22% (11/50) at 2 years and 43% (16/37) at 3 years. As for the virological and biochemical response at 6 months of therapy, 163 (86%) of the patients achieved IVR, whereas IBR was seen in 146 (77%) of patients.

When the various patient characteristics were compared between IVR-positive and -negative patients (Table 2), HBV DNA at baseline tended to be lower in patients showing IVR (median 6.5 [range 3.0 to 7.6<] logcopies/mL) than in those who did not show IVR (median 7.3 [range 4.3 to 7.6<] logcopies/mL) ($P < 0.0001$). IVR-negative patients had higher HBeAg positivity at baseline than IVR-positive patients (81% vs 40%, $P = 0.01$). As for the emergence of LAM-resistant mutant virus during follow-up, it was detected more frequently in IVR-negative patients (21/27, 78%) than in IVR-positive patients (65/163, 40%) ($P = 0.002$).

Among the 190 patients examined in this study, the emergence of LAM-resistant YMDD mutant virus occurred in 86 (45%) patients during follow-up. The cumulative probabilities of the emergence of the YMDD mutant virus were 10% at 1 year, 30% at 2 years and 46% at 3 years.

Factors affecting the emergence of LAM-resistant mutant virus

Factors affecting the cumulative probability of the emergence of the YMDD mutant virus were investigated using

Table 2 Comparison of patient characteristics between IVR-positive and -negative patients

	IVR ($n = 163$)	Non-IVR ($n = 27$)	*P*-value
Gender (male/female)	118/45	21/6	NS
Age (years)	50 ± 11	48 ± 12	NS
Chronic hepatitis/liver cirrhosis	91/72	22/5	NS
Hepatocellular carcinoma	13 (8.0%)	1 (4%)	NS
AST (IU/L)	131 ± 167	69 ± 34	NS
ALT (IU/L)	190 ± 252	100 ± 55	NS
ALT (≤1/1–2/2–5/>5 × ULN)	21/43/52/47	1/10/13/3	NS
HBV DNA (logcopies/mL)	6.5 (3.0–7.6<)	7.3 (4.3–7.6<)	<0.0001
HBeAg (positive/negative)	65/98	22/5	0.01
Combination with interferon	27 (17%)	6 (22%)	NS
Emergence of LAM-resistant viruses	65 (40%)	21 (78%)	0.002
Duration of treatment (months)	39.2 ± 17.2	37.3 ± 19.1	NS

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IVR, initial viral response; LAM, lamivudine; NS, not significant; ULN, upper limit normal.

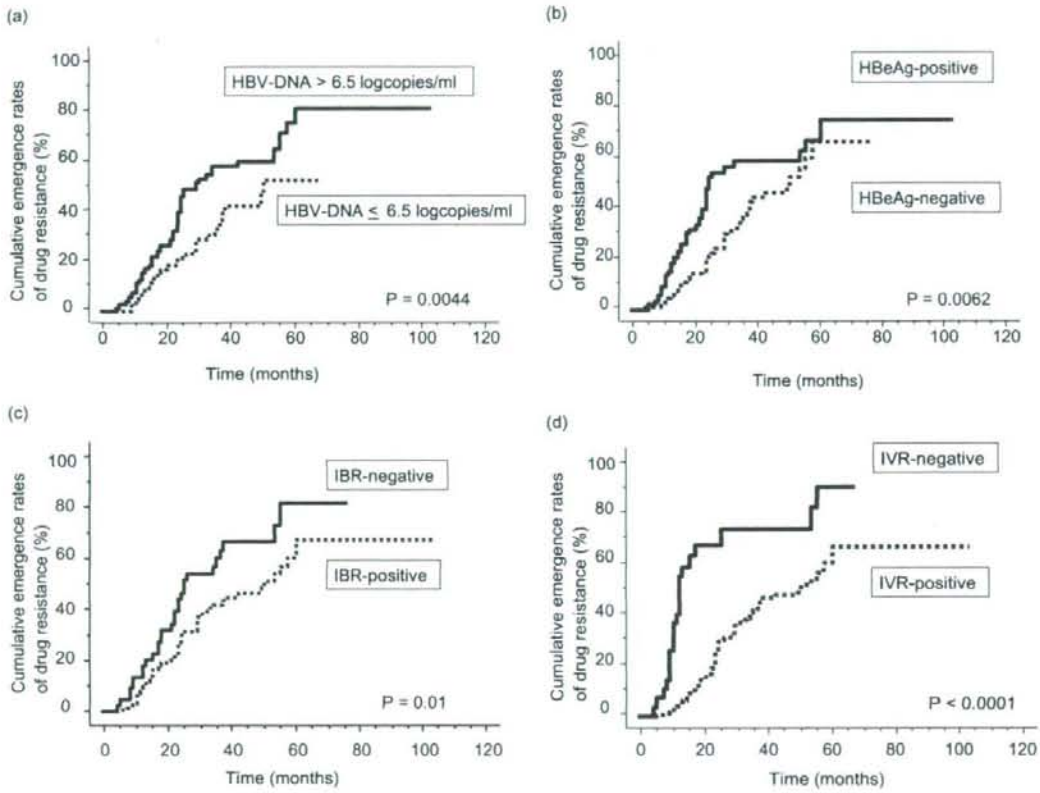

Figure 1 Cumulative emergence rate of lamivudine (LAM)-resistant virus in patients with chronic hepatitis B virus (HBV) infection treated with LAM according to: (a) HBV DNA at baseline; (b) hepatitis B e antigen (HBeAg) status; (c) the presence or absence of initial biochemical response (IBR); and (d) the presence or absence of initial viral response (IVR).

both univariate and multivariate analyses. Nine baseline and on-treatment factors – gender, age, liver disease (chronic hepatitis or cirrhosis), ALT at baseline, HBeAg positivity, HBV DNA at baseline, combination therapy with IFN-α, presence of IBR and presence of IVR – were examined. The cumulative emergence of LAM-resistant virus was significantly higher in patients with baseline HBV DNA > 6.5 logcopies/mL than in those with HBV DNA ≤ 6.5 logcopies/mL ($P = 0.0044$) (Fig. 1a). HBeAg-positive patients revealed a significantly higher emergence rate of the LAM-resistant virus than HBeAg-negative patients ($P = 0.0062$) (Fig. 1b). A significant difference was also seen in the cumulative emergence of the YMDD mutant virus between IBR-positive and -negative patients ($P = 0.01$) (Fig. 1c). Furthermore, the cumulative emergence of LAM-resistant mutant virus was much higher in the IVR-negative patients than in the IVR-positive patients ($P < 0.0001$) (Fig. 1d). The cumulative emergence rates of LAM-resistant virus in the IVR-positive and -negative patients were 4% and 41% at 1 year, 25% and 69% at 2 years, and 41% and 79% at 3 years, respectively. Gender, age, liver disease, ALT at baseline and combination therapy of IFN-α did not show a significant relation with the emergence of the YMDD mutant virus. When factors influencing the higher cumulative emergence of LAM-resistant virus were searched for by multivariate analysis, only the absence of IVR was selected as a significant independent factor ($P < 0.001$) (Table 3), with high HBV DNA, HBeAg positivity and the absence of IBR not being selected.

Table 3 Factors associate with emergence of LAM-resistant virus determined by multivariate analysis

	Hazard ratio	95% confidence interval	*P*-value
Gender			
0: male	1	0.497–1.455	0.55
1: female	1.176		
Age			
0: ≤50	1	0.640–1.700	0.87
1: >50	0.959		
Chronic hepatitis/liver cirrhosis			
0: CH	1	0.656–1.740	0.79
1: LC	0.935		
Pretreatment ALT (IU/L)			
0: ≤200	1	0.605–1.818	0.87
1: >200	0.953		
HBV DNA (logcopies/mL)			
0: ≤6.5	1	0.394–1.125	0.13
1: >6.5	1.502		
HBeAg			
0: negative	1	0.499–1.337	0.42
1: positive	1.225		
Combination therapy with interferon			
0: no	1	0.410–1.303	0.29
1: yes	1.368		
IBR			
0: positive	1	0.483–1.312	0.37
1: negative	1.256		
IVR			
0: positive	1	0.159–0.536	<0.001
1: negative	3.425		

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; IBR, initial biochemical response; IVR, initial viral response; LAM, lamivudine.

DISCUSSION

IN LAM THERAPY for patients with chronic HBV infection, the emergence of a LAM-resistant YMDD mutant virus is a serious problem, because it inevitably restricts the antiviral efficacy of LAM. To resolve this, detailed studies are needed to identify factors related to the emergence of the YMDD mutant virus. To date, a few investigators have suggested male gender, advanced age, high baseline ALT, the presence of severe acute exacerbation of the liver disease, high baseline HBV DNA and HBeAg-positivity as possible predictors of the emergence of LAM-resistant virus.[7,17,18] Lower body surface area was also reported as a significant factor for virological and biochemical therapeutic effect.[19] In the present study, we studied 190 patients with chronic hepatitis B treated with LAM and investigated baseline and on-treatment factors affecting the emergence of LAM-resistant mutant virus. Univariate analysis revealed that two baseline factors, high HBV DNA and HBeAg positivity, had a relation to the high incidence of the YMDD mutant virus, which is consistent with previous reports.[7,17,18] In addition, two on-treatment factors, IBR and IVR, were found to be correlated with the emergence of LAM resistance. Patients who did not show IVR had a 3.4-fold higher incidence of the emergence of the YMDD mutant virus than those who did show IVR. This agrees with a previous report that the HBV DNA level after 6 months of therapy may be a determinant for subsequent occurrence of a LAM-resistant mutant virus.[20] Multivariate analysis showed that only the absence of IVR was a significant factor contributing to the emergence of LAM-resistant virus. Baseline HBV DNA and HBeAg status were not selected as significant factors by multivariate analysis probably because of the tendency for higher HBV DNA and high frequency of HBeAg positivity in IVR-negative patients compared with IVR-positive patients. It is particularly interesting that the absence of IVR, rather than other baseline and on-treatment factors, was a powerful independent pre-

dictor for the emergence of the YMDD mutant virus in LAM therapy for chronic HBV infection. This means that IVR of an on-treatment factor is very important for good therapeutic effect and the stage for the next therapeutic strategy can thus be set in a new light with this information.

Our results showed that approximately one-seventh of the patients with chronic hepatitis B treated with LAM did not achieve IVR. In the non-IVR patients, the antiviral therapeutic regimen should be amended due to the frequent emergence of LAM-resistant virus. Recently, new nucleos(t)ide analogs have become available for the treatment of chronic HBV infection. ETV has been reported to be more effective for the reduction of HBV DNA and the less frequently induced drug-resistant mutant virus than LAM in "naïve" patients with chronic hepatitis B who had not previously received nucleos(t)ide analog therapy.[10,11] ETV was also effective in patients with chronic HBV infection showing LAM resistance,[21] but the emergence rate of the ETV-resistant virus was considerably higher in LAM-resistant patients than in naïve patients.[13,22] This is because the ETV-resistant HBV strain is established by LAM-resistant YMDD mutation plus additional mutation(s) at the amino acid position(s) 184, 202 and/or 250 within the reverse transcriptase domain of HBV.[22] According to these findings, switching from LAM to ETV may be useful for treating patients who do not achieve IVR on LAM administration. This should be done before the emergence of LAM-resistant YMDD mutant virus so as not to reduce the therapeutic efficacy of ETV. In clinical practice, there are still a number of patients who have already been on continuous LAM therapy, although the current first choice drug for patients with chronic HBV infection is ETV. In our opinion, foregoing patients without IVR or YMDD mutant viruses should be switched from LAM to ETV. The therapeutic efficacy of switching from LAM to ETV in non-IVR patients should be assessed by further study with a larger number of patients.

ADV and tenofovir disoproxil fumarate (TDF) have also been shown to exert antiviral efficacy in patients with chronic HBV infection with less frequent occurrence of drug-resistant mutant virus compared to LAM.[23] In addition, unlike the case of ETV, both ADV and TDF are known to be effective in LAM-refractory patients with chronic hepatitis B, as well as naïve patients.[23] Using ADV and TDF may be helpful for the treatment of non-IVR patients, especially after the establishment of LAM-resistant mutant virus.

In conclusion, our findings indicate that IVR may be a useful factor for predicting the emergence of LAM-resistant mutant virus in patients with chronic HBV infection treated with LAM. For patients who do not achieve IVR, therapeutic options other than LAM monotherapy should be promptly implemented because of the high incidence of the subsequent emergence of the YMDD mutant virus.

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JOURNAL OF VIROLOGY, Aug. 2008, p. 8241–8242
0022-538X/08/$08.00+0 doi:10.1128/JVI.00793-08

Vol. 82, No. 16

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.

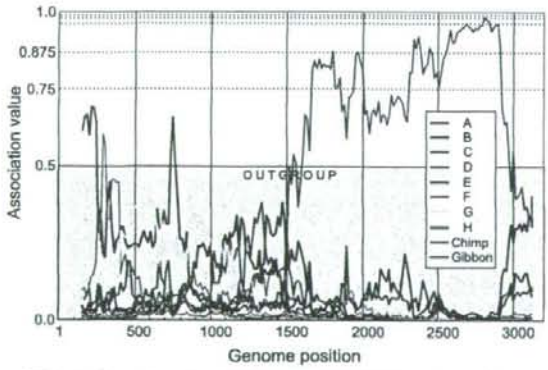

FIG. 1. GroupScanning analysis (10) of VH24 against reference groups of nonrecombinant HBV sequences of human genotypes A to H and nonhuman ape-derived variants (chimpanzee/gibbon) (n = 288), incorporating all HBV sequences used for recombination detection in the Huy et al. study (3). Association values 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).

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.

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

Virology 376 (2008) 408–415

Contents lists available at ScienceDirect

Virology

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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.

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).

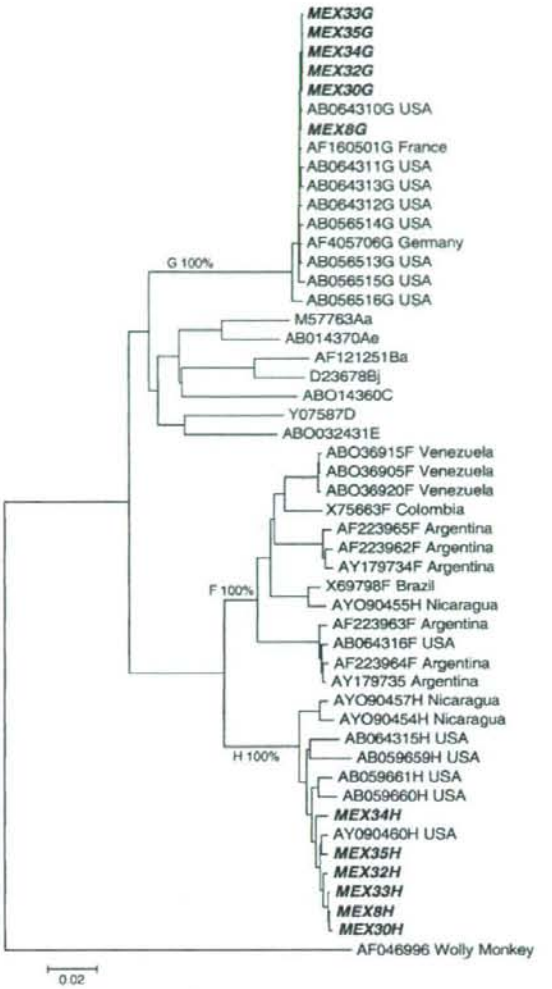

Fig. 1. A phylogenetic tree constructed using the complete nucleotide sequences of 50 HBV strains. The 6 HBV/G strains coinfected with HBV/H in Mexico (MEX), shown in bold italic, were compared with reference sequences recruited from GenBank/DDBJ/EMBL databases; 10 HBV/G, 7 HBV/H, 13 HBV/F, 7 representing 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.

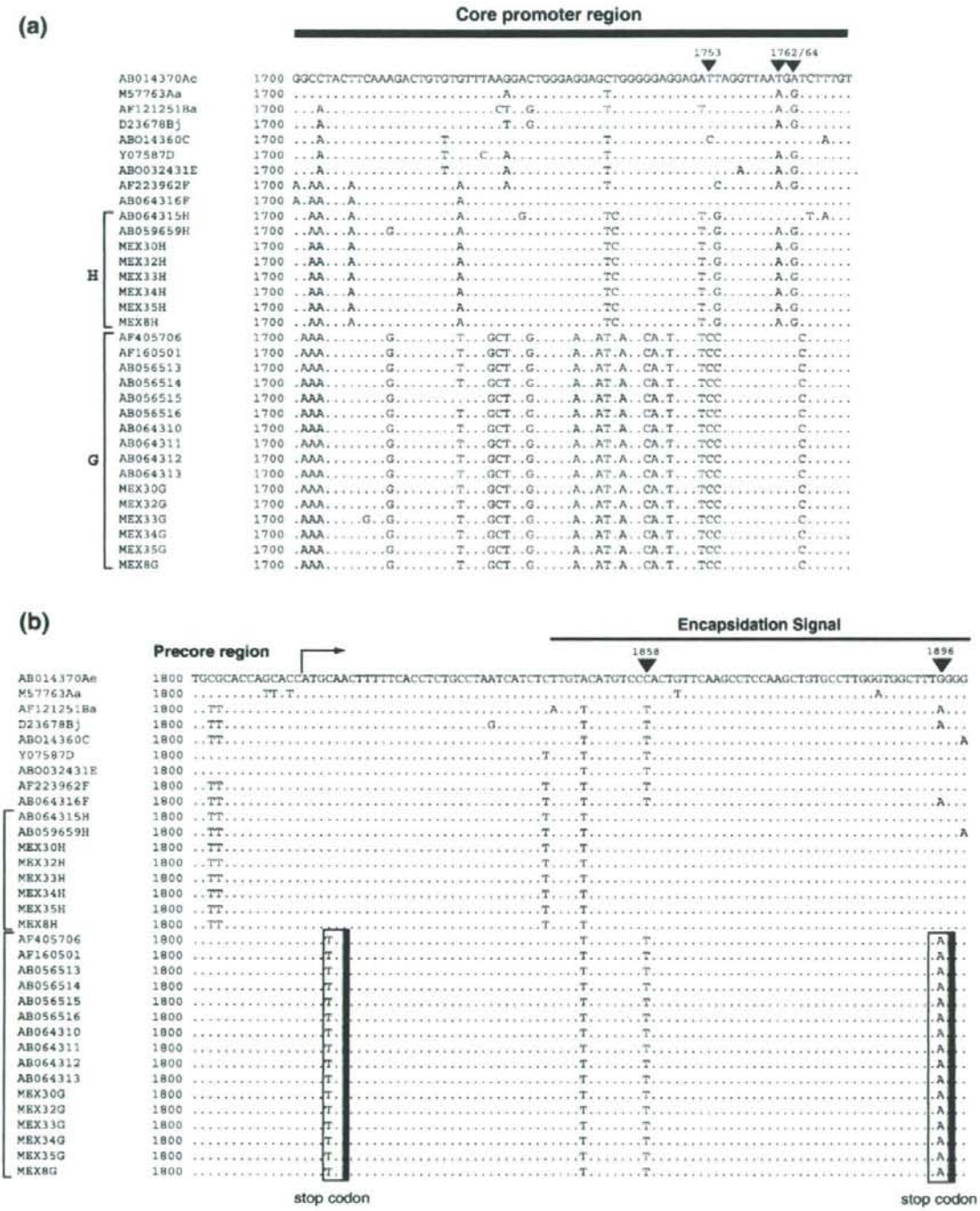

Fig. 2. Nucleotide sequences spanning (a) core promoter and (b) precore gene in HBV genotypes A–H. The reference sequence is shown for the AB014370Ae. The precore region includes pregenome encapsidation signal. Positions of the double mutation (A1762T and G1764A), T1753C, and C1858T making a pair with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

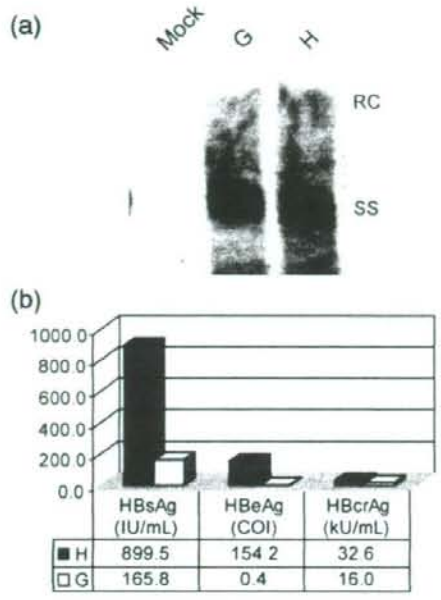

Fig. 3. Intracellular expression of HBV DNA and antigens. (a) The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. RC; relaxed circular double stranded DNA forms. (b) HBsAg, HBeAg and HBcrAg levels were also compared.

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

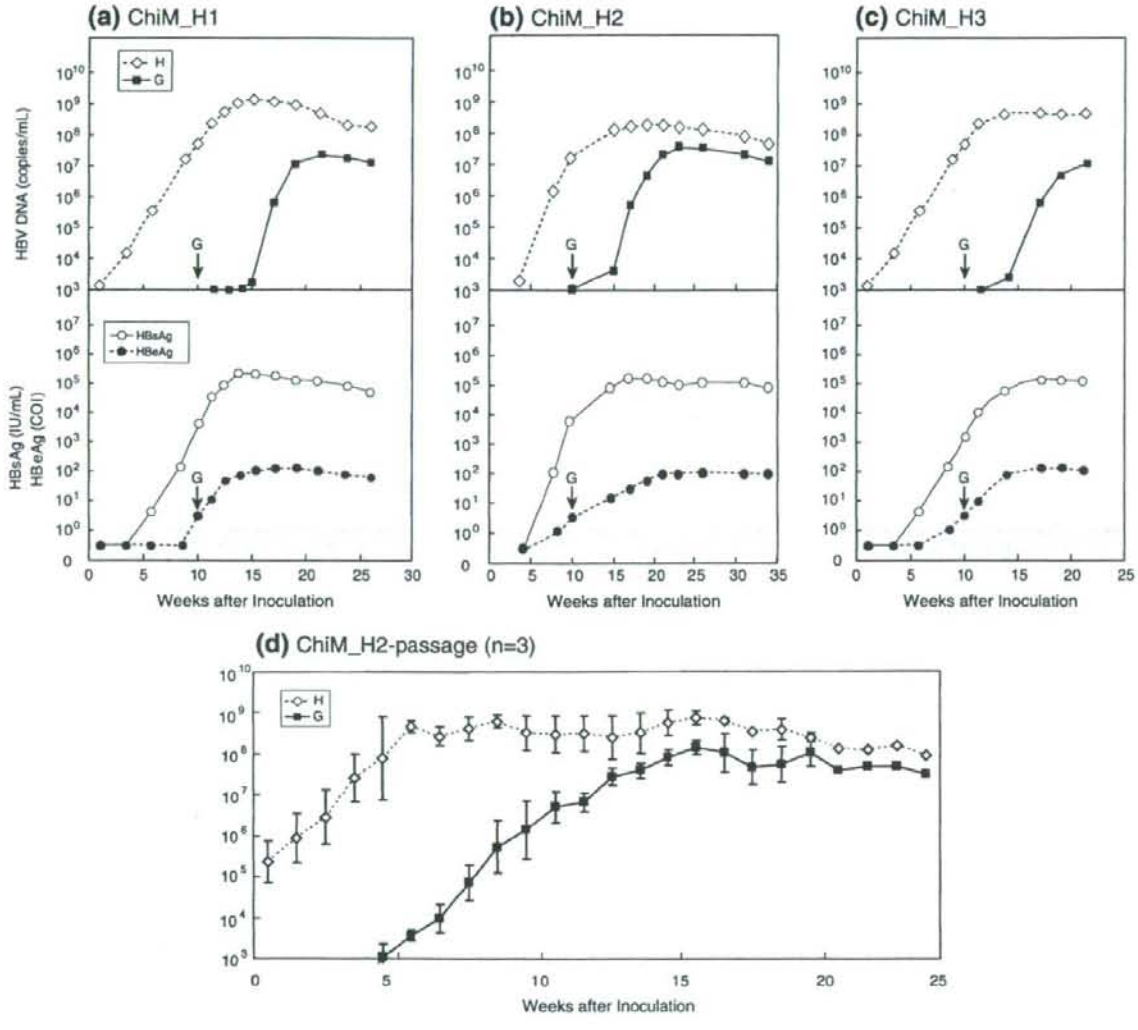

Fig. 4. Superinfection with HBV/G on chimeric mice infected with HBV/H [(a) ChiM_H1, (b) ChiM_H2, (c) ChiM_H3]. Inoculation with genotype G is indicated by large arrows. The dynamics of HBV DNA, HBsAg and HBeAg assessed superinfection of 3 chimeric mice (ChiM_H1–H3) are shown in panels a–c. Another 3 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.

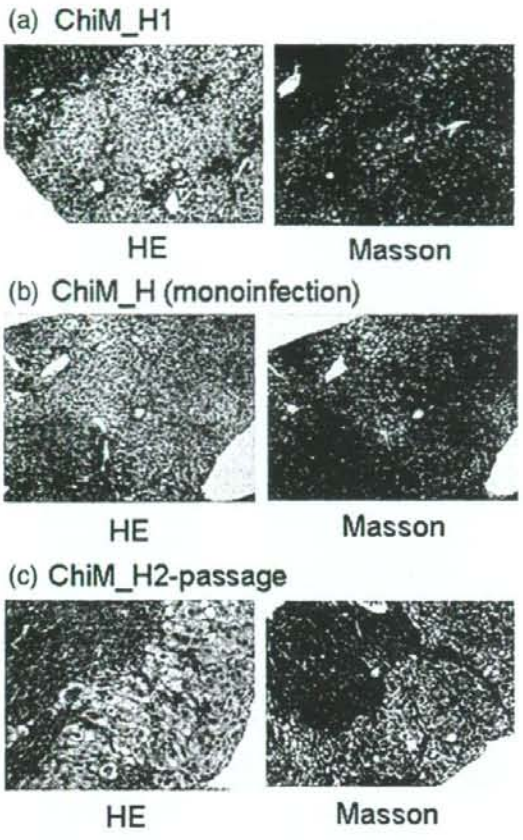

Fig. 5. Liver histology in chimeric mice superinfected with HBV/G on H. (a) A chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1) revealed F1A2 with Hematoxlin–Eosin (HE) and Masson's trichrome stain. (b) A chimeric mouse 24 weeks after monoinfection with HBV/H had no fibrosis. The 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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 doi:10.1111/j.1365-2893.2008.00997.x

Two simultaneous hepatitis B virus epidemics among injecting drug users and men who have sex with men in Buenos Aires, Argentina: characterization of the first D/A recombinant from the American continent

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Received February 2008; accepted for publication April 2008

SUMMARY. Previous studies have revealed that hepatitis B virus (HBV)/D and HBV/F predominate among blood donors from Buenos Aires, Argentina. In the present study, blood samples from two high-risk groups were analysed: 160 corresponding to street- and hospital-recruited injecting drug users [81.2% showing the 'anti-hepatitis B core antigen (anti-HBc) only' serological pattern] and 20 to hepatitis B surface antigen $(HBsAg)^+$/$anti\text{-}HBc^+$ men who have sex with men. HBV genotypes were assigned by polymerase chain reaction amplification followed by restriction fragment length polymorphism and confirmed by nucleotide sequencing of two different coding regions. HBV DNA was detected in 27 injecting drug users (16.9%, occult infection prevalence: 7.7%), and 14 men who have sex with men (70%). HBV/A prevailed among injecting drug users (81.8%) while HBV/F was predominant among men who have sex with men (57.1%). The high predominance of HBV/A among injecting drug users is in sharp contrast to its low prevalence among blood donors ($P = 0.0006$) and men who have sex with men ($P = 0.0137$). Interestingly, all HBV/A S gene sequences obtained from street-recruited injecting drug users encoded the rare serotype *ayw*1 and failed to cluster within any of the known A subgenotypes. Moreover, one of the HBV strains from a hospital-recruited injecting drug user was fully sequenced and found to be the first completely characterized D/A recombinant genome from the American continent. Data suggest that two simultaneous and independent HBV epidemics took place in Buenos Aires: one spreading among injecting drug users and another one sexually transmitted among the homosexual and heterosexual population.

Keywords: Argentina, hepatitis B virus, HBV recombinant, molecular epidemiology.

Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, injecting drug user; MSM, men who have sex with men; NJ, neighbour-joining; pre-C/C, Precore/core; RFLP, restriction fragment length polymorphism.

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INTRODUCTION

Eight major genotypes of HBV (A to H) with distinct geographical distribution have been defined [1]. Some of these genotypes are subdivided into subgenotypes. For example, genotype A is classified into three major subgroups: A1 (in Asia and Africa), A2 (in Europe and the USA) and A3 (in Central West Africa) [2]. Likewise, genotype F is represented by four subgenotypes: F1 which includes subclades 1a (Central America) and 1b (Alaska and Argentina), F2 (widely present in Central and South America), F3 (Venezuela and Colombia) and F4 (Bolivia and Argentina) [2–4]. On the other hand, prior to the definition of the genotypes, HBV strains were distinguished by serological analysis into nine different HBsAg subtypes named *adw2*, *adw4*, *ayw1*, *ayw2*, *ayw3*, *ayw4*, *adrq+*, *adrq-* and *ayr* [1].

Previous reports have shown that genotype F is the most prevalent among Argentine chronic patients [5,6]. Furthermore, França *et al.* [22] observed in the same country a prevalence of 64% for genotype F, 17.3% each for genotypes A and D, and 1.3% for genotype C among blood donors. Noteworthy, the distribution of HBV genotype F is uneven throughout Argentina since it predominates in the northern part of the country (88.9%), in contrast with Buenos Aires city area where 40.9% of the 22 samples analysed were classified as HBV/D, 31.8% as HBV/F, 22.7% as HBV/A and 4.6% as HBV/C.

HBV molecular epidemiology data regarding risk groups, such as injecting drug users (IDUs) and men who have sex with men (MSM), are still lacking in Argentina and scarce in the rest of the world.

Bearing in mind both the emerging evidence of HBV genotype relevance in the course of the infection [7], as well as the lack of information associating a given HBV genotype with its route of transmission, the aims of the present study were to retrospectively assess the HBV genotype prevalence among the above mentioned at risk populations from Argentina and to analyse their genetic diversity and phylogenetic relatedness.

MATERIALS AND METHODS

Informed consent and Ethics Committee

All subjects had provided their informed written consent to carry out this study. This protocol received approval of the Independent Ethics Committee on Research (CIEI-FM-UBA).

Samples

During the period 2000–2001, serum samples and epidemiological data were collected from 80 street-recruited subjects from Buenos Aires with past or ongoing history of injecting drug addiction. Recruitment was done by the so-called snowball sampling procedure [8].

In order to rule out the possibility that results corresponding to street-recruited IDUs were merely the direct consequence of their behaviour as a close community due to the sampling technique chosen in this study, we decided to include a second group consisting of an identical number of unrelated IDUs, who individually visited public hospitals and treatment centres in Buenos Aires city and its suburbs. Their serum samples were obtained between 1995 and 2006 (most of them-except 16-during 2001–2002). IDUs patients were only enrolled if either HBsAg or total immunoglobulin to hepatitis B core antigen (total anti-HBc) were detected.

In addition, 20 MSM from Buenos Aires, previously enrolled in a cohort study [9] and whose serum samples had been collected between February and December 2003, were also included in this study. MSM were only included if both markers (HBsAg and total anti-HBc) were positive and no current or past history of injecting drug use was reported.

Serological assays

Serum samples had been stored at −70 °C until use. HBsAg and total anti-HBc were tested with commercially available kits (AxSYM, Abbot, IL, USA). Serological tests for human immunodeficiency virus (HIV) (Bio-Rad, Fujirebio, Tokyo, Japan) and hepatitis C virus (HCV) (AxSYM, Abbot, IL, USA) were also performed following the manufacturer's instructions.

HBV DNA PCR amplification, nucleotide sequencing and genotype assignment

By using a DNA extraction kit (Macherey, Nagel, Germany), HBV DNA was extracted from 200 μL of serum from all patients.

Initially, the HBV S gene was partially amplified by a previously described nested PCR protocol [10], yielding an amplicon of 585 bp (nucleotide position 203 to 787). HBV DNA was also looked for by three further methods: (i) another partial S gene PCR [11], yielding an amplicon of 541 bp (nucleotide position 256 to 796), and exceptionally; (ii) a nested PCR [10] followed by a boosted PCR (by using the same primers employed during the nested step) – as an attempt to make very faint bands useful for DNA sequencing-, as well as (iii) a nested PCR performed by combination of primers used by Zeng *et al.* (first round) and by Lindh *et al.* (second round) and its corresponding boosted PCR (third round).

Amplicons of expected sizes were detected in 1% agarose gels and subsequently subjected to restriction fragment length polymorphism (RFLP) [10,11]. In some samples, very faint bands were observed; consequently, the RFLP pattern was under the level of detection for proper HBV genotype assignment and the sample was considered to be untypeable.

In order to examine the possibility of an HBV recombination event, the Precore/core (pre-C/C) region was partially

amplified as well (nucleotide position 1756–2451), as previously described [12].

Subsequently, full-length HBV sequencing [13] was attempted in all DNA samples showing either inconsistencies/ambiguities of phylogenetic analysis of partial S and pre-C/C sequences or discrepancy between S gene RFLP and phylogenetic genotype assignment. However, full length sequences were only obtained from five samples (S-IDU4, S-IDU8, S-IDU11, S-IDU19 and H-IDU7) due to a combination of technical factors (low sera availability and very faint bands). Due to the retrospective nature of the study and the limited serum volume available, HBV DNA viral load was not measured.

Appropriate precautions and procedures were strictly followed to avoid cross-contamination [14,15]. PCR products were bidirectionally sequenced by using the Big-Dye Termination chemistry (Applied Biosystem, USA).

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are: EU185765–EU185767, EU185781–EU185789 (street-recruited IDUs), EU185768–EU185780, EF467999 (hospital-recruited IDUs) and EU185741-EU185764 (MSM).

Sequence analysis

DNA alignments of the nucleotide sequences obtained and GenBank sequences corresponding to HBV genotypes A–H were generated with the CLUSTAL X program. Phylogenetic trees were constructed using neighbour-joining (NJ) analysis included within the PHYLIP package version 3.5 c [16]. A sequence identity matrix was recorded for each the pre-C/C and S gene partial sequences, by using the BioEdit Sequence Alignment Editor, version 7.0.1.

Detection of HBV DNA recombination and identification of recombination sites

All complete genome sequences from Argentine strains were examined with other HBV genotypes for the presence of potential recombination event(s) by running the bootscan analysis available in the SIMPLOT (version 3.5.1) [17] and by using the Grouping Scan included in the Simmonic 2005 Sequence Editor Package (version 1.5) [18]. Moreover, and in order to confirm the results obtained, a third method to detect recombinants developed by one of the authors (P. D. Ghiringhelli, unpublished data) was used as well. In this method, alignments were carried out with the CLUSTAL X program (default parameters: [19,20]) between sequence pairs, always involving the putative recombinant candidate and one representative sequence of each one of the putative parental genotypes. The relative similarities were calculated using the CLUSTAL X consensus symbol (* and blank space) as the input sequence, in an overlapping windows-based strategy. Arbitrary values of +1 for identical (*) and −1 for nonidentical (blank spaces) residues, to obtain the similarity profiles were assigned. The sum of assigned values for each residue in each window (101 nucleotides) was divided by the window width and allotted to the central position to generate the plots. Pairs of profiles were superimposed and analysed with the aim of detecting cross points between them. In order to find a good relation between graph complexity and cross-point detection sensitivity, windows length of 101 residues were scanned.

The breakpoints were estimated by mapping the informative sites using SIMPLOT (version 3.5.1) [17] and further confirmed by means of chi-square analysis [21].

Statistical analysis

Statistical differences were evaluated by two methods: (a) the chi-square calculation with Yates' correction, and (b) the two-tailed *t*-test. A P value < 0.05 was used as an indicator of statistical significance.

RESULTS

Serological status and HBV genotypes of street-recruited IDUs (S-IDUs)

Street-recruited IDUs were divided into three groups according to different HBV serological patterns. The first group included six IDUs (83.3% male; mean age ± SD = 27.6 years ± 6.9) who were HBsAg positive but total anti-HBc negative. The second one included 10 IDUs (90% male; 28.7 years ± 5.0) and showed positivity for both HBsAg as well as total anti-HBc. Finally, a third group encompassed 64 IDUs (82.8% male; 32.5 years ± 6.3) who exhibited the sole presence of total anti-HBc. The serological status for HCV and HIV from all HBV DNA PCR positive samples is depicted in Table 1.

HBV S gene was amplified in 20 out of 80 samples (25%): three patients from the first group (50%), eight patients from the second group (80%) and nine from the third one (14.1%). In 5 of these 20 cases, negligible yields of DNA products were obtained from PCR, even after performing a third round of amplification. Therefore, only 15 samples out of these 20 were appropriate for an RFLP analysis [11]. Two out of 15 samples (13.3%), which belonged to the 'anti-HBc only' group, were classified as genotype C. The remaining samples (86.7%) were characterized as genotype A, whose assignment was further confirmed by performing a second RFLP method, originally described for characterization of genotypes A–F [11] (Table 1).

Furthermore, the HBV S coding region was successfully sequenced in 9 out of these 15 samples and all of them were confirmed as belonging to genotype A. These nine Argentine HBV strains clustered separately from HBV/A1, HBV/A2 and HBV/A3 strains (Fig. 1a). The phylogenetic analysis revealed that all the samples were closely related to each other, with (mean ± SD) 98.7 ± 0.8 nucleotide identity, as