

Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CPS§	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.
 †Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).
 ‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).
 §Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2).

In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than "other substitutions" (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

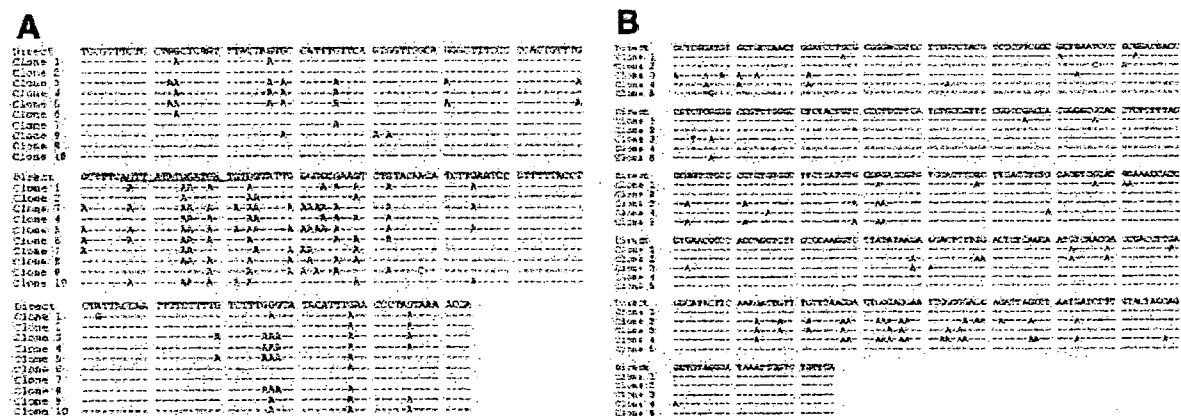


Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (top line). The target sequence of PNA annealing is underlined. (B) DNA sequence alignment in the x region of the HBV.

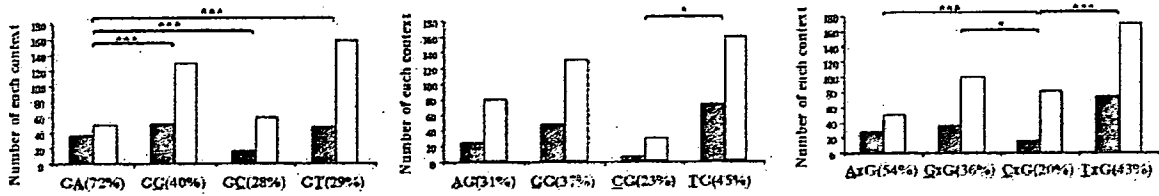


Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. Open bars: number of occurrences of each context in the sequence analyzed. Gray bars: number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues. * $P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible

for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Expression of Deaminases in HepG2 Cell Lines. We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.²³ The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.²³ The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,²¹ we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions*		No. of Clones†	eAg	HBs Ag	HBV DNA
	G to A	Other				
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

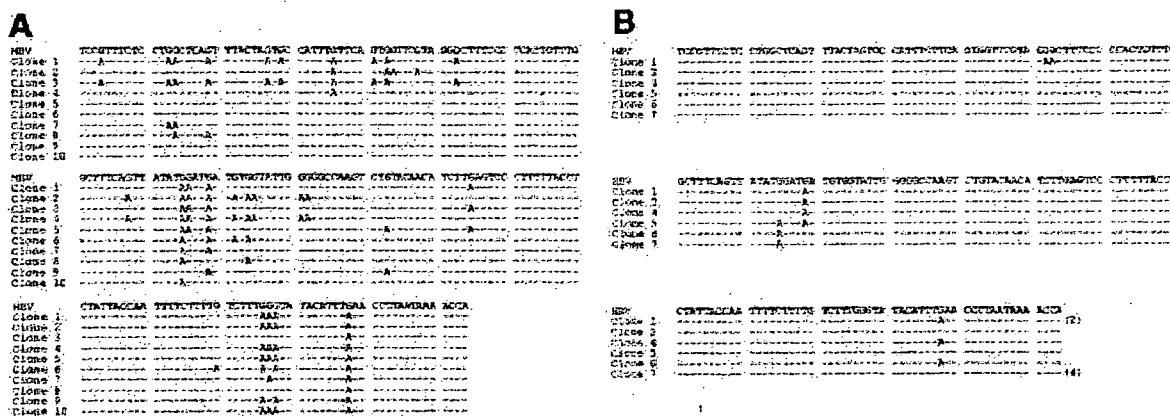


Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (top line). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,¹⁹ in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.²⁴⁻²⁸ However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation

is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOVE3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.^{20,29} suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.³⁰ reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,³⁰ suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

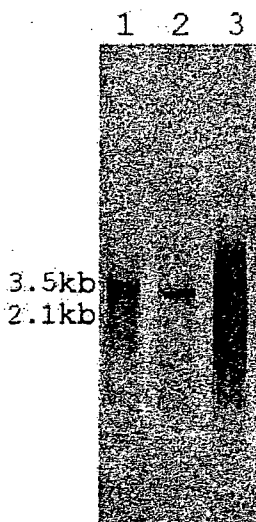


Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

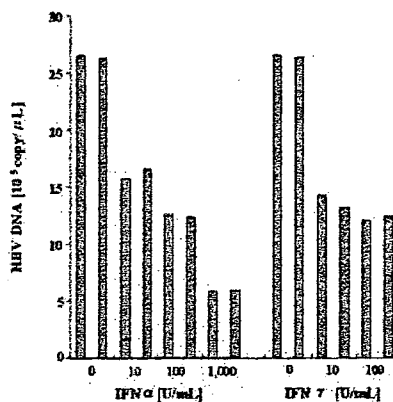


Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.

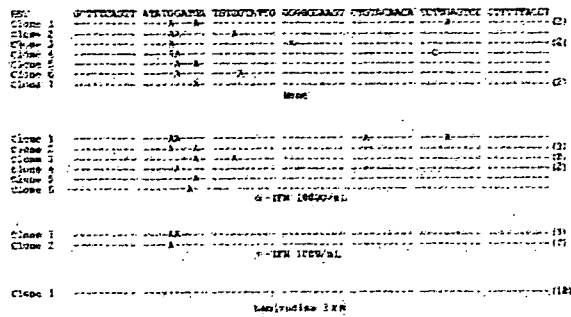


Fig. 6. Nucleotide sequence substitutions around YMDD motif of reverse transcriptase detected by PCR with PNA clamping after treating a HepG2 cell line (cell line 2 in Table 3). The nucleotide sequence of the transfected clone was used as a reference sequence (top line). Cells were treated with interferons and lamivudine as shown in Figs. 5 and 7, respectively.

noticeable increase in HepG2 cells by our detection method (C. Noguchi and K. Chayama, unpublished data). However, the method employed to detect hypermutation is not quantitative. Moreover, no antibody to detect APOBEC3G is available. Measurement of activity of this enzyme might be necessary to address this issue.

Because the patterns of hypermutations found in patients as well as cell lines are in agreement with strong dinucleotide preferences of a retroviral genome³¹⁻³⁵ edited by APOBEC3G,⁷⁻⁹ we assume that hypermutations might also be induced by a similar enzyme. As pointed out by Turelli et al.,²⁰⁻²⁹ another deaminase including APOBEC3F might be responsible for the generation of hypermutation. We actually detected the expression of deaminases in HepG2 cell lines. The expression levels of these deaminases are very low because they were detected by only two-stage PCR with one exception (only APOBEC3F was detected by a single-stage PCR).

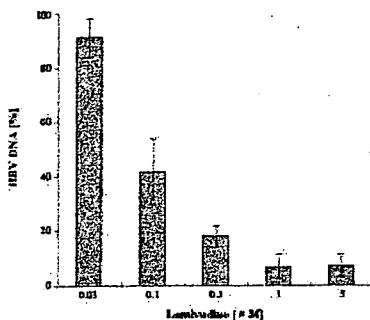


Fig. 7. Effects of lamivudine on production of HBV DNA by cell line 1. After 5 days of lamivudine treatment, the HBV DNA in core particles was immunoprecipitated and quantitated by real-time PCR. Data are mean \pm SD of 4 independent experiments.

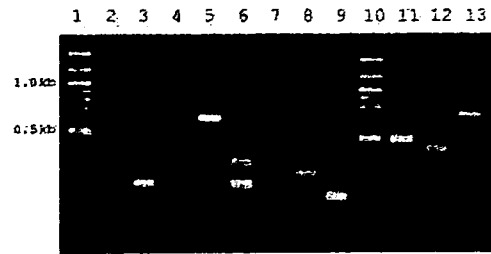


Fig. 8. Agarose gel electrophoresis of mRNAs of known deaminases amplified by reverse transcription-polymerase chain reaction. Lane 1: molecular weight size marker; lane 2: APOBEC1; lane 3: APOBEC2; lane 4: APOBEC3A; lane 5: APOBEC3B; lane 6: APOBEC3C; lane 7: APOBEC3D; lane 8: APOBEC3F; lane 9: APOBEC3G; lane 10: molecular weight size marker. Only mRNA of APOBEC3F was detected by one-stage PCR. To confirm the predictability of the assay, 3 negative mRNAs in Hep3G (APOBEC1, 3A and 3D) were amplified by using mRNAs from tissues known to express it. Lanes 11 and 12: APOBEC1 and APOBEC3A from the ileum; lane 13: APOBEC3D from the duodenum. All detected cDNAs were cloned, and nucleotide sequences were confirmed.

However, other possibilities should not be ignored. For example, some viral proteins might prevent such editing activity of deaminase by associating with this enzyme, as virion infectivity factor does in HIV-1-infected cells. Possibly the edited HBV genomes are degraded in liver cells rapidly by removal of the U residues by uracil DNA glycosylase followed by cellular nucleases.³⁶

We found hypermutated genomes only in patients positive for eAb. The G to A nucleotide substitution of codon 28 of pre-core protein, which induces premature stop of this protein and basal core promoter mutations (A1762T/G1764A), might be related to the clearance of eAg.²⁸ Further studies should be conducted to investigate the relationship between G to A substitutions in these regions by deaminase(s), production of eAg, and replication efficacy of the virus.

A recent study showed that the amount of HBV DNA reduction occurs noncytopathologically through the action of cytokines, especially interferon alpha/beta and gamma.^{37,38} We thus examined whether interferon can alter the occurrence of hypermutation. However, the results showed no increase in the number of hypermutation in HepG2-derived cell lines treated by interferon alpha and gamma (Fig. 6). Thus, the antiviral action of the mechanism responsible for G to A substitution in liver cells is likely to be independent of the action of interferon.

In conclusion, numerous innate intracellular defense systems exist, and the precise pathways of such systems are not fully understood. The role of editing of the HBV genome in such defense systems should be further investigated to understand the natural antiviral mechanisms and to develop an antiviral strategy against HBV.

Acknowledgment: A part of this work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, and Liver Research Project Center, Hiroshima University, Hiroshima, Japan. The authors thank Eiko Okutani, Yukiji Tonouchi and Kiyomi Toyota for their excellent technical assistance.

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Infection of Human Hepatocyte Chimeric Mouse With Genetically Engineered Hepatitis B Virus

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Studies of hepatitis B virus (HBV) mutants have been hampered by the lack of a small animal model with long-term infection of cloned HBV. Using a mouse model in which liver cells were highly replaced with human hepatocytes that survived over a long time with mature human hepatocyte function, we performed transmission experiments of HBV. Human serum containing HBV and the virus produced in HepG2 cell lines that transiently or stably transfected with 1.4 genome length HBV DNA were inoculated. Genetically modified e-antigen-negative mutant strain also was produced and inoculated into the mouse model. A high-level ($\approx 10^{10}$ copies/mL) viremia was observed in mice inoculated with HBV-positive human serum samples. The level of viremia tended to be high in mice with a continuously high human hepatocyte replacement index. High levels and long-lasting viremia also were observed in mice injected with the *in vitro* generated HBV. The viremia continued up to 22 weeks until death or killing. Passage experiments showed that the serum of these mice contained infectious HBV. Genetically engineered hepatitis B e antigen-negative mutant clone also was shown to be infectious. Lamivudine effectively reduced the level of viremia in these infected mice. **In conclusion**, this mouse model of HBV infection is a useful tool for the study of HBV virology and evaluation of anti-HBV drugs. Our results indicate that HBeAg is dispensable for active viral production and transmission. (HEPATOLOGY 2005;42:1046-1054.)

Hepatitis B virus (HBV) is a small enveloped DNA virus and causes chronic infection of the liver that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.¹⁻⁴ The lack of a

practical small animal model has impeded the study of the biology of this virus and the development of effective antiviral therapies. Chimpanzee is the only natural host that allows active replication of HBV.⁵⁻⁷ Although this animal is a valuable model for the study of hepatitis viruses,⁸ the practical use of chimpanzees is severely limited both ethically and economically.

Several small animal models of HBV infection have been reported. The HBV transgenic mouse is a very useful model for the study of virology and evaluation of antiviral drugs.⁹⁻¹² However, the liver cells of this model are not permissive for HBV infection; therefore, studying virus-cell interactions such as receptor binding and entry is not possible. The HBV-trimera mouse is another useful mouse model.¹³ In this model, *ex vivo* HBV-infected human liver fragments are implanted into lethally irradiated mice after SCID mouse bone marrow transplantation. Approximately 80% of the mice develop viremia 2 to 3 weeks after infection. However, the rate of positivity subsequently decreases to less than 20% 6 weeks after infection. The level viremia is approximately 10^5 copies/mL. More recently, HBV-containing human serum samples were used to infect human hepatocyte repopulated mice.¹⁴ A high-level viremia (4.5 and 10×10^8 copy/mL) and HBs antigenemia are observed 8 weeks after injection. This mouse model is promising because HBV replicates in natural host cells, human hepatocytes. However,

Abbreviations: HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; HSA, human serum albumin; PCR, polymerase chain reaction; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; DMEM, Dulbecco's modified Eagle's medium.

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Received March 20, 2005; accepted August 14, 2005.

Supported in part by a Grant-in-Aid for Scientific Research from Japanese Ministry of Health, Labor and Welfare. A part of this work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University.

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

DOI 10.1002/hep.20892

Potential conflict of interest: Nothing to report.

Table 1. Table Inoculum Used for Infection Experiments

Inoculum	Source	Transfection	HBs Antigen	HBe Antigen	HBV DNA (LGE/mL)
Serum 1	HBV carrier 1	—	+	130	10.8
Serum 2	HBV carrier 2	—	+	150	8.7
CA59	pCAG-HB-wt	Stable	6.3 ± 2.8	15 ± 7	8.0 ± 0.2
CM3	pTRE-HB-wt	Transient	3.9 ± 1.5	105 ± 7	8.3 ± 0.4
Fresh CM3	pTRE-HB-wt	Transient	ND	ND	8.0
e-Negative	pTRE-HB (PC)	Transient	2.9 ± 0.2	0.5 ± 0.3	8.1 ± 0.3
Mouse CM3	CM3-infected mouse	—	ND	ND	ND

NOTE. Fifty microliters of each serum or cell culture supernatant was used for injection except for fresh CM3 (500–1,000 μ L) and mouse CM3 (5 μ L). Data are mean \pm SD.

Abbreviations: LGE, log genome equivalent; ND, not determined.

long-term high-level viremia has not been reported so far in this model, probably because of technical difficulties in maintaining large quantities of human hepatocytes in these mice.

Long-term HBV viremia was reported after subcutaneous transplantation of immortalized human hepatocytes in RAG-2–deficient mice after transfection of circularized full-length HBV genome.¹⁵ Viremia of up to 3×10^8 copy/mL was still observed in these mice at least 5 months after transplantation. This long-term viremia model should be useful for *in vivo* HBV studies. However, the production and selection of HBV-secreting immortalized human hepatocytes takes a long time, and the level of viremia in the transplanted animal depends on the volume of live immortalized cells in mice. The mode of viremia might be different from natural infection because the pregenome RNA is transcribed from integrated HBV. Whether the produced HBV re-infects implanted immortalized human hepatocytes has not been confirmed.

A useful woodchuck hepatitis virus (WHV) infection model was established by Petersen et al.¹⁶ They showed high-level replacement of uPA/Rag-2 knockout mice liver with woodchuck hepatocytes and development of high-level (1×10^{11} virion/mL) WHV viremia. Dandri et al.¹⁷ transplanted *Tupaia* hepatocyte into uPA/RAG-2 mice and showed up to 8.2×10^7 genome equivalent/mL viremia. This model is useful because viremia continued up to 29 weeks. However, probably because of different host cells, the replication levels of HBV are lower than those of woolly monkey HBV. Using SCID mouse homozygous for Alb-uPA transgene, the group of Mercer and colleagues¹⁸ were the first group to report high-level replacement of mouse liver with human hepatocytes and successful infection of these mice with hepatitis C virus. Recently, we also created a human hepatocyte chimeric mouse in which the hepatocytes were highly replaced by implanted human liver cells.¹⁹ The repopulation index calculated from serum human serum albumin (HSA) concentrations exceeded 70% in 32% of the transplanted mice, and these animals survived up to 80 days after transplanta-

tion with high replacement index. Using this chimeric mouse, we performed transmission experiments of HBV. Using serum samples obtained from patients with chronic HBV infection, high-level viremia (approximately 10^{10} copies/mL) was observed up to 22 weeks in mice inoculated with HBV-positive human serum samples. We also performed infection study using *in vitro*–generated HBV. Infectious HBV was produced in HepG2 cell lines by transfecting with 1.4 genome length HBV DNA. Because mice injected with this *in vitro*–produced virus developed viremia, we further performed passage study. In addition, we introduced point mutations in HBV genome to create an HBe antigen–negative variant. The mice inoculated with this HBe antigen–negative variant developed viremia. Lamivudine effectively suppressed replication of HBV in mice inoculated with human serum samples and wild-type *in vitro*–created HBV. This model is a useful tool for the study of the nature of HBV mutants and development of anti-viral drugs.

Materials and Methods

Human Serum Samples. Serum samples were obtained from four HBV carriers after obtaining written informed consent. Inocula for mice were obtained from two patients who tested positive for HBs and HBe antigens with slightly elevated levels of serum alanine aminotransferase and high-level viremia (Table 1). Serum samples for extraction and cloning of HBV were obtained from the remaining two patients who were positive for hepatitis B e antigen (HBeAg) and had high-level HBV DNA (6.9×10^9 and 9.8×10^{10} copies/mL by real-time polymerase chain reaction [PCR], respectively). All of these HBV belonged to genotype C.

Analysis of HBV Markers. Hepatitis B surface antigen (HBsAg) and HBeAg were measured by commercially available ELISA (Abbott Japan, Osaka, Japan). For quantitative analysis of HBV DNA, 100 μ L serum samples or culture supernatants were used. DNA was extracted from these samples by SMITEST (Genome

Science Laboratories, Tokyo, Japan) and was dissolved in 20 μ L H₂O. One microliter DNA solution was amplified by Light Cycler (Roche Diagnostics, Japan, Tokyo) for quantitation of HBV. The primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds, extension at 72°C for 6 seconds. The lower detection limit of this assay is 300 copies. Nested PCR was used to detect a small amount of HBV DNA with the outer primers X1F1 (5'-CGCGGGACGTCCTTTGTCTA-3') and X2R1 (5'-GTTACGGTGGTCTCCATGC-3') and inner primers X1F2 (5'-TACGTCCCGTCGGCGCTGAA-3') and X2R2 (5'-CAGAGGTGAAGCGAAGTGCA-3'). The amplification condition included 35 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes after 2 minutes of initial denaturation at 94°C followed by 7 minutes of final extension using Gene Taq (Wako Pure Chemicals, Tokyo, Japan) with anti-Taq high (TOYOBO Co., Osaka, Japan) according to the instructions provided by TOYOBO.

Cloning of HBV DNA and Plasmid Construction.

Full-length HBV DNA was amplified using these HBV DNA samples by the method of Gunther et al.²⁰ and cloned into pBluescript SK+ (Stratagene, La Jolla, CA). HBV DNA, 1.4 genome length, obtained from one of these two patients was cloned into pcDNA3 (Invitrogen, San Diego, CA) after replacement of CMV promoter with CAG to yield pCAG-HB-wt. Similarly, 1.4 genome length HBV DNA from the other patient was cloned into a plasmid vector pTRE2 (BD Biosciences, Franklin Lakes, NJ) and designated pTRE-HB-wt. A modified plasmid pTRE-HB-PC was generated by introducing a G-to-A point mutation to nucleotide 1896 to create pre-core stop codon (TTG to TAG). The substitution was introduced by a commercially available site directed mutagenesis kit (QuickChange Site-Directed Mutagenesis Kit, Stratagene). Nucleotide sequences of the HBV cloned into plasmids pCAG-HB-wt and pTRE-HB-wt were deposited into the GenBank database under accession numbers AB206817 and AB206816, respectively.

Transfection of HepG2 Cell Lines With 1.4 Genome Length HBV DNA and Endogenous Polymerase Reaction Analysis. HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C and under 5% CO₂. The cells were seeded to semi-confluence in 6-well tissue culture plates. For transient transfection experiments, two plasmids; pTRE-HB-wt and pTRE-HB-PC, were used. Two micrograms of each plasmid was transfected using Fu-

gene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the instructions provided by the supplier. Three to five days after transfection, the culture supernatant was collected for infection of mice and quantitative analysis of HBV DNA by real-time PCR. Alternatively, calcium phosphate precipitation was performed to prepare fresh supernatant for large-dose administration experiments. Concentrated supernatants were prepared by using Microsep 10K spin filter, according to the instructions provided by the manufacturer (Pall Life Sciences., Ann Arbor, MI). The HBV particles produced in the supernatants were immunoprecipitated with protein A sepharose and mouse anti-HBs monoclonal antibody 2Z824Z (Institute of Immunology, Tokyo, Japan) and subjected to endogenous polymerase reaction²¹ and Southern blot analysis after sodium dodecyl sulfate/proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was electrophoresed in a 1% agarose gel and transferred onto a nylon membrane. The transferred DNA was detected with full-length HBV DNA probe synthesized with the PCR DIG probe synthesis kit and the DIG Nucleic Acid Detection kit and CSPD, ready-to-use (Roche Diagnostics) in the Fluor-S Max MultiImager (BIO-RAD Laboratories, Hercules, CA).

For the production of stably transfected cell lines, HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Cells were seeded into 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pCAG-HB-wt was transfected by calcium precipitation. Twenty-four hours after transfection, the cells were split and cultured in G418 selection DMEM (1 mg/mL). One hundred fifty colonies were isolated and amplified for identification of virus-producing cell lines. Clones positive for both HBs and HBe antigens were selected and further analyzed for production of HBV particles. Finally, one of five cell lines that produced more than 10⁵ copy/mL HBV DNA in supernatant were selected and used for further experiments. This cell line produced stable levels of HBV DNA for more than 12 months (data not shown).

Analysis of HBV Produced in the Supernatant of Transfected HepG2 Cell Lines by Sucrose Density Gradient. Five milliliters HBV-positive serum (10⁸ copy/mL) or 100 mL cell culture supernatant (10⁷ copy/mL) was layered on a 20% (wt/wt) sucrose gradient, and centrifuged at 24,000 rpm for 1 hour at 4°C with a Beckman SW28 rotor Beckman Coulter, Fullerton, CA). The precipitate was resuspended with 500 μ L phosphate-buffered saline. These HBV samples were layered on a linear 20% to 50% (wt/wt) sucrose gradient. Centrifugation was carried out at 24,000 rpm for 21 hours at 4°C with a Beckman SW40 rotor. The gradients were frac-

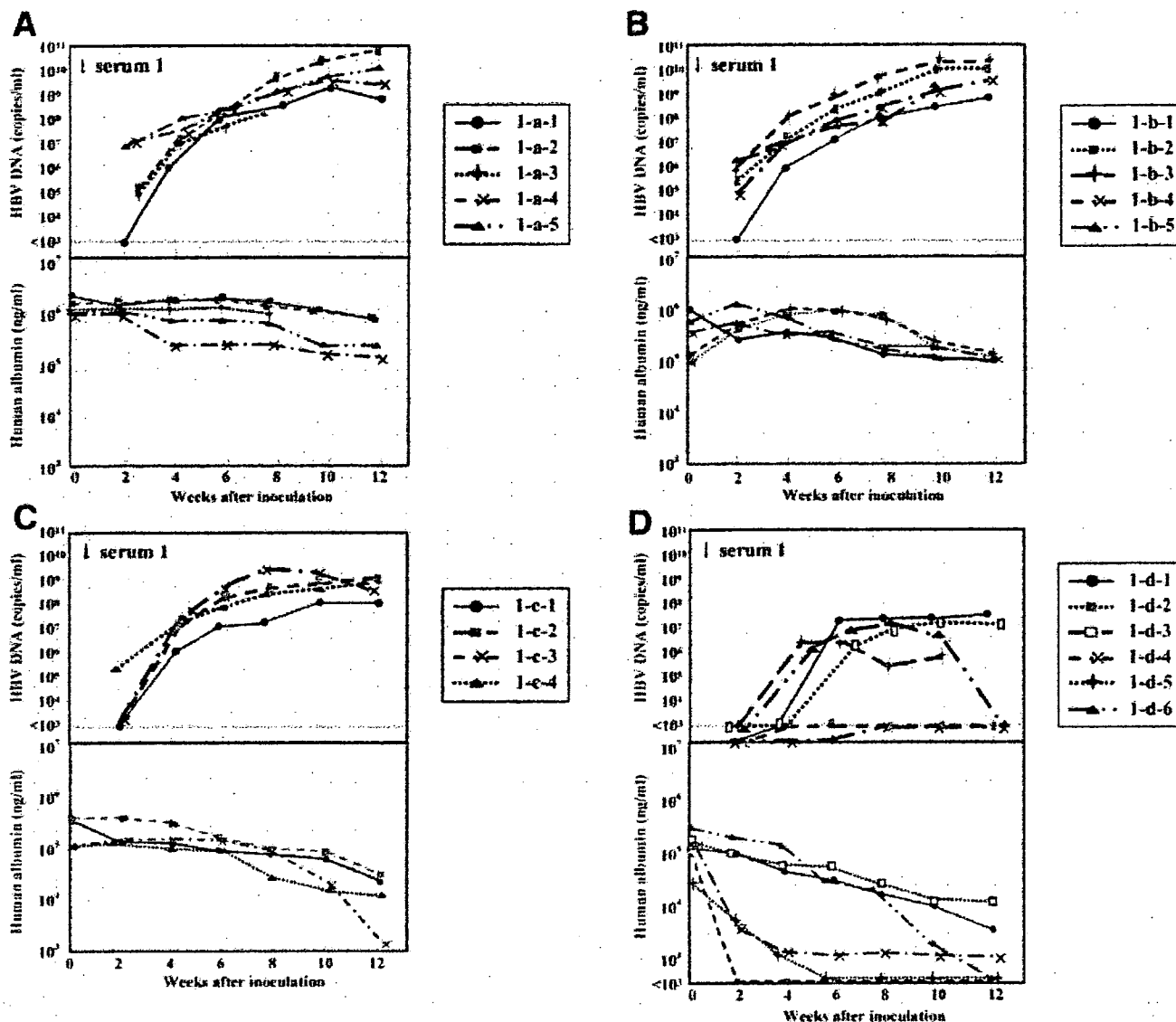


Fig. 1. Time course studies in 20 mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 1, Table 1) was intravenously injected into each mouse. The upper half of each panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin (HSA). Mice were divided according to the levels of HSA; (A) initial HSA > 1,000,000 ng/mL with only slight decline (n = 5); (B) initial HSA > 500,000 ng/mL, with slight decline (n = 5); (C) initial HSA > 200,000 ng/mL, but declined to less than 100,000 ng/mL during observation (n = 4); (D) initial HSA = 100,000 ng/mL, but diminished to less than 30,000 ng/mL (n = 6).

tionated into 500- μ L samples, and the density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBV DNA by real-time PCR.

Generation of Human Hepatocyte Chimeric Mice and Analysis of Serum Samples. Generation of the uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described previously by our group.¹⁹ Animal protocols were performed in accordance with the guidelines of the local committee for animal experiments. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. HSA was measured with a Human Albumin ELISA Quantita-

tion kit (Bethyl Laboratories Inc., Montgomery, TX) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquoted and stored in liquid nitrogen until use.

Histochemical Analysis of Mouse Liver. The liver specimens of infected mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. The liver sections were stained with hematoxylin-eosin or subjected to immunohistochemical staining by using an antibody against hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3% H₂O₂

and methanol. Immunoreactive materials were visualized by using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo) and diaminobenzidine.

Results

Human Hepatocyte Chimeric Mice Develop High-Level and Long-Term Viremia After Inoculation of Serum Samples Obtained From Carriers. Twenty chimeric mice were inoculated with 50 μ L serum 1 (Table 1). We used mice that had relatively low-level HSA because we had previously found that mice with low-level replacement are susceptible to HBV (Chayama K and Tatenno C, unpublished results). The HSA of these mice was 300,000 ng/mL (median, range, 40,000-3,090,000, Fig. 1A). All 20 mice tested positive for HBV DNA by nested PCR 2 to 4 weeks after inoculation. Eighteen of 20 mice developed quantitatively measurable viremia, but two mice showed very low-level viremia that was detectable only by nested PCR. Mice with persistently high-level HSA tended to show high virus titer (Fig. 1A-C). The maximum level of viremia was 9.5×10^{10} copy/mL. The viremia reached a plateau 4 to 6 weeks after infection. In contrast, mice with a rapid decrease in HSA or persistently low-level HSA showed low virus titer (Fig. 1D). We also performed infection experiments using serum 2 (Table 1). Of the five mice inoculated with this serum, all developed quantitatively measurable viremia 2 to 4 weeks after inoculation (Fig. 2). The level of viremia reached 1×10^7 to 1×10^9 copies/mL. The level of viremia also tended to be high in mice with high HSA levels.

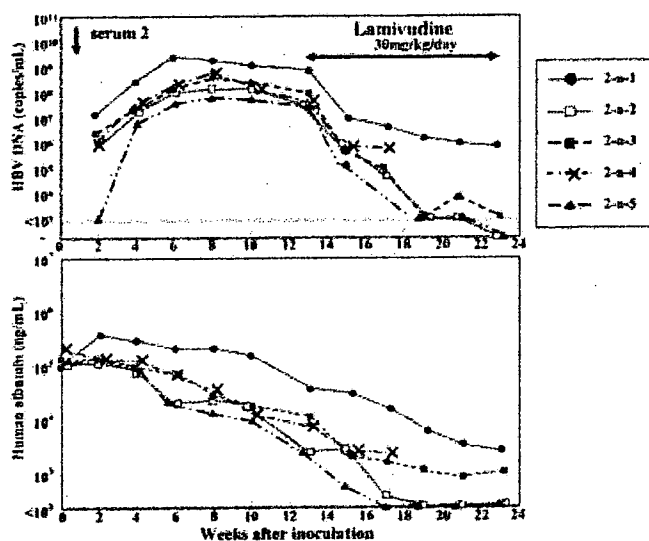


Fig. 2. Time course studies in five mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 2, Table 1) was intravenously injected into each mouse. The upper panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin. The effects of lamivudine are shown in the upper panels.

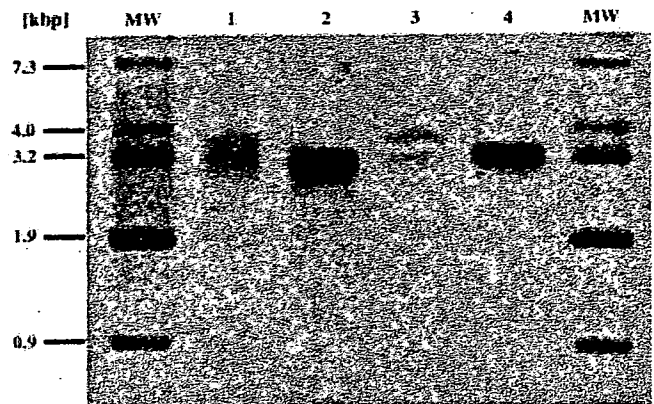


Fig. 3. Formation of fully repaired, relaxed circular hepatitis B virus (HBV) DNA after endogenous polymerase reaction. HBV particles produced into supernatant were immunoprecipitated with a monoclonal antibody against HBs antigen and subjected to Southern blot analysis before (lanes 1 and 2) and after (lanes 3 and 4) endogenous polymerase reaction. The undigested (lanes 1 and 3) and unique *Sma*I-digested DNA was electrophoresed in a 1% agarose gel and detected by Southern blot hybridization.

HBV Generated in HepG2 Cell Lines Are Infectious to Human Hepatocyte Chimeric Mice. HBV markers and endogenous polymerase experiments with Southern blot analysis of HBV produced by transiently or stably transfected HepG2 cell lines are shown in Table 1 and Fig. 3. The results indicated that these cell lines produced the expected HBV antigens and HBV DNA into the supernatant. Using virus particles produced by transient transfection of plasmid pTRE-HB-wt, we performed endogenous polymerase chain reaction experiments. Formation of fully double-stranded, relaxed circular DNA was observed after the reaction (Fig. 3). Sucrose density gradient analysis of HBV produced by stably transfected cell line (CA59, Table 1) showed that the produced viruses were sedimented to similar fractions of HBV obtained from the serum of the HBV carrier (Fig. 4), suggesting that HBV particles similar to those in serum are produced in these cell lines.

In the next step, we inoculated each chimeric mouse with 50 μ L of the supernatants produced by transiently or stably transfected cell lines (Table 1). Three mice were inoculated with CM3 (Fig. 5A). Four weeks later, one of these three mice developed measurable viremia. It reached a high level (7.3×10^8 copy/mL) at week 14. A serum sample obtained from this mouse at week 6 was stored in liquid nitrogen and used in the subsequent passage experiments. The other two mice developed viremia, but its level was so low that HBV was only detectable by nested PCR. At week 13, these two mice (5-a-2, 5-a-3, Fig. 5A) were inoculated with serum 1, which induced high-level viremia in mice with high HSA levels (Fig. 1). These mice did not develop measurable viremia, suggesting that the

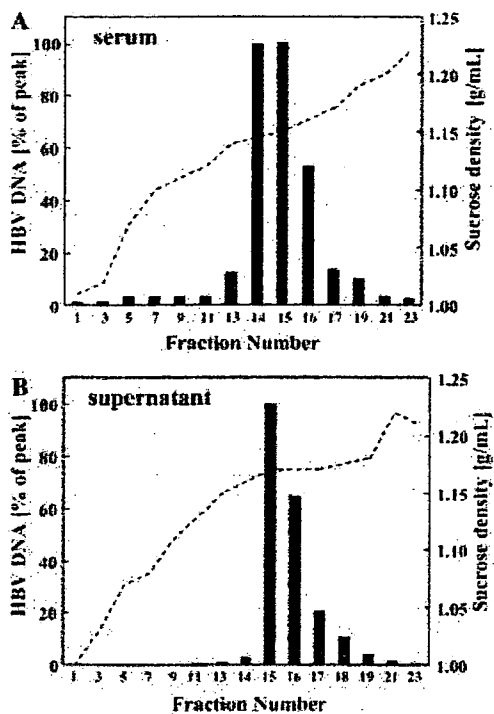


Fig. 4. Sucrose density gradient analysis of hepatitis B virus (HBV) produced into the supernatant of the transfected HepG2 cell line (CA59). Sucrose density is indicated by the dotted line. The amount of HBV DNA in each fraction was measured by real-time PCR.

low-level viremia in the latter two mice was due to low-levels of human hepatocyte replacement. Similarly, six mice were inoculated with supernatant CA59. One of these six mice developed quantitatively measurable viremia (peak, 2.6×10^9 copies/mL) (Fig. 5B). Two of the six mice developed viremia only detectable by nested PCR. For a more efficient reverse genetics infection procedure, we used mice with higher human albumin concentrations and inoculated each with 500 to 1,000 μ L freshly prepared high-titer virus particles. This resulted in infection of all 10 mice (Fig. 5C). Thus, we established a highly effective infection procedure of reverse genetics of HBV.

Infection of Genetically Engineered Mutant Viruses. Four mice were inoculated with the supernatant of genetically engineered e-antigen-negative HBV generated in a pTRE-HBV-PC-transfected HepG2 cell line (e-negative, Table 1). Three of these four mice developed quantitatively measurable, but relatively low-level (less than 10^7 copies/mL) viremia, 2 to 6 weeks after inoculation (Fig. 6). Nucleotide sequence analysis of the precore region showed that the sequence obtained from the infected mice was completely in agreement with the transfected plasmid with precore stop codon at 1896.

Passage Experiment of HBV From a Mouse Infected by In Vitro Generated HBV to Naïve Chimeric Mice. Each of four naïve mice was injected with 5 μ L serum

samples obtained from a mouse that developed HBV viremia after inoculation of *in vitro* generated virus (CM3, Table 1). All four mice developed viremia at 2 to 6 weeks after inoculation (Fig. 7). One of the four mice that developed measurable viremia died at week 5 (7-a-3, Fig. 7). Another mouse (7-a-2) was weak and was sacrificed at week 13. The high-level viremia in the third mouse (7-a-1) increased further to 8.5×10^9 copies/mL. The remaining mice developed viremia detectable only by nested PCR (7-a-4).

Histochemical Analysis of the Liver of Mice Infected With HBV. Liver specimens from mice that became positive for HBV DNA after the inoculation of the described passage experiment were subjected to histological and immunohistochemical analyses. Multiple foci of replaced human hepatocytes were noted in hematoxylin-eosin-stained sections (Fig. 8A) that were positive for HSA (Fig. 8B). Such positive human hepatocytes were also positive for the HBV core antigen in serial sections (Fig. 8C).

Effect of Lamivudine Treatment in Mice Infected With HBV. Five mice that became positive for HBV DNA by inoculation with serum 2 (Fig. 2) were fed lamivudine (30 mg/kg/day)-containing food. A rapid reduction of HBV DNA level was observed in all 5 mice. Although two of five mice showed graft failure reflected by a decrease in HSA levels to the lower limits of the assay (2-a-2 and 2-a-5), the reduction of HBV DNA levels appeared before the decrease in HSA in these mice, suggesting that the decrease in HBV DNA was due to both the effect of lamivudine and the loss of virus replicating human hepatocytes (Fig. 2). Similarly, 1 mouse with high-level viremia as described in the above passage experiment (7-a-1, Fig. 7) showed a marked reduction of HBV DNA.

Discussion

The major finding of the current study was the successful establishment of a model of HBV infection with long-term and high-level HBV viremia in the human hepatocyte chimeric mouse. The level of viremia correlated with the degree of human hepatocyte replacement indicated by HSA levels. We also showed that HBV created *in vitro* using HepG2 cell lines are infectious to this mouse model. Thus, a combination of chimeric mouse and molecularly cloned virus enabled us to prepare a practical model for the study of HBV virology. Chimpanzee is also a useful model for the study of HBV virology. Injection of molecularly cloned HBV into the liver of chimpanzee induced HBV infection and hepatitis.²² However, there might be some difference between hepatocytes of human and chimpanzee that could affect the nature of infection and the replication of this narrow host virus.

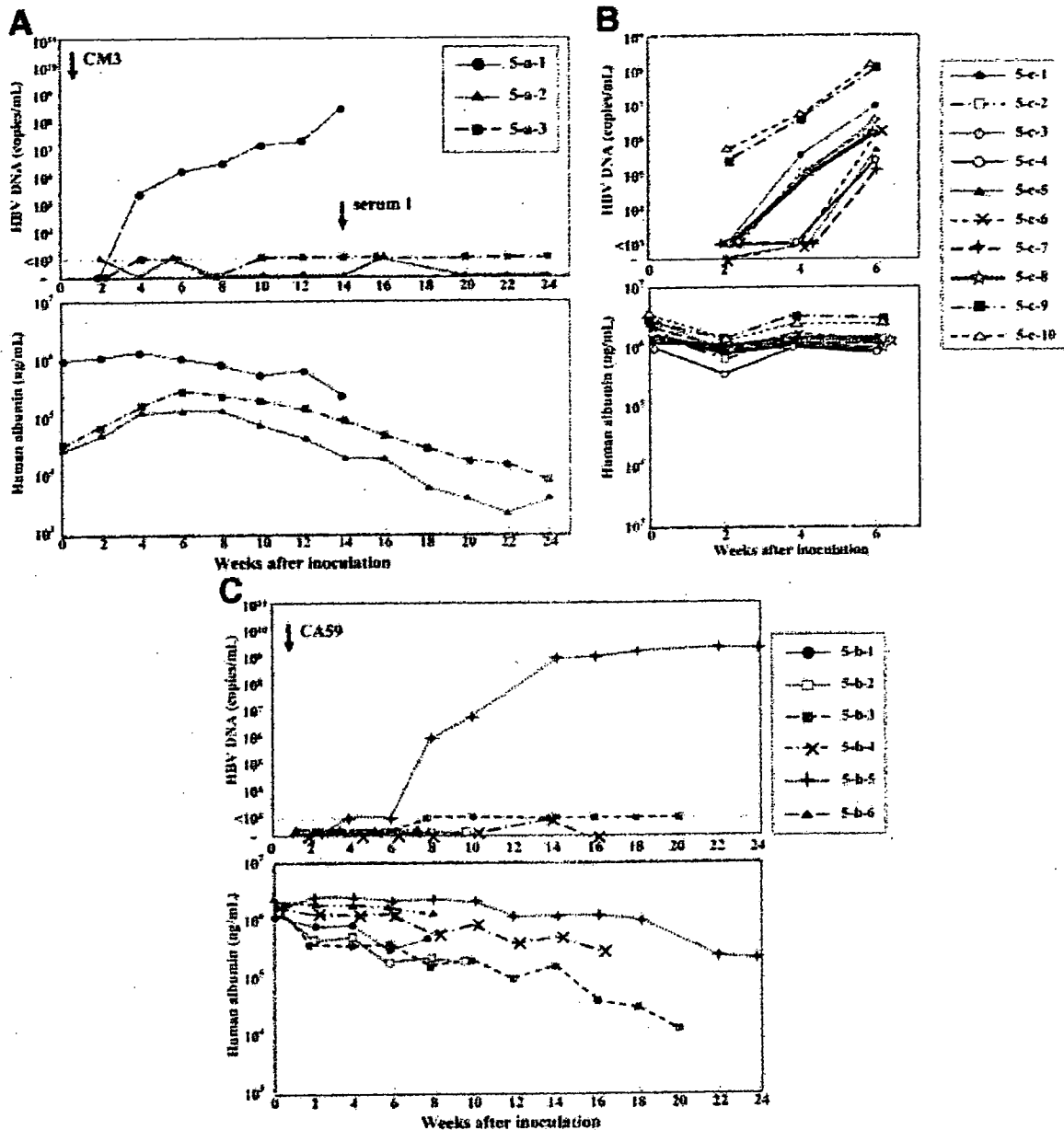


Fig. 5. (A) Time course studies in three mice inoculated with supernatants of HepG2 cell lines transfected with hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (CM3, Table 1) was inoculated intravenously into each mouse. Upper panel: HBV DNA; lower panel: concentrations of human serum albumin. (B) Time course studies in 6 mice inoculated with supernatants of HepG2 cell lines transfected with HBV DNA. Fifty microliters culture supernatant (CA59, Table 1) was inoculated intravenously into each mouse. (C) Time course studies in 10 mice inoculated with freshly prepared supernatants of HepG2 cell lines transfected with HBV DNA. Five hundred (mice 5-c-1 to 5-c-4) and 1,000 μ L culture supernatants (5-c-5 to 5-c-8) and 500 μ L concentrated (from 1.1×10^9 copies/mL to 1.3×10^{10} copy/mL by ultrafiltration) supernatant (5-c-9 and 5-c-10) were inoculated intravenously into each mouse.

A critical difference between the chimeric mouse model reported here and chimpanzee is that there is no immune system active for HBV in the mouse model. Although the chimpanzee model is known to cause hepatitis and is suitable for the study of HBV-induced hepatitis,²³ the mouse model is expected to be free from inflammation because these mice are SCID and do not have any human cytotoxic T lymphocytes. Actually, we observed no lymphocyte infiltration or focal necrosis of human hepatocytes in our

mouse model. Recently, similar morphological changes in a similar model were reported by Meuleman et al.²⁴; they also observed no alteration of liver architecture by HBV and hepatitis C virus infections. Interestingly, however, we observed a poor increase in the viral titer during the early phase of infection in some mice (Figs. 5A, 6). This might represent some innate anti-viral defense mechanism of liver cells themselves against viral infection. Further investigation is necessary to explore this issue.

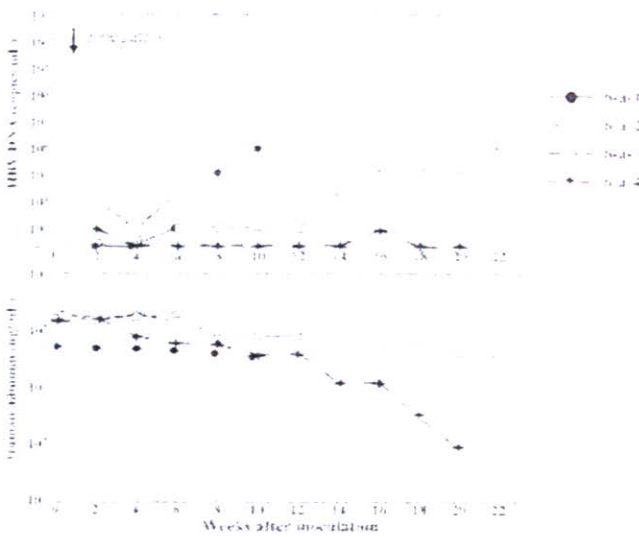


Fig. 6. Time course studies in four mice inoculated with supernatants of HepG2 cell lines transfected with e-antigen-negative (G1896A) hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (e-negative, Table 1) was inoculated intravenously into each mouse. The upper panel shows HBV DNA, and the lower panel shows the concentrations of human serum albumin.

A mouse model without any inflammation is an advantageous phenotype because it allows the study of HBV replication without any influence of immunological reaction. The model is also beneficial for studying the effects of drugs without any influence of fluctuation of the virus by immunological reaction. The HBV-infected mouse described here opens the way to create a long desired practical small animal model that overcomes economical

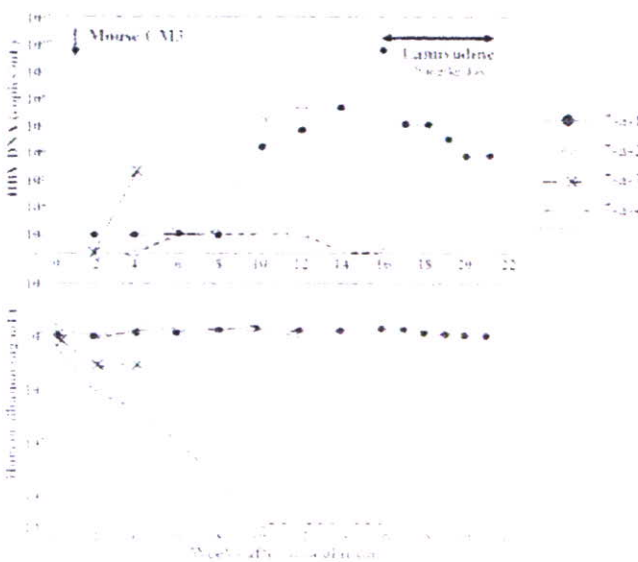


Fig. 7. Passage experiments in four mice. Five microliters mouse serum (mouse CM3, Table 1) was intravenously inoculated into each of four naive mice. Upper panel: HBV DNA; lower panel: concentrations of human serum albumin.

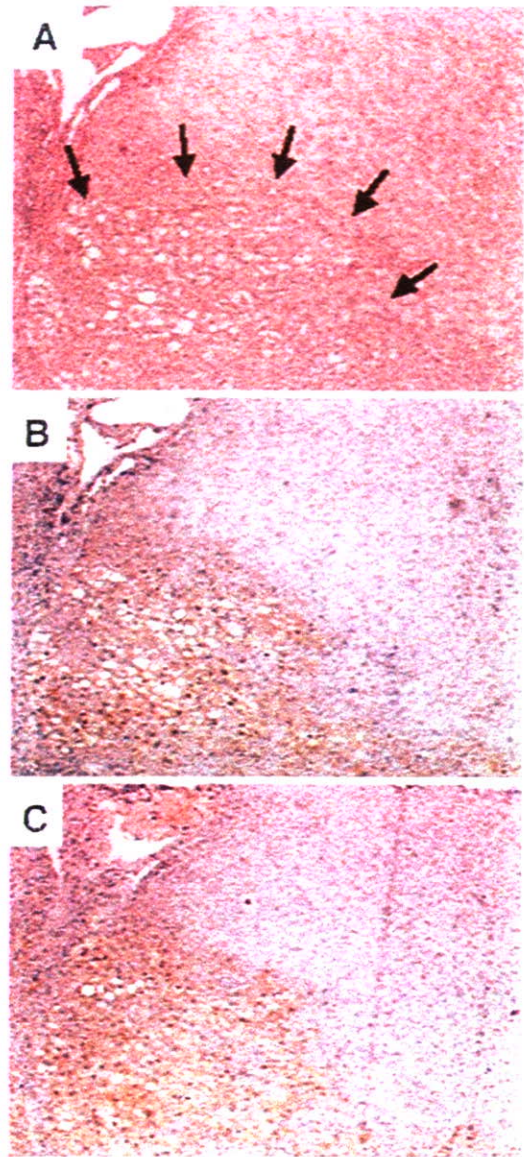


Fig. 8. Histochemical analysis of liver samples obtained from mice infected with hepatitis B virus generated in the 1.4 genome transfected HepG2 cell line. (A) Hematoxylin-eosin staining. Human hepatocytes are indicated by arrows. Immunohistochemical staining with anti-human serum albumin antibody (B) and anti-HBc-Ag antibody (C) (original magnification, $\times 40$).

and ethical problems associated with the chimpanzee model.

We showed in this study that reverse genetics of HBV can be achieved highly efficiently by using mice with high human albumin levels and inoculating mice with large amounts of freshly prepared virus particles (Fig. 5C). We further showed in this study that e-antigen is completely dispensable for infection and replication. The e-antigen-negative HBV-containing serum was previously used in chimpanzee and is known to induce more severe hepatitis.¹³ However, it is difficult to exclude the possible presence of a small amount of e-antigen-producing virus that might help infection and

replication of HBe antigen-negative HBV strain. Our results clearly demonstrate that HBV can infect and replicate in the complete absence of e-antigen-producing species. However, the level of viremia was relatively low (less than 1×10^7 copies/mL) in these mice. Whether this is due to lack of e-antigen should be further confirmed in a larger number of mice with high replacement index.

Because the mice treated with lamivudine showed a reduction of viremia (Figs. 2, 7), our infected mouse is suitable for the study of new drugs. Lamivudine is a potent anti-HBV drug that reduces the virus and induces clinical remission and histological improvement.²⁶⁻²⁸ Emergence of drug-resistant HBV mutants against this drug as well as other anti-viral drugs is a serious problem in the treatment of HBV,²⁹⁻³¹ as has been seen in the therapy of human immunodeficiency virus infection. Our model is especially useful for the study of the biology and drug susceptibility of such mutants, because almost all such drug resistances are based on only one or two point mutation(s).^{29,31}

In conclusion, the mouse model presented in this study is very useful for the study of HBV biology and evaluating of anti-HBV drugs. Furthermore, many applications of this model are expected because we can easily create, manipulate, and modify the model compared with other models.

Acknowledgment: The authors thank Eiko Okutani, Asako Yoshizato, Hiromi Ishino, Kana Kunihiro, and Kiyomi Toyota for their excellent technical assistance.

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EDITORIAL

Integration of hepatitis B virus DNA and hepatocellular carcinoma

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See article in *J. Gastroenterol. Hepatol.* 2005; 20: 1206–1214.

Hepatocellular carcinoma (HCC) is one of the most common malignant human tumors worldwide.¹ Hepatitis B virus (HBV) and the hepatitis C virus (HCV) infections are etiologically linked to this cancer. HCC often emerges after the development of cirrhosis in patients with chronic HCV infection. In contrast, in patients with the HBV infection, HCC sometimes develop in non-cirrhotic patients, although the incidence is much lower than in patients with cirrhosis.² The HBV is a DNA virus that integrates into the chromosome of the host. In contrast, the HCV is a RNA virus that does not integrate into the host genome. It seems that the integration of the HBV into host chromosome(s) plays an important role in the development of HCC, especially in non-cirrhotic patients with HBV infection.

There are two major possible carcinogenic effects of HBV on the development of HCC. One is the direct effect of viral proteins such as HBx. In fact, this protein has been proven to be a transactivator that activates a variety of viral and cellular promoters. It has also been reported that HBx binds to p53 and inhibits p53-mediated cellular processes, including DNA binding, transcriptional transactivation and apoptosis. The viral proteins that might be produced as a result of integration and truncation of preS2/S and hepatitis B spliced proteins have also been reported to function as transcriptional modulators.³ These proteins have been shown to modulate the transcriptional activation of cellular growth-regulating genes, to modify apoptosis and to inhibit nucleotide excision and the repair of damaged cellular DNA. The other possible carcinogenic effect of HBV is the integration of HBV-DNA into a host chromosome. This might interrupt cellular tumor suppressor genes, or cause alterations in the expression of cellular growth factors and/or apoptosis regulating

factors. Although frequent activation of the N-myc oncogene has been reported in woodchuck hepatitis virus integration,⁴ no apparent favored locus of human oncogene/tumor suppressor gene has been found. However, there are many reports that describe the alteration of cellular proliferation pathways (important in the control of cell signaling, proliferation and viability) as a result of the insertion of HBV-DNA. Recent reports have shown that in at least some hepatocellular carcinomas, the human telomerase reverse transcriptase (hTERT) gene is a non-random integration site of the HBV genome, which activates the hTERT transcription *in cis*.⁵

In this issue of the Journal, Huang *et al.*⁶ reported a high incidence of HBV-DNA integration in tumor and non-tumor tissues of children with chronic HBV infection. They detected a higher incidence of the HBV integration using a sensitive inverse polymerase chain reaction (IPCR). This method was developed to amplify unknown sequences that flank a region of a known sequence (Fig. 1)^{7,8}. This technique is very sensitive because a nested polymerase chain reaction is used for amplification of DNA. However, in some instances, it fails to detect integrated HBV-DNA. For example, integrants that do not contain a full four site of primer escape detection. Alternatively, the flanking region that does not contain an employed restriction enzyme digestion site near the integration portion fails to be amplified. The latter possibility is less likely because the employed restriction enzymes are four base recognizing cutters, the restriction site often appearing in common nucleotide sequences. However, it sometimes the restriction enzyme site is too near to the integration site and consequently, the amplified flanking sequences are too short to analyze. This could be avoided if one were to use several different pairs of enzymes in order to analyze the samples. It is, thus, highly likely that the incidence of integration of HBV-DNA in a host chromosome is higher than that detected.

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Accepted for publication 4 February 2005.

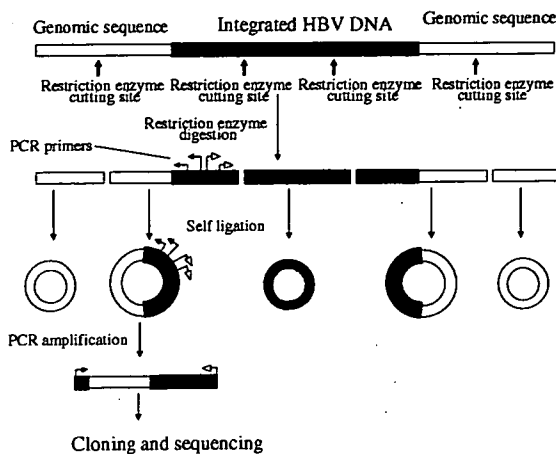


Figure 1 Alu-polymerase chain reaction to detect the integrated hepatitis B virus DNA in host chromosomes.

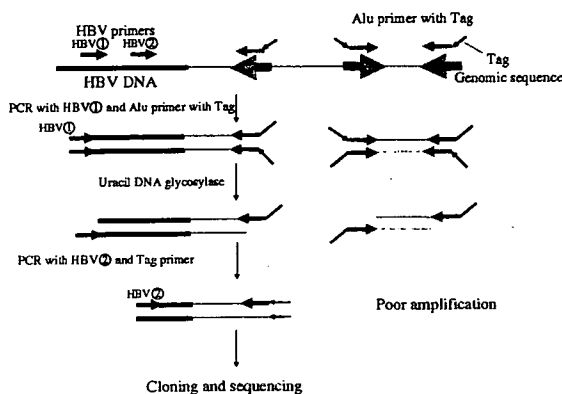


Figure 2 Cellular DNA was amplified using primer HBV1 and Alu specific primer with 5'-Tag sequence. Primers were synthesized using dUTP. The amplified products were then digested with uracil DNA glycosylase. Primers in the amplified DNA were digested with this enzyme leaving complementary sequences. The second round PCR was then carried out using HBV2 and a Tag primer. Only sequences that contain HBV-DNA and Alu sequence were effectively amplified.

An alternative method used to detect integration of HBV-DNA is Alu PCR (Fig. 2).⁹ A recent study showed that this method is very sensitive, detecting more than 70% of HCC related to HBV containing integrated HBV-DNA.¹⁰ However, this approach might fail to detect integrated HBV-DNA if the site of integration is too far from the Alu sequences. Also, it is possible that the flanking sequence contains a PCR resistant secondary structure or guanine-cytosine rich regions. Furthermore, similarly to IPCR, if the integrated sequence does not contain a sequence for primers to anneal, the examined integration rate might be underestimated.

The high integration rate (related or unrelated to carcinogenesis) and the fact that the related hepadna virus, the duck hepatitis B virus (DHBV), integrates into a host genome very early after infection, show us that

almost all infected cells contain integrated HBV-DNA.¹¹ However, previous studies have shown that a greater proportion of HBV-DNA integrations are not related to cancer development. Although it is obvious that chronic inflammation and fibrosis are main factors for accelerated cell proliferation and transformation, there are many steps to the development of HCC. Although it remains unclear if the integration of HBV plays an important role in hepatocarcinogenesis, it might open up new avenues of research for hepatocarcinogenesis, for example, to improve the detection of HBV-DNA integration (see Huang *et al.*⁶), and to continue the analysis of the effect of integrated HBV-DNA to intracellular biological phenomenon. Tissues obtained from HCC in children might be good candidates for such analysis because they might cause a serious alteration in chromosomal genetic functions.

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Clinical Studies

Liver International

DOI: 10.1111/j.1478-3231.2005.01200.x

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsunami H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.

Liver International 2006; 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Received 19 June 2005,
accepted 8 August 2005

Prediction of lamivudine resistance

Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retro-

spectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

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over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 µL serum was mixed with 50 µL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 µL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 µL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance		<i>P</i>
	Negative (n = 54)	Positive (n = 27)	
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	> 0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	> 0.2‡
HBV genotype (A/B/C)	2/2/50	0/1/26	> 0.2‡
HBe antigen (positive %)	59%	70%	> 0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	> 0.2‡
At 6 months	27 (11–115)	30 (15–92)	> 0.2‡
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	> 0.2‡
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073‡
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡ χ^2 -test.

antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it (*P* > 0.2).

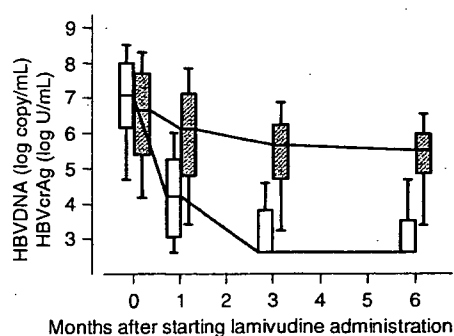


Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, *P* < 0.001), 3 (3.60 log copy/ml vs. 0.83 log U/ml, *P* < 0.001) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, *P* < 0.001) after the initiation of lamivudine administration.

Prediction of lamivudine resistance

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P < 0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine

resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

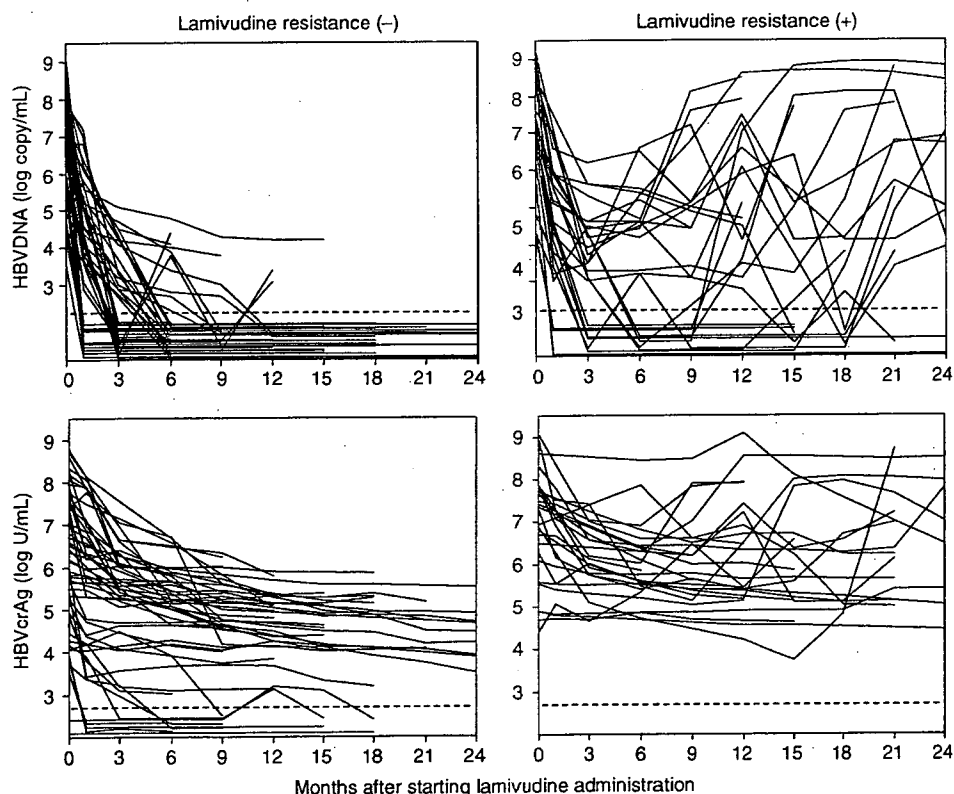


Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.