

図7 HCV TgM における鉄代謝異常機構の模式図

HCV 蛋白により産生される ROS は CHOP の発現を亢進させ、これにより C/EBP α の DNA 結合能が低下し、hepcidin の転写が抑制される。hepcidin の発現低下は ferroportin (FPN) の発現を増加させ、十二指腸からの鉄吸収ならびにマクロファージからの鉄放出が増加し、結果的に肝の鉄蓄積を引き起こす。

レス増強因子、さらには HCV による肝脂肪化や鉄蓄積による酸化ストレスの増強が絡み合って C 型肝炎からの高率な肝発癌の一因になっていると考えられる。一方で酸化ストレスの増強はアポトーシスを誘導しやすいが、冒頭でも述べたように ROS を消去する抗酸化系の各種遺伝子は代償性に亢進しており⁴⁾、いわば酸化的 DNA 障害をもつ細胞が排除されない状態は高癌化状態ともいえる。

筆者らは複合的な酸化ストレス状態でどのように肝発癌を来すかについて検討したのでその成績を紹介する²⁸⁾。HCV TgM あるいはコントロールマウス (C57BL/6) に通常餌 (carbonyl iron 25 mg/kg 餌) または鉄過剰餌 (carbonyl iron 225 mg/kg 餌) を与える 4 群を設定し、6ヵ月目と 12ヵ月目に肝内組織学的変化ならびに肝腫瘍の有無について検索した。鉄負荷 6ヵ月目、12ヵ月目の HCV TgM の肝内鉄濃度は C 型慢性肝炎患者

のそれとほぼ同等であった。鉄負荷 6ヵ月目の HCV TgM は他の 3 群に比べて肝内の脂肪沈着が亢進し、中心静脈周囲には小滴性脂肪沈着も目立った (図 8)。さらにミトコンドリアの超微形態異常 (図 9-A) を認め、脂肪酸分解も低下した。鉄負荷 12ヵ月目になると HCV TgM は肝内脂質過酸化物質とともに 8-OHdG (図 9-B, C) の蓄積が亢進した。この際に特記すべき点は鉄負荷 HCV TgM のみならず、他の 3 群もマウスも加齢とともに肝内の 8-OHdG 量が有意に増加していることである。最終的には鉄負荷 HCV TgM のみに肝細胞癌を含む肝腫瘍を 45 % (5/11) 認めた (表 1)。このように HCV TgM は HCV 蛋白に加えて自らが引き起こす鉄過剰やさらには加齢が加わることでミトコンドリア障害とともに肝内の脂肪沈着が亢進し、遂には酸化ストレスが亢進するとともに酸化的 DNA 障害が進行し肝発癌を来すことが明らかになった。

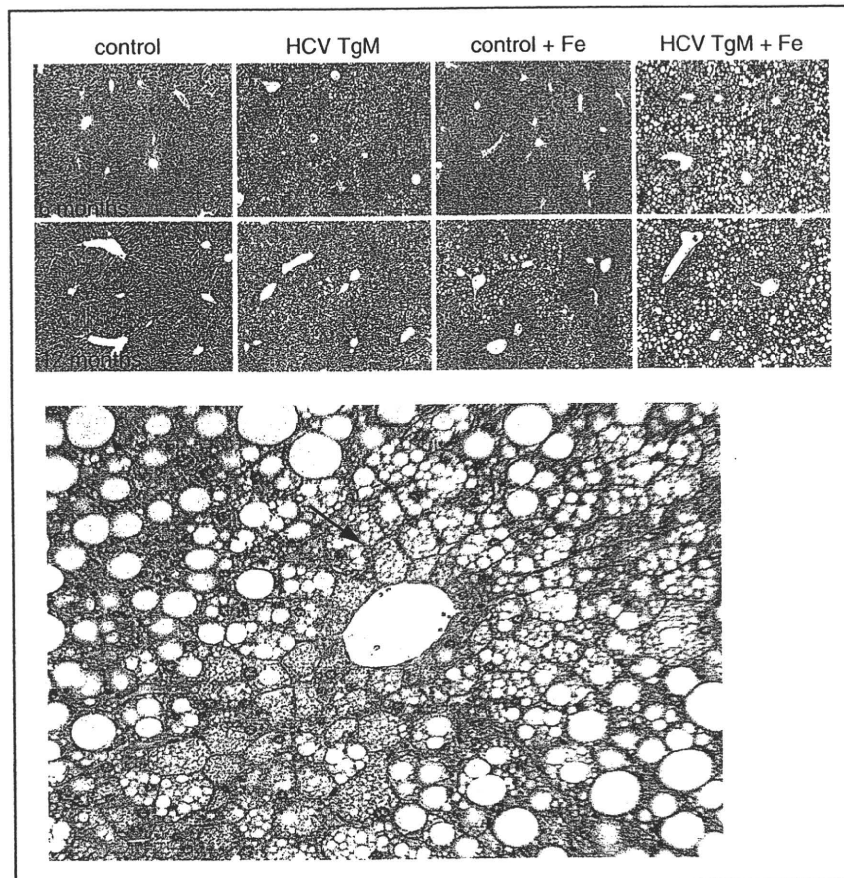


図8 鉄負荷 HCV TgM (HCV TgM+Fe), 鉄負荷コントロールマウス (control+Fe), 通常餌の TgM (HCV TgM), および通常餌のコントロールマウス (control) の肝組織像 (HE 染色)

中心静脈周囲には矢印で示すような小滴性脂肪の沈着が顕著である。



おわりに

本稿では酸化ストレスからみた C 型肝炎の病態について解説した。酸化ストレスの引き金となる HCV を排除することが治療の基本であることはいうまでもないが、現在のペグインターフェロンとリバビリン併用療法をもってしても約半数の患者では HCV の排除が困難である。非アルコール性脂肪性肝炎が生活習慣病として認識されているが、C 型肝炎も酸化ストレスを軽減するという点においては同様な認識が重要と考えられる。すなわち禁酒はもちろんのこと体重減少やバランスの取れた食生活、さらには治療介入としての酸化

ストレス軽減は病変の進展阻止のための基本的な戦略であることを認識すべきである。

References

- 1) Farinati F, Cardin R, De Maria N, et al : Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J Hepatol* 22 : 449-456, 1995
- 2) Moriya K, Fujie H, Shintani Y, et al : The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 4 : 1065-1067, 1998
- 3) Moriya K, Nakagawa K, Santa T, et al : Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 61 : 4365-4370, 2001
- 4) Okuda M, Li K, Beard MR, et al : Mitochondrial injury, oxidative stress, and antioxidant gene expression

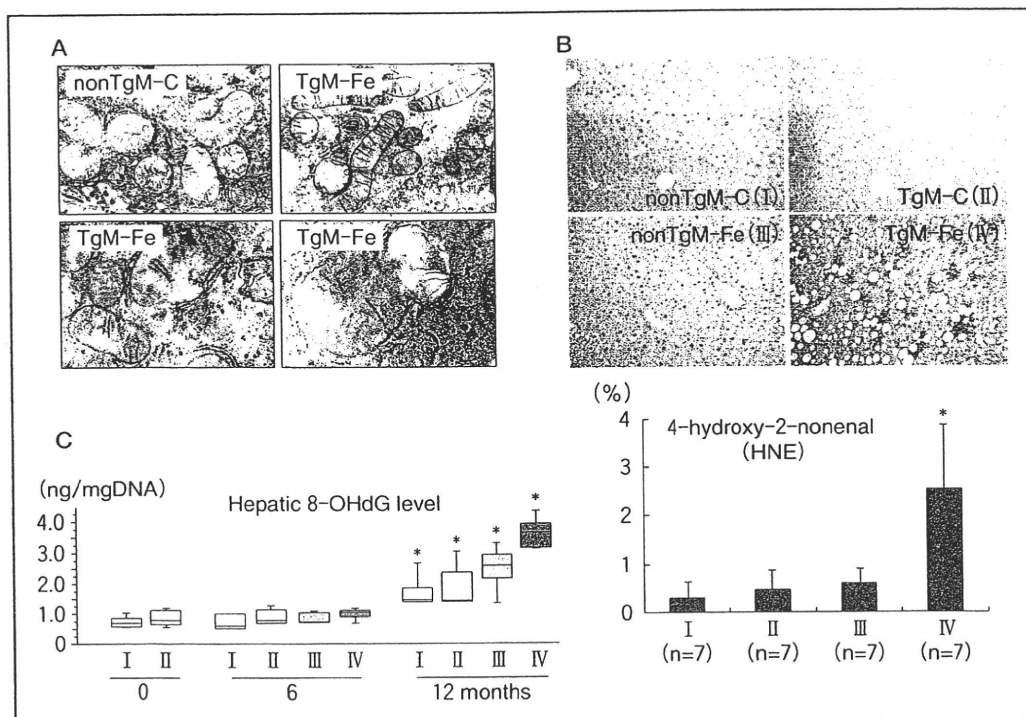


図9 鉄負荷6ヵ月後のHCV TgM (TgM-Fe)と鉄負荷なしのコントロールマウス(nonTgM-C)の肝ミトコンドリア電子顕微鏡像(A), 12ヵ月目の肝組織内脂質過酸化物質(4-hydroxy-2-nonenal : HNE)の比較(B), および12ヵ月目の肝組織内8-hydroxy-2'-deoxyguanosine (8-OHdG)量の比較(C)

A : 鉄負荷HCV TgMの肝ミトコンドリアはコントロールに比べて大小不同や膜の膨化, クリステの乱れ, 内膜腔のbulgingなどの形態異常が認められる。B : HNE量は免疫染色による陽性面積の割合で比較した。C : 鉄負荷HCV TgMは他の3群に比べて8-OHdG量が多く, また他の3群もそれぞれ飼育開始時, 6ヵ月目と比べて8-OHdG量は多かった。

are induced by hepatitis C virus core protein. *Gastroenterology* 122 : 366-375, 2002

- 5) Otani K, Korenaga M, Beard MR, et al : Hepatitis C virus core protein, cytochrome P 450 2 E 1, and alcohol produce combined mitochondrial injury and cytotoxicity in hepatoma cells. *Gastroenterology* 128 : 96-107, 2005
- 6) Korenaga M, Wang T, Li Y, et al : Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 280 : 37481-37488, 2005
- 7) Schwer B, Ren S, Pietschmann T, et al : Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* 78 : 7958-7968, 2004
- 8) Li Y, Boehning DF, Qian T, et al : Hepatitis C virus core protein increases mitochondrial ROS production by stimulation of Ca²⁺ uniporter activity. *FASEB J* 21 : 2474-2485, 2007
- 9) Piccoli C, Scrima R, Quarato G, et al : Hepatitis C virus protein expression causes calcium-mediated mito-

chondrial bioenergetic dysfunction and nitro-oxidative stress. *Hepatology* 46 : 58-65, 2007

- 10) Murakami C, Hino K, Korenaga M, et al : Factors predicting progression to cirrhosis and hepatocellular carcinoma in patients with transfusion-associated hepatitis C virus infection. *J Clin Gastroenterol* 28 : 148-152, 1999
- 11) Thabut D, Le Calvez S, Thibault V, et al : Hepatitis C in 6,865 patients 65 yr or older : a severe and neglected curable disease? *Am J Gastroenterol* 101 : 1260-1267, 2006
- 12) Tanguy S, de Leiris J, Besse S, Boucher F : Ageing exacerbates the cardiotoxicity of hydrogen peroxide through the Fenton reaction in rats. *Mech Ageing Dev* 124 : 229-235, 2003
- 13) Hourigan LF, Macdonald GA, Purdie D, et al : Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. *Hepatology* 29 : 1215-1219, 1999
- 14) Kitase A, Hino K, Furutani T, et al : In situ detection

表1 鉄負荷 TgM (TgM+Fe), コントロールマウス (Control+Fe), 通常餌 TgM (TgM+C), コントロールマウス (Control+C) の肝腫瘍発生率

Feeding period	Mice group	Liver tumor
6 months	Control + C	0/10
	Control + Fe	0/10
	TgM + C	0/10
	TgM + Fe	0/10
9 months	Control + C	0/7
	Control + Fe	0/6
	TgM + C	0/5
	TgM + Fe	0/6
12 months	Control + C	0/10
	Control + Fe	0/8
	TgM + C	0/9
	TgM + Fe	5/11

of oxidized n-3 polyunsaturated fatty acids in chronic hepatitis C : correlation with hepatic steatosis. *J Gastroenterol* 40 : 617-624, 2005

- 15) Hayashi H, Takikawa T, Nishimura N, et al : Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. *Am J Gastroenterol* 89 : 986-988, 1994
- 16) Kato J, Kobune M, Nakamura T, et al : Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. *Cancer Res* 61 : 8697-8702, 2001
- 17) Perlemuter G, Sabile A, Letteron P, et al : Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion : a model of viral-related steatosis. *FASEB J* 16 : 185-194, 2002
- 18) Moriishi K, Mochizuki R, Moriya K, et al : Critical role of PA 28 gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci U S A* 104 : 1661-1666, 2007
- 19) Tanaka N, Moriya K, Kiyosawa K, et al : PPARalpha activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice. *J Clin Invest* 118 : 683-694, 2008
- 20) Nishina S, Hino K, Korenaga M, et al : Hepatitis C virus-induced reactive oxygen species raise hepatic iron level in mice by reducing hepcidin transcription. *Gastroenterology* 134 : 226-238, 2008
- 21) Nemeth E, Tuttle MS, Powelson J, et al : Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306 : 2090-2093, 2004
- 22) Lin L, Valore EV, Nemeth E, et al : Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP 2/4. *Blood* 110 : 2182-2189, 2007
- 23) Babitt JL, Huang FW, Wrighting DM, et al : Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* 38 : 531-539, 2006
- 24) Pietrangelo A, Dierssen U, Valli L, et al : STAT 3 is required for IL-6-gp 130-dependent activation of hepcidin *in vivo*. *Gastroenterology* 132 : 294-300, 2007
- 25) Miura K, Taura K, Kodama Y, et al : Hepatitis C virus-induced oxidative stress suppresses hepcidin expression through increased histone deacetylase activity. *Hepatology* 48 : 1420-1429, 2008
- 26) Nagashima M, Kudo M, Chung H, et al : Regulatory failure of serum prohepcidin levels in patients with hepatitis C. *Hepatology Res* 36 : 288-293, 2006
- 27) Fujita N, Sugimoto R, Motonishi S, et al : Patients with chronic hepatitis C achieving a sustained virological response to peginterferon and ribavirin therapy recover from impaired hepcidin secretion. *J Hepatol* 49 : 702-710, 2008
- 28) Furutani T, Hino K, Okuda M, et al : Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 130 : 2087-2098, 2006

Distribution of Hepatitis B Virus Genotypes among Patients with Chronic Infection in Japan Shifting toward an Increase of Genotype A[∇]

Kentaro Matsuura,^{1,2} Yasuhito Tanaka,^{1*} Shuhei Hige,³ Gotaro Yamada,⁴ Yoshikazu Murawaki,⁵ Masafumi Komatsu,⁶ Tomoyuki Kuramitsu,⁷ Sumio Kawata,⁸ Eiji Tanaka,⁹ Namiki Izumi,¹⁰ Chiaki Okuse,¹¹ Shinichi Kakumu,¹² Takeshi Okanoue,¹³ Keisuke Hino,¹⁴ Yoichi Hiasa,¹⁵ Michio Sata,¹⁶ Tatsuji Maeshiro,¹⁷ Fuminaka Sugauchi,² Shunsuke Nojiri,² Takashi Joh,² Yuzo Miyakawa,¹⁸ and Masashi Mizokami^{1,19}

Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan¹; Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan²; Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan³; Department of Basic Laboratory Sciences, Kawasaki Medical School, Kawasaki Hospital, Okayama, Japan⁴; Division of Medicine and Clinical Science, Faculty of Medicine, Tottori University, Tottori, Japan⁵; Department of Gastroenterology, Akita City Hospital, Akita, Japan⁶; Kuramitsu Clinic, Akita, Japan⁷; Department of Gastroenterology, Yamagata University School of Medicine, Yamagata, Japan⁸; Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan⁹; Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan¹⁰; Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kawasaki, Japan¹¹; Department of Gastroenterology, Aichi Medical University School of Medicine, Aichi, Japan¹²; Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan¹³; Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan¹⁴; Department of Gastroenterology and Metabolism, Ehime University Graduate School of Medicine, Ehime, Japan¹⁵; Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan¹⁶; First Department of Internal Medicine, University Hospital, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan¹⁷; Miyakawa Memorial Research Foundation, Tokyo, Japan¹⁸; and Research Center for Hepatitis and Immunology, Kohnodai Hospital International Medical Center of Japan, Ichikawa, Japan¹⁹

Received 29 October 2008/Returned for modification 17 December 2008/Accepted 2 March 2009

Acute hepatitis B virus (HBV) infection has been increasing through promiscuous sexual contacts, and HBV genotype A (HBV/A) is frequent in patients with acute hepatitis B (AHB) in Japan. To compare the geographic distribution of HBV genotypes in patients with chronic hepatitis B (CHB) in Japan between 2005 and 2006 and between 2000 and 2001, with special attention to changes in the proportion of HBV/A, a cohort study was performed to survey changes in genotypes of CHB patients at 16 hospitals throughout Japan. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses. Of the 1,271 patients, 3.5%, 14.1%, and 82.3% were infected with HBV/A, -B, and -C, respectively. In comparison with our previous survey during 2000 and 2001, HBV/A was twice as frequent (3.5% versus 1.7%; $P = 0.02$). The mean age was lower in the patients with HBV/A than in those with HBV/B or -C. Based on phylogenetic analyses of 11 full-length genomes and 29 pre-S2/S region sequences from patients, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India. They clustered with HBV/A from AHB patients and have spread throughout Japan. HBV/A has been increasing in CHB patients in Japan as a consequence of AHB spreading in the younger generation through promiscuous sexual contacts, aided by a tendency of HBV/A to induce chronic hepatitis. The spread of HBV/A infection in Japan should be prevented by universal vaccination programs.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae*, is a circular, partially double-stranded DNA virus and is one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

The HBV genome is composed of approximately 3,200 nucleotides. HBV is classified into eight genotypes, designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence (3, 23, 26, 37). They have dis-

tinct geographical distributions and are associated with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (7, 8, 12, 13, 22, 28). Furthermore, subgenotypes have been reported for HBV/A, -B, and -C and named A1 to -3 (17, 38), B1 to -6 (31, 32, 40), and C1 to -6 (20, 31, 45). Equally, other genotypes are classified into subgenotypes. There have been increasing lines of evidence to indicate influences of HBV subgenotypes on the outcome of liver disease and the response to antiviral therapies (1, 39, 44).

In 2001, we reported the geographic distribution of HBV genotypes in Japan (27). Of the 720 Japanese patients with chronic HBV infection (CHB), 12 (1.7%) harbored HBV/A, 88 (12.2%) HBV/B, 610 (84.7%) HBV/C, 3 (0.4%) HBV/D, and 7 (1.0%) mixed genotypes. HBV/C was detected in over 94%

* Corresponding author. Mailing address: Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Phone: 81-52-853-8292. Fax: 81-52-842-0021. E-mail: ytanaka@med.nagoya-cu.ac.jp.

[∇] Published ahead of print on 18 March 2009.

of patients on the Japanese mainland, while HBV/B was found in 64% of those in Okinawa, the southernmost islands, and 44% of those in the Tohoku area in the northern part of the mainland.

Recently, acute HBV infection (AHB) has been increasing in Japan, predominantly through promiscuous sexual contacts. In addition, it was reported that HBV/A was more frequent in patients with acute hepatitis than in those with chronic hepatitis (29, 41, 49). Recent studies suggest that the chances for progression to chronic disease may differ among patients acutely infected with HBV of distinct genotypes (21, 25); patients infected with HBV/A run an increased risk of becoming HBV carriers. Hence, it is of utmost concern whether chronic HBV/A infection is increasing in Japan.

In the present study, we compared the geographic distribution of HBV genotypes in Japan during 2005 and 2006 with 2000 and 2001, with special attention to changes in the proportion of HBV/A. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses.

MATERIALS AND METHODS

Patients. From September 2005 to October 2006, sera were collected from 1,370 consecutive patients with CHB at 16 representative hospitals that were liver centers in their respective regions throughout Japan for the purpose of investigating the geographic distribution of HBV genotypes in Japan. All of the patients were diagnosed after they had been followed for at least 12 months. Patients diagnosed with AHB were excluded from the study; they had a sudden onset of clinical symptoms of hepatitis, along with high-titer antibody to HBV core antigen of the immunoglobulin M class in serum. Their sera were tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP), and hepatitis B e antigen (HBeAg), as well as antibody to HBeAg (anti-HBe) (Dinabot, Tokyo, Japan). Four clinical diagnoses were established for them. The inactive carrier state was defined by the presence of HBV surface antigen (HBsAg) with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/liter]) persisting over 6 months (with at least three bimonthly tests). Cirrhosis was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism), platelet counts of $<100,000/\text{cm}^3$, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

The study protocol conformed to the 1975 declaration of Helsinki and was approved by the ethics committees of the respective institutions. Every patient or his/her next of kin gave informed consent to the purpose of the study.

Genotypes and subgenotypes of HBV. The six HBV genotypes (A to F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV Genotype EIA; Institutes of Immunology Co., Ltd., Tokyo, Japan). The method depends on the combination of epitopes on pre-S2 region products detected by monoclonal antibodies that were specific for each of them (46, 47). Subgenotypes of HBV/A, designated A1 and A2, were determined by direct sequencing of the pre-S2/S gene, followed by a phylogenetic analysis.

Quantification of HBV DNA and sequencing. HBV DNA levels in sera were quantitated with a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a detection range from 2.6 to 7.6 log copies/ml. Nucleic acids were extracted from 100 μ l of serum using the Qiaamp DNA Blood Minikit (Qiagen GmbH, Hilden, Germany). Eleven complete HBV/A genomes and 29 pre-S2/S region sequences were amplified by PCR with appropriate primer sets, as described previously (40). The amplified HBV DNA fragments were directly sequenced using the ABI Prism Big Dye kit version 3.0 (Applied Biosystems, Foster City, CA) in an ABI 3100 automated DNA sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genome sequences were aligned using GENETYX version 11.0 (Software Development Co., Ltd., Tokyo, Japan).

TABLE 1. Characteristics of 1,271 CHB patients

Parameter	Value
Characteristic	
Male gender [no. (%)]	766 (60.3)
Age (yr; mean \pm SD)	51.4 \pm 14.0
Diagnosis	
Inactive carrier state [no. (%)]	206 (16.2)
Chronic hepatitis [no. (%)]	786 (61.8)
Cirrhosis [no. (%)]	175 (13.8)
HCC [no. (%)]	104 (8.2)
Antiviral treatment [no. (%)]	577 (45.4)
Blood tests	
Platelets ($10^4/\text{mm}^3$)	21.4 \pm 30.2
ALT (IU/liter)	59.8 \pm 103.0
ALP (IU/liter)	270.4 \pm 136.0
γ -GTP (IU/liter)	47.4 \pm 66.1
HBV markers	
HBeAg [no. (%)]	399 (31.4)
HBV DNA (median [range] [log copies/ml])	4.2 (<2.6 to >7.6)

Molecular evolutionary analysis of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. To investigate the relationship between HBV isolates from patients with chronic and acute hepatitis B in Japan, HBV/A isolates (A1 to -10) were randomly retrieved from them and sequenced in our previous study (29). Nucleotide sequences of HBV DNA were aligned by the program CLUSTAL X, and genetic distance was estimated by the six-parameter method (10) in the Hepatitis Virus Database (36). Based on these values, phylogenetic trees were constructed by the neighbor-joining method (30) with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1,000 times.

Statistical analysis. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test and noncategorical variables by the Mann-Whitney U test. A *P* value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates JPN_CH1 to -11 are AB453979 to AB453989.

RESULTS

Distribution of HBV genotypes among patients with CHB.

Of the 1,370 serum samples, the genotype could not be determined for 99 (7.2%) by EIA due to low HBsAg levels, leaving 1,271 for analysis in this study (Table 1). Of these, 206 (16.2%) were inactive carriers, 786 (61.8%) had chronic hepatitis, 175 (13.8%) cirrhosis, and 104 (8.2%) HCC. They had a mean age of 51.4 \pm 14.0 years and included 766 (60.3%) men. They had a median HBV DNA level of 4.2 log copies/ml, and 399 (31.4%) of them were positive for HBeAg. Antiviral treatment had been given to 577 (45.4%) of them with interferon, lamivudine, adefovir pivoxil, or entecavir.

The genotypes were HBV/A in 44 (3.5%), HBV/B in 179 (14.1%), HBV/C in 1,046 (82.2%), and HBV/D in 2 (0.2%) (Table 2). In comparison with our previous report on the distribution of genotypes in Japan in 2001 (27), HBV/A was more frequent in this study (3.5% versus 1.7%; *P* = 0.02). Of the 16 hospitals in this study, 10 overlapped with those in our previous report from 2001. In these 10 hospitals, HBV/A was more frequent in the present than in the previous survey (3.6% versus 1.7%; *P* = 0.04).

The distribution of HBV genotypes in Japan differed by

TABLE 2. Distribution of HBV Genotypes

Genotype	No. (%)	
	2005–2006 (n = 1,271)	2000–2001 ^a (n = 720)
A	44 (3.5 ^b)	12 (1.7)
B	179 (14.1)	88 (12.2)
C	1,046 (82.3)	610 (84.7)
D	2 (0.2)	3 (0.4)
Mixed	0 (0.0)	7 (1.0)

^a From Orito et al. (27).^b $P = 0.02$.

geographic location (Fig. 1). HBV/C was the most prevalent in the majority of areas. In the Tohoku area, the northern part of the Japanese mainland (Honshu), HBV/B was more prevalent than in the other areas of the Japanese mainland. In Okinawa, the southernmost islands of Japan, HBV/B was predominant. Of note, HBV/A was more frequent in the Kanto area (9.5%), the metropolitan area, and Okinawa (9.1%) than in the other areas.

Clinical differences among HBV/A, -B, and -C. Clinical backgrounds were compared among the patients infected with HBV/A, -B, and -C (Table 3). HBeAg was significantly less prevalent in the patients infected with HBV/B than in those infected with HBV/A or -C ($P < 0.01$ for each). When the positivity of HBeAg was stratified by age, HBeAg was markedly less common in patients infected with HBV/B than in those infected with HBV/A or -C who were older than 40 years of age (7/157 [4.5%] versus 4/19 [21.1%] [$P < 0.05$] or 215/755 [28.5%] [$P < 0.01$]) (Fig. 2). There were no significant differences in HBV DNA levels among patients infected with the three genotypes. As antiviral treatments might have influenced the severity of liver disease, clinical states were compared among patients infected with HBV/A, -B, and -C who did and

did not receive it; antiviral treatments did not affect the above-mentioned trends represented in Table 3 in age, diagnosis, and HBeAg, as well as ALT and HBV DNA levels (data not shown).

Additionally, we compared the distributions of age and liver diseases in patients infected with HBV/A, -B, and -C. In patients infected with HBV/C, the prevalence of cirrhosis and HCC increased in those older than 50 years of age compared to younger patients (Fig. 3), whereas in the patients infected with HBV/B, cirrhosis and HCC were rare in elderly patients. The proportion of patients younger than 40 years of age was higher in those infected with HBV/A than in those infected with HBV/B or -C (25/44 [56.8%] versus 22/179 [12.3%] or 288/1,046 [27.5%]; $P < 0.01$ for each), while cirrhosis and HCC were also found in those older than 50 years of age infected with HBV/A.

Coinfection with human immunodeficiency virus type 1 (HIV-1) was found in 6 of the 44 (13.6%) patients infected with HBV/A compared to only 3 of the 1,046 (0.3%) patients infected with HBV/C ($P < 0.0001$); it occurred in none of the 179 patients infected with HBV/B.

Phylogenetic analyses. Among the 44 HBV/A isolates, the complete genome was sequenced successfully in 11 (JPN_CH1 to -11). Seven of them were classified as HBV/A2 and four as HBV/A1. A phylogenetic tree was constructed based on the complete genome sequences of these 11 isolates, along with those from two patients with AHB and those from 40 HBV/A isolates retrieved from the database (Fig. 4). Of the seven HBV/A2 isolates, the four from patients with CHB in this study formed a cluster with the Japanese isolates retrieved from the database and two from patients with AHB. Of the other three isolates, JPN_CH5 clustered with French and U.S. isolates, JPN_CH6 with German isolates, and JPN_CH7 with

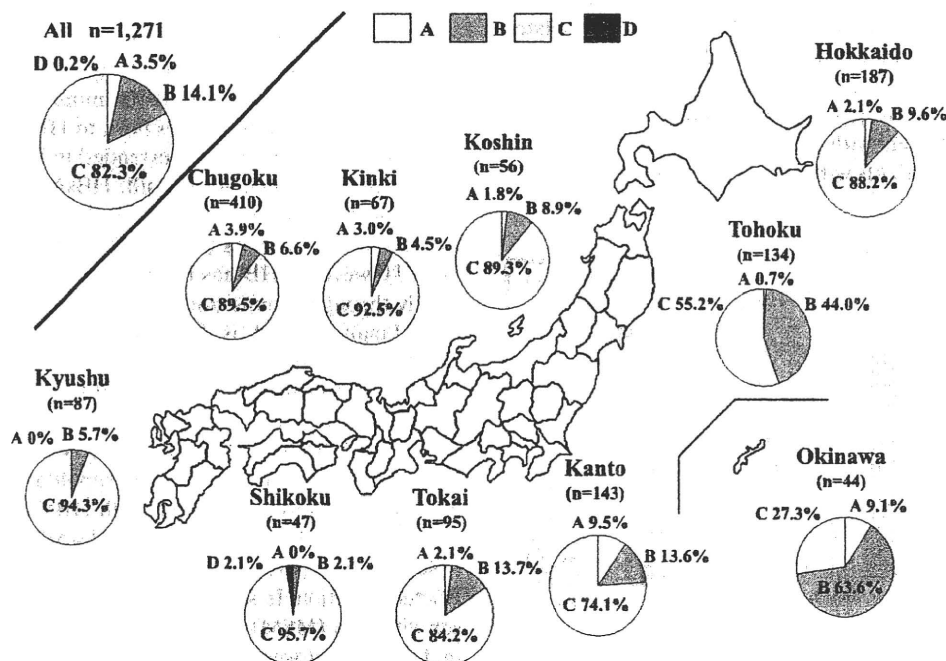


FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

TABLE 3. Clinical characteristics of individuals chronically infected with HBV of different genotypes

Parameter	Value for genotype:		
	A (n = 44)	B (n = 179)	C (n = 1,046)
Male gender [no. (%)]	32 (72.7)	112 (62.6)	621 (59.4)
Age (yr [mean \pm SD])	41.3 \pm 14.9 ^a	55.8 \pm 13.7 ^b	48.8 \pm 13.3
Diagnosis			
Inactive carrier state [no. (%)]	13 (29.5) ^c	63 (35.2) ^b	129 (12.3)
Chronic hepatitis [no. (%)]	26 (59)	103 (57.5)	656 (62.7)
Cirrhosis [no. (%)]	3 (6.8)	10 (5.6) ^b	162 (15.5)
HCC [no. (%)]	2 (4.5)	3 (1.7) ^b	99 (9.5)
Anti viral treatment [no. (%)]	13 (29.5) ^d	48 (26.8) ^b	516 (49.3)
Blood tests			
Platelet (10 ⁴ /mm ³)	23.3 \pm 21.9	25.9 \pm 35.9 ^e	20.6 \pm 29.5
ALT (IU/liter)	56.2 \pm 83.8	42.2 \pm 104.2 ^e	63.0 \pm 103.3
ALP (U/liter)	247.1 \pm 123.0	255.5 \pm 97.9	273.9 \pm 141.9
γ -GTP (U/liter)	39.6 \pm 34.6	49.3 \pm 63.4	47.5 \pm 67.6
HBV markers			
HBeAg [positive rate(%)]	15 (34.0) ^f	17 (9.5) ^b	367 (35.1)
HBV DNA (median [range]) (log copies/ml)	4.2 (<2.6-7.6)	4.1 (<2.6-7.6)	4.2 (<2.6-7.6)

^a $P < 0.01$, A versus B or C.

^b $P < 0.01$, B versus C.

^c $P < 0.01$, A versus C.

^d $P < 0.05$, A versus C.

^e $P < 0.05$, B versus C.

^f $P < 0.01$, A versus B.

Spanish and Italian isolates. All four HBV/A1 isolates in this study formed a cluster with Philippine and Indian isolates.

In addition, the pre-S2/S region sequences of a total of 29 isolates were determined, including the 11 isolates whose complete genomes were sequenced. Of these, 21 (72%) were classified as HBV/A2 and the remaining 8 as HBV/A1. A phylogenetic tree was constructed based on the pre-S2/S region sequences from the 29 isolates, along with those from 10 patients with AHB infected with HBV/A and 47 HBV/A isolates retrieved from the database (Fig. 5). The 21 HBV/A2 isolates in the present study formed a cluster with Japanese, American, and European isolates retrieved from the database and those from patients with acute hepatitis. In addition, some of them were highly homologous with each other. Likewise, HBV/A1 isolates from eight patients with chronic hepatitis in this study

were highly homologous with those from two patients with acute hepatitis and isolates from the Philippines and India. Based on the phylogenetic analyses, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India, and had infiltrated throughout Japan.

DISCUSSION

Perinatal transmission from carrier mothers to their babies has been the principal route for establishing persistent HBV infection in Asian countries (19). In Japan, passive and active immunoprophylaxis with HBV immune globulin and vaccine has been mandated for babies born to HBeAg-positive carrier mothers since 1986; this was extended to HBeAg-negative carrier mothers in 1995. As a result, HBsAg has become rare in Japanese born after 1986; it was detected in only 0.2% of first-time blood donors younger than 19 years of age in 2000 (24). However, AHB has been increasing in Japan, predominantly through promiscuous sexual contacts.

In Japan, HBV/A is detected rarely among patients with CHB but is frequent in those with acute hepatitis (14, 25, 29, 41, 43). Yotsuyanagi et al. reported the distribution of genotypes in 145 Japanese patients with AHB and found HBV/A in 27 (19%), HBV/B in 8 (5%), and HBV/C in 109 (75%) (49). HBV/A is more frequent in metropolitan areas than other areas. The majority of patients with HBV/A infection in metropolitan areas have had extramarital sexual contacts with multiple irregular partners, through which they could have contracted infection. In support of this view, among men who have sex with men (MSM) who are coinfecting with HBV and HIV-1 in Tokyo, most were infected with HBV/A (15, 35).

In Japan, AHB in adulthood becomes chronic in only ~1%

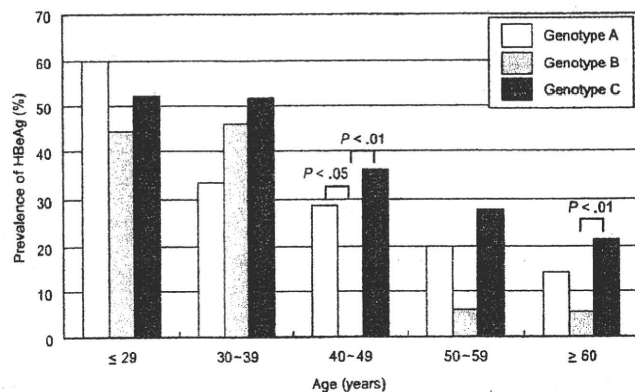


FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

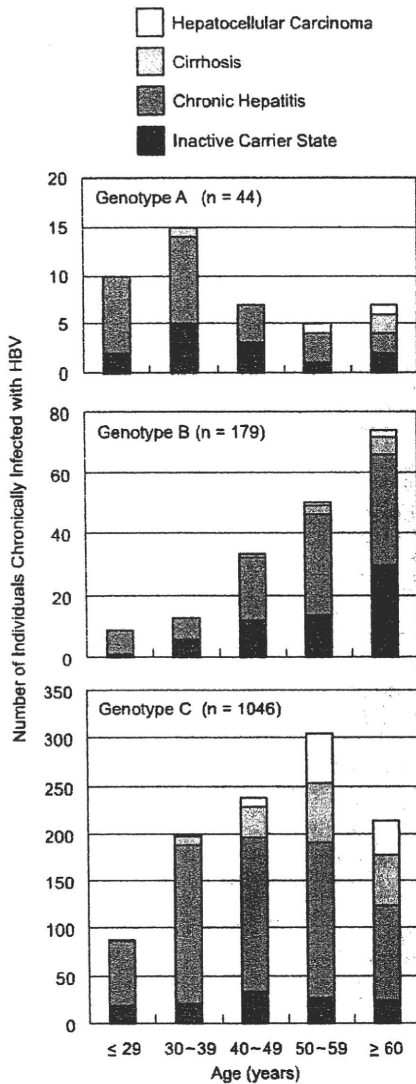


FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

of cases. This is much less than the progression to chronic disease (close to 10%) in Europe and the United States, where HBV/A prevails (34). Recent studies have suggested that the chances for persistence may differ among patients acutely infected with HBV of distinct genotypes (21, 25). In particular, acute infection with HBV/A may bring about an increased risk of progression to chronic disease. Therefore, an increase of acute infection with HBV/A would result in a surge of HBV/A among patients with CHB in Japan. In actuality, in comparison with our previous results during 2000 and 2001 (27), HBV/A was twice as frequent in this study (3.5% versus 1.7%; $P = 0.02$). HBV/A has been increasing in patients with CHB in the Kanto area, where HBV/A in patients with acute hepatitis is more frequent than in the other areas. In the islands of Okinawa, also, HBV/A was found to be prevalent in this study. Of the four patients infected with HBV/A there, two were coinfecting with HIV-1. They were both MSM, and they were sus-

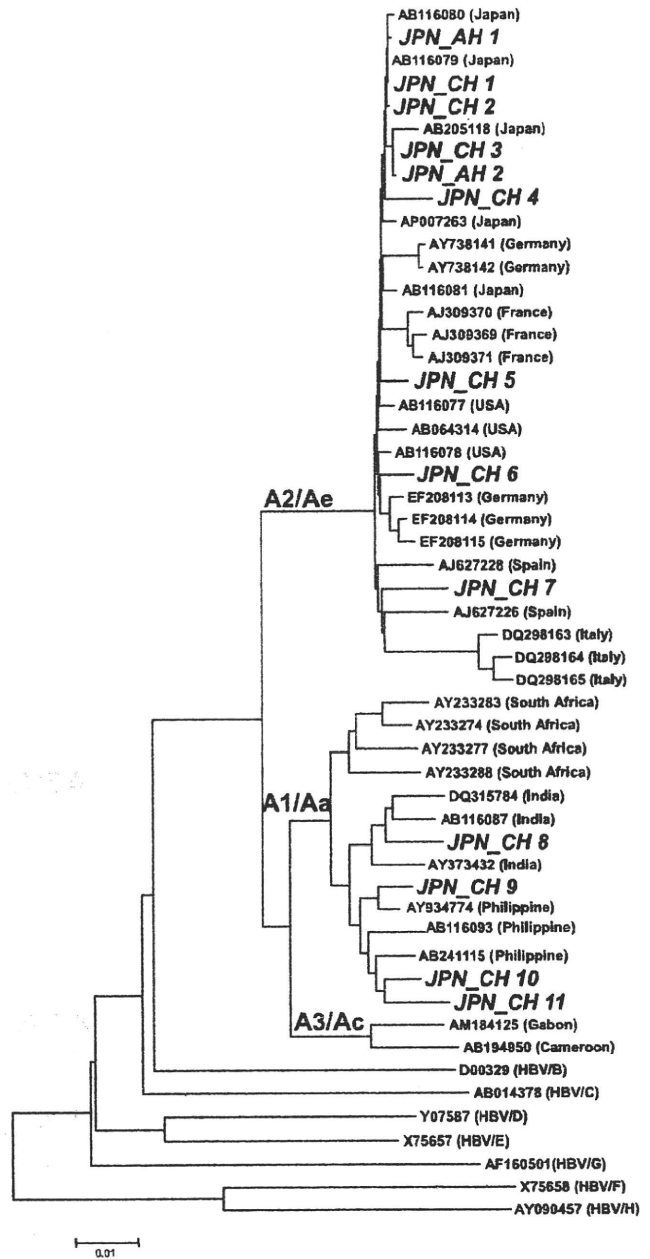


FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 21 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates, along with 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

pected to have been infected with HIV through sexual contacts on the Japanese mainland. It has been reported that HIV infection increases the probability that AHBs will become chronic (2, 11, 33, 48). Because they share routes of transmission and the risk for HIV-1 and HBV infections, approximately

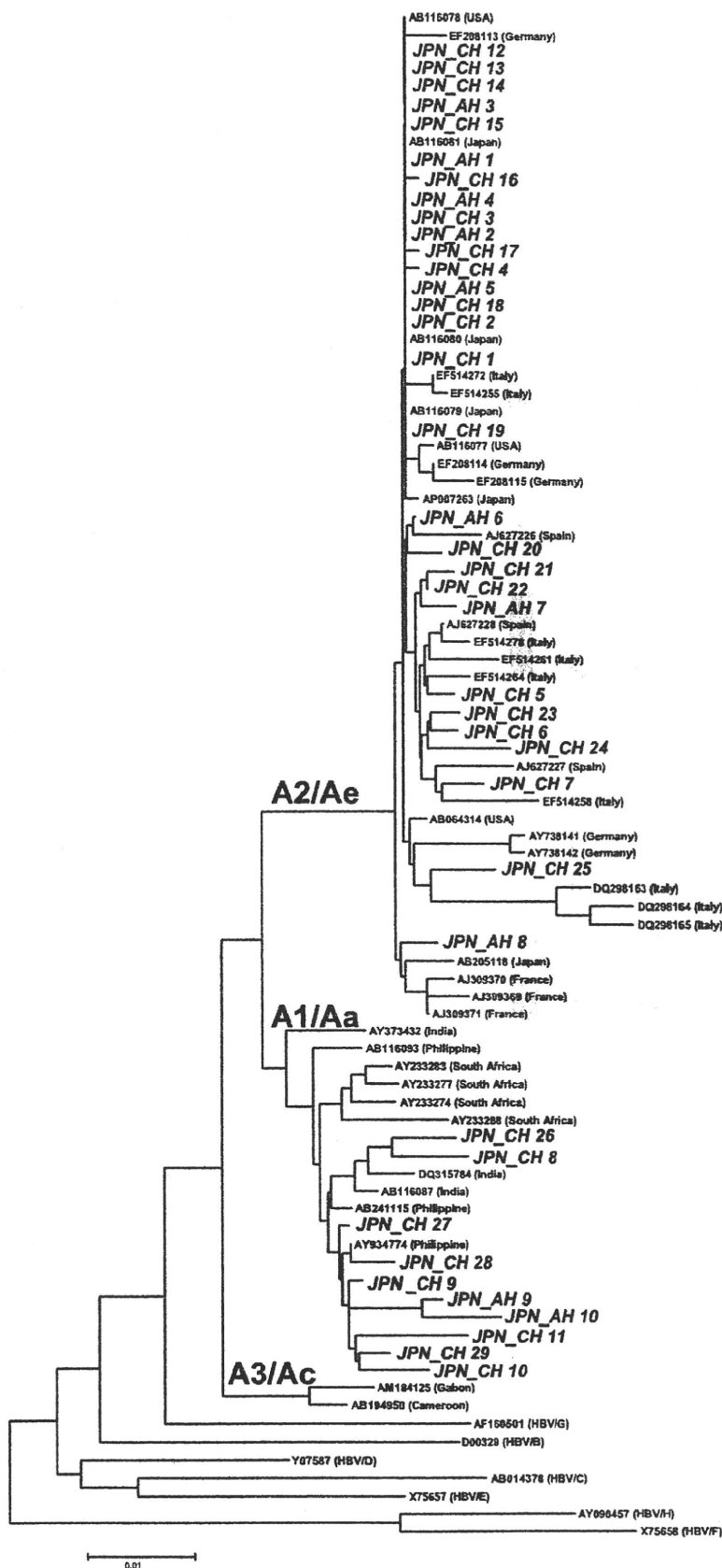


FIG. 5. Phylogenetic tree constructed based on pre-S2/S region sequences of HBV/A isolates. Those from 29 patients with chronic infection in this study are shown in boldface italic (*JPN_CH 1 to -29*), along with 10 isolates (*JPN_AH 1 to -10*) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 28 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates and 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

90% of patients with AIDS have markers of past or ongoing HBV infection (18). Thus, HBV carriers are more frequent in the HIV-1-positive than in the HIV-1-negative population (4, 9). Among patients with HIV infection in Japan, 6.3% are HBeAg positive, in particular, 8.3% of HIV-infected MSM (16). In this study, coinfection with HIV was found in 6 of the 44 (13.6%) patients infected with HBV/A. All of them were men. Their median age was 27.7 ± 4.1 years, and five patients were positive for HBeAg. Thus, there is a possibility that HIV-1 and HBV/A coinfections are increasing among young people in Japan, and the high rate of HBeAg positivity may be influenced by immune suppression due to HIV infection.

In the phylogenetic analysis, the HBV/A2 isolates recovered in this study were homologous to those from Europe and the United States, and some of them clustered with the Japanese isolates. On the other hand, there were HBV/A1 isolates that formed a cluster with those from the Philippines and India. Furthermore, some isolates from patients with acute hepatitis who were infected with HBV/A in Japan were highly homologous to HBV/A isolates from patients with chronic hepatitis. This invites speculation that some HBV/A isolates were introduced into Japan from foreign countries, while others have already settled down there and spread from patients with chronic infection to their contacts. HBV/A would have been infiltrating throughout Japan by these two different routes.

Clinical differences among patients infected with HBV/A, -B, and -C were observed. The mean age was lower in the patients infected with HBV/A than in those infected with HBV/B or -C. As mentioned above, AHB patients infected with HBV/A have been increasing in the younger generation in Japan, and around 10% of them would have progressed to chronic infection. This is one of the reasons why the patients infected with HBV/A are younger than those infected with HBV/B or -C. Most patients infected with HBV/B were negative for HBeAg, while a high proportion of the patients infected with HBV/A and -C had it. In particular, this difference was remarkable in the patients who were older than 40 years of age. Thus, the seroconversion rate for the loss of HBeAg among younger people may be higher in infection with HBV/B than in that with HBV/A or -C. Inactive carriers were commoner in HBV/A than in HBV/C infection, as well.

These lines of evidence indicate that the activity of hepatitis is lower in HBV/B than HBV/C infection, and patients with HBV/B seroconvert from HBeAg to anti-HBe at young ages. In addition, cirrhosis and HCC were less frequent in the patients infected with HBV/B than in those infected with HBV/C. Therefore, the prognosis would be better in the patients infected with HBV/B than in those infected with HBV/C. These results are in accord with previous reports (5, 13, 28, 42). There have been few reports on the clinical features of patients with chronic hepatitis infected with HBV/A in Japan. Chu et al. have reported the distribution of HBV genotypes with reference to clinical characteristics in the United States (6). They have shown that HBV/A and HBV/C infections are accompanied by a higher frequency of HBeAg than HBV/B infection, while HBV/B is associated with a lower rate of hepatic decompensation than HBV/A and -C. In our study, inactive carriers were commoner, while cirrhosis and HCC were found less often in HBV/A than in HBV/C infection. HBeAg was more prevalent in the patients infected with HBV/A than in those

infected with HBV/B who were older than 40 years of age. Therefore, it can be said that the prognosis is better for patients infected with HBV/A than for those infected with HBV/C; it may be poorer than for those infected with HBV/B.

In conclusion, HBV/A has been increasing among CHB patients in Japan. On the basis of phylogenetic analyses, some HBV/A isolates appear to have been imported from foreign countries. They clustered with HBV/A from AHB patients and have infiltrated throughout Japan. It is very likely that acute and chronic infections with HBV/A have been increasing in Japan. Obviously, immunoprophylaxis of perinatal HBV infection, implemented since 1986 on a national basis, has been insufficient to prevent horizontal HBV/A infection diffusing among high-risk groups by transmission routes shared by HIV infection. The foreseeable spread of HBV/A infection in Japan should be prevented by universal vaccination programs extended to high-risk groups or the general population.

ACKNOWLEDGMENTS

The study was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare of Japan and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology.

We thank T. Kimura and K. Sato, Institutes of Immunology Co., Ltd. (Tokyo, Japan), for determining HBV genotypes in this study and Takashi Saito, Yamagata University Hospital; Akihiro Matsumoto, Shinshu University Hospital; Yasuhiro Asahina, Musashino Red Cross Hospital; Yoshito Ito, University Hospital, Kyoto Prefectural University of Medicine; Keiko Hoshio, Tottori University Hospital; Morikazu Onji, Ehime University Hospital; Tatsuya Ide, Kurume University Hospital; and Hiroshi Sakugawa, Hospital, University of the Ryukyus, for their help throughout this work.

Kentarō Matsuura wrote the study protocol and the first draft of the manuscript and performed the experiments and statistical analysis. Yasuhiro Tanaka contributed to the experimental work and the final version of the manuscript. Shuhei Hige, Gotaro Yamada, Yoshiyazu Murawaki, Masafumi Komatsu, Tomoyuki Kuramitsu, Sumio Kawata, Eiji Tanaka, Namiki Izumi, Chiaki Okuse, Shinichi Kakumu, Takeshi Okanoue, Keisuke Hino, Yoichi Hiasa, Michio Sata, and Tatsuji Maeshiro contributed to the collection of the samples and clinical data from patients and to the final version of the manuscript. Fuminaka Sugauchi, Shunsuke Nojiri, Takashi Joh, and Yuza Miyakawa contributed to the final version of the manuscript. Masashi Mizokami had the original idea and did the planning of the study and contributed to the final version of the manuscript. All of the authors have seen and approved the final draft of the manuscript.

REFERENCES

1. Akuta, N., F. Suzuki, M. Kobayashi, A. Tsubota, Y. Suzuki, T. Hosaka, T. Someya, S. Saitoh, Y. Arase, K. Ikeda, and H. Kumada. 2003. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. *J. Hepatol.* 38:315-321.
2. Alter, M. J. 2006. Epidemiology of viral hepatitis and HIV co-infection. *J. Hepatol.* 44:S6-S9.
3. Arauz-Ruiz, P., H. Nordler, B. H. Robertson, and L. O. Magnius. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Gen. Virol.* 83:2059-2073.
4. Bodsworth, N. J., D. A. Cooper, and B. Donovan. 1991. The influence of human immunodeficiency virus type 1 infection on the development of the hepatitis B virus carrier state. *J. Infect. Dis.* 163:1138-1140.
5. Chu, C. J., M. Hussain, and A. S. Lok. 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 122:1756-1762.
6. Chu, C. J., E. B. Keeffe, S. H. Han, R. P. Perrillo, A. D. Min, C. Soldevilla-Pico, W. Carey, R. S. Brown, Jr., V. A. Luketic, N. Terrault, and A. S. Lok. 2003. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 125:444-451.
7. Chu, C. J., and A. S. Lok. 2002. Clinical significance of hepatitis B virus genotypes. *Hepatology* 35:1274-1276.
8. Ding, X., M. Mizokami, G. Yao, B. Xu, E. Orito, R. Ueda, and M. Nakanishi. 2001. Hepatitis B virus genotype distribution among chronic hepatitis B virus carriers in Shanghai, China. *Intervirology* 44:43-47.

9. Fujii, T., H. Taguchi, H. Katano, S. Mori, T. Nakamura, N. Nojiri, K. Nakajima, K. Tadokoro, T. Juji, and A. Iwamoto. 1999. Seroprevalence of human herpesvirus 8 in human immunodeficiency virus 1-positive and human immunodeficiency virus 1-negative populations in Japan. *J. Med. Virol.* 57:159-162.
10. Gojbori, T., K. Ishii, and M. Nei. 1982. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J. Mol. Evol.* 18:414-423.
11. Hadler, S. C., F. N. Judson, P. M. O'Malley, N. L. Altman, K. Penley, S. Buchbinder, C. A. Schable, P. J. Coleman, D. N. Ostrow, and D. P. Francis. 1991. Outcome of hepatitis B virus infection in homosexual men and its relation to prior human immunodeficiency virus infection. *J. Infect. Dis.* 163:454-459.
12. Kao, J. H. 2002. Clinical relevance of hepatitis B viral genotypes: a case of *deja vu*? *J. Gastroenterol. Hepatol.* 17:113-115.
13. Kao, J. H., P. J. Chen, M. Y. Lai, and D. S. Chen. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118:554-559.
14. Kobayashi, M., Y. Arase, K. Ikeda, A. Tsubota, Y. Suzuki, S. Saitoh, F. Suzuki, N. Akuta, T. Someya, M. Matsuda, J. Sato, K. Takagi, Y. Miyakawa, and H. Kumada. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. *J. Med. Virol.* 68:522-528.
15. Koibuchi, T., A. Hitani, T. Nakamura, N. Nojiri, K. Nakajima, T. Jyuji, and A. Iwamoto. 2001. Predominance of genotype A HBV in an HBV-HIV-1 dually positive population compared with an HIV-1-negative counterpart in Japan. *J. Med. Virol.* 64:435-440.
16. Koike, K., Y. Kikuchi, M. Kato, J. Takamatsu, Y. Shintani, T. Tsutsumi, H. Fujie, H. Miyoshi, K. Moriya, and H. Yotsuyanagi. 2008. Prevalence of hepatitis B virus infection in Japanese patients with HIV. *Hepatol. Res.* 38:310-314.
17. Kurbanov, F., Y. Tanaka, K. Fujiwara, F. Sugauchi, D. Mbanya, L. Zekeng, N. Ndembu, C. Ngansop, L. Kaptue, T. Miura, E. Ido, M. Hayami, H. Ichimura, and M. Mizokami. 2005. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J. Gen. Virol.* 86:2047-2056.
18. Lebovics, E., B. M. Dworkin, S. K. Heier, and W. S. Rosenthal. 1988. The hepatobiliary manifestations of human immunodeficiency virus infection. *Am. J. Gastroenterol.* 83:1-7.
19. Lok, A. S. 1992. Natural history and control of perinatally acquired hepatitis B virus infection. *Dig. Dis.* 10:46-52.
20. Lusida, M. I., V. E. Nugrahaputra, Soetjipto, R. Handajani, M. Nagano-Fujii, M. Sasayama, T. Utsumi, and H. Hotta. 2008. Novel subgenotypes of hepatitis B virus genotypes C and D in Papua, Indonesia. *J. Clin. Microbiol.* 46:2160-2166.
21. Mayerat, C., A. Mantegani, and P. C. Frei. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J. Viral Hepat.* 6:299-304.
22. Miyakawa, Y., and M. Mizokami. 2003. Classifying hepatitis B virus genotypes. *Intervirology* 46:329-338.
23. Norder, H., B. Hammas, S. Lofdahl, A. M. Courouce, and L. O. Magnus. 1992. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J. Gen. Virol.* 73:1201-1208.
24. Noto, H., T. Terao, S. Ryou, Y. Hirose, T. Yoshida, H. Ookubo, H. Mito, and H. Yoshizawa. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980-1994. *J. Gastroenterol. Hepatol.* 18:943-949.
25. Ogawa, M., K. Hasegawa, T. Naritomi, N. Torii, and N. Hayashi. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. *Hepatol. Res.* 23:167-177.
26. Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastrosewignjo, M. Imai, Y. Miyakawa, and M. Mayumi. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.* 69:2575-2583.
27. Orito, E., T. Ichida, H. Sakugawa, M. Sata, N. Horiike, K. Hino, K. Okita, T. Okanoue, S. Iino, E. Tanaka, K. Suzuki, H. Watanabe, S. Hige, and M. Mizokami. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 34:590-594.
28. Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, S. Iino, et al. 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology* 33:218-223.
29. Ozasa, A., Y. Tanaka, E. Orito, M. Sugiyama, J. H. Kang, S. Hige, T. Kuramitsu, K. Suzuki, E. Tanaka, S. Okada, H. Tokita, Y. Asahina, K. Inoue, S. Kakumu, T. Okanoue, Y. Murawaki, K. Hino, M. Onji, H. Yatsushashi, H. Sakugawa, Y. Miyakawa, R. Ueda, and M. Mizokami. 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 44:326-334.
30. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
31. Sakamoto, T., Y. Tanaka, E. Orito, J. Co, J. Clavio, F. Sugauchi, K. Ito, A. Ozasa, A. Quino, R. Ueda, J. Sollano, and M. Mizokami. 2006. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. *J. Gen. Virol.* 87:1873-1882.
32. Sakamoto, T., Y. Tanaka, J. Simonetti, C. Osiowy, M. L. Borresen, A. Koch, F. Kurbanov, M. Sugiyama, G. Y. Minuk, B. J. McMahon, T. Joh, and M. Mizokami. 2007. Classification of hepatitis B virus genotype B into 2 major types based on characterization of a novel subgenotype in Arctic indigenous populations. *J. Infect. Dis.* 196:1487-1492.
33. Salmon-Ceron, D., C. Lewden, P. Morlat, S. Bevilacqua, E. Jouglu, F. Bonnet, L. Heripret, D. Costagliola, T. May, and G. Chene. 2005. Liver disease as a major cause of death among HIV infected patients: role of hepatitis C and B viruses and alcohol. *J. Hepatol.* 42:799-805.
34. Sherlock, S. D. J. 1997. Virus hepatitis, p. 265-392. *In* S. D. J. Sherlock (ed.), *Diseases of the liver and biliary system*, 10th ed. Blackwell Scientific Publications, London, United Kingdom.
35. Shibayama, T., G. Masuda, A. Ajisawa, K. Hiruma, F. Tsuda, T. Nishizawa, M. Takahashi, and H. Okamoto. 2005. Characterization of seven genotypes (A to E, G and H) of hepatitis B virus recovered from Japanese patients infected with human immunodeficiency virus type 1. *J. Med. Virol.* 76:24-32.
36. Shin, I. T., Y. Tanaka, Y. Tateno, and M. Mizokami. 2008. Development and public release of a comprehensive hepatitis virus database. *Hepatol. Res.* 38:234-243.
37. Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi, and R. Rossau. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* 81:67-74.
38. Sugauchi, F., H. Kumada, S. A. Acharya, S. M. Shrestha, M. T. Gamutan, M. Khan, R. G. Gish, Y. Tanaka, T. Kato, E. Orito, R. Ueda, Y. Miyakawa, and M. Mizokami. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J. Gen. Virol.* 85: 811-820.
39. Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. G. Gish, R. Ueda, Y. Miyakawa, and M. Mizokami. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 124:925-932.
40. Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. Ueda, Y. Miyakawa, and M. Mizokami. 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J. Virol.* 76:5985-5992.
41. Sugauchi, F., E. Orito, T. Ohno, Y. Tanaka, A. Ozasa, J. H. Kang, J. Toyoda, T. Kuramitsu, K. Suzuki, E. Tanaka, Y. Akahane, T. Ichida, N. Izumi, K. Inoue, H. Hoshino, S. Iino, H. Yotsuyanagi, S. Kakumu, E. Tomita, T. Okanoue, S. Nishiguchi, Y. Murawaki, K. Hino, M. Onji, H. Yatsushashi, M. Sata, Y. Miyakawa, R. Ueda, and M. Mizokami. 2006. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. *Hepatol. Res.* 36:107-114.
42. Sumi, H., O. Yokosuka, N. Seki, M. Arai, F. Imazeki, T. Kurihara, T. Kanda, K. Fukai, M. Kato, and H. Saisho. 2003. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 37:19-26.
43. Suzuki, Y., M. Kobayashi, K. Ikeda, F. Suzuki, Y. Arase, N. Akuta, T. Hosaka, S. Saitoh, T. Someya, M. Matsuda, J. Sato, S. Watabiki, Y. Miyakawa, and H. Kumada. 2005. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J. Med. Virol.* 76:33-39.
44. Tanaka, Y., I. Hasegawa, T. Kato, E. Orito, N. Hirashima, S. K. Acharya, R. G. Gish, A. Kramvis, M. C. Kew, N. Yoshihara, S. M. Shrestha, M. Khan, Y. Miyakawa, and M. Mizokami. 2004. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 40:747-755.
45. Tanaka, Y., E. Orito, M. F. Yuen, M. Mukaide, F. Sugauchi, K. Ito, A. Ozasa, T. Sakamoto, F. Kurbanov, C. L. Lai, and M. Mizokami. 2005. Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. *Hepatol. Res.* 33:216-224.
46. Usuda, S., H. Okamoto, H. Iwanari, K. Baba, F. Tsuda, Y. Miyakawa, and M. Mayumi. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J. Virol. Methods* 80:97-112.
47. Usuda, S., H. Okamoto, T. Tanaka, K. Kidd-Ljunggren, P. V. Holland, Y. Miyakawa, and M. Mayumi. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. *J. Virol. Methods* 87:81-89.
48. Weinbaum, C. M., K. M. Sabin, and S. S. Santibanez. 2005. Hepatitis B, hepatitis C, and HIV in correctional populations: a review of epidemiology and prevention. *AIDS* 19(Suppl. 3):S41-S46.
49. Yotsuyanagi, H., C. Okuse, K. Yasuda, E. Orito, S. Nishiguchi, J. Toyoda, E. Tomita, K. Hino, K. Okita, S. Murashima, M. Sata, H. Hoshino, Y. Miyakawa, and S. Iino. 2005. Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. *J. Med. Virol.* 77:39-46.

BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Direct Cytopathic Effects of Particular Hepatitis B Virus Genotypes in Severe Combined Immunodeficiency Transgenic With Urokinase-Type Plasminogen Activator Mouse With Human Hepatocytes

MASAYA SUGIYAMA,* YASUHITO TANAKA,* FUAT KURBANOV,* ISAO MARUYAMA,† TAKASHI SHIMADA,‡ SATORU TAKAHASHI,§ TOMOYUKI SHIRAI,§ KEISUKE HINO,|| ISAO SAKAIDA,¶ and MASASHI MIZOKAMI*,#

*Department of Clinical Molecular Informative Medicine, and †Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, ‡PhoenixBio Co, Ltd, Higashi-Hiroshima, Japan; †Department of Basic Laboratory Sciences, ‡Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan, and †Research Center for Hepatitis & Immunology, Kohnodai Hospital, International Medical Center of Japan, Chiba, Japan

Background & Aims: Little is known about the direct cytopathic effect of hepatitis B virus (HBV) and its association with particular viral genotypes or genetic mutations. We investigate HBV genotype-related differences in viral replication, antigen expression, and histopathology in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mice harboring human hepatocytes. **Methods:** Mice were inoculated with wild-type of different genotype strains (3 for each HBV/A2, B1, and C2) recovered from preinfected-mice sera or patient sera. **Results:** Histologic analysis of mice infected with HBV/C2 for 22–25 weeks showed abundant ground-glass appearance of the hepatocytes and fibrosis in the humanized part of the murine liver owing to the activation of hepatic stellate cells mediated by oxidative stress through transforming growth factor- β 1 signaling, whereas neither was observed with HBV/A2 and B1. The HBV-DNA level in sera was the highest in mice infected with HBV/C2 compared with those with HBV/A2 and HBV/B1 (10^9 , 10^7 , and 10^4 log copies/mL, respectively, $P < .05$) during 6–8 weeks postinoculation. HB core-related antigen excretion had a similar trend among the genotypes, whereas secretion of HB surface antigen was more pronounced for HBV/A2 followed by HBV/C2 and much less for HBV/B1. Introduction of precore stop-codon mutation in the HBV/B1 caused a significant increase in viral replication, antigen expression, and a histopathologic picture similar to HBV/C2. **Conclusions:** By using a humanized in vivo model, we show that different HBV genotypes and even particular mutations resulted in different virologic and histopathologic outcomes of infection, indicating that particular genetic variants of HBV may be directly cytopathic in immunosuppressive conditions.

With an estimated 420 million chronic carriers, hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections of human beings. The chronic infection often leads to cirrhosis and/or hepatocellular carcinoma, which is responsible for at least 1 million deaths annually worldwide.¹ The precise mechanism by which chronic viral hepatitis results in hepatocellular carcinoma (HCC) is not known. However, evidence now is available concerning the direct effects of HBV in this process.^{2,3} The important issue of a distinct impact of the various HBV genotypes on the virulence has not been addressed directly so far.^{4,5}

Genotypes are subdivided further into subgenotypes on the basis of phylogenetic relationships.⁶ Evidence for the influence of HBV genotypes/subgenotypes on liver diseases in acute, fulminant, and chronic infection have been reported increasingly.^{7–13} Involvement of genetic mutations of HBV in its pathogenesis is another open question. Previous reports have indicated that mutations in basal core promoter, precore/core, envelope, and X coding regions may be associated with HCC.¹⁴ The term *precore mutants* refers to HBV strains with nonsense frameshift or initiation codon mutation in the precore region that prevent translation of hepatitis B e antigen (HBeAg) precursor and are associated with an increase of viral replication via stabilization of the pregenomic encapsidation signal.¹⁵ However, little is known about the histopathologic implication of the mutants. Complexity

Abbreviations used in this paper: α -SMA, α -smooth muscle actin; PCm, precore stop-codon mutation; HBcAg, antigens related to hepatitis B virus core; HSC, hepatic stellate cell; 8-OHdG, 8-hydroxydeoxyguanosine; PCR, polymerase chain reaction; ROS, reactive oxygen species; TGF- β 1, transforming growth factor- β 1.

© 2009 by the AGA Institute
0016-5085/09/\$36.00

doi:10.1053/j.gastro.2008.10.048

Table 1. Inoculum Profiles on HBV Isolates of Distinct Genotypes/Subgenotypes

Genotype (Subgenotype)	Isolates	Mice (n)	Accession No.	Precore (1896)	HBeAg
A (A2/Ae)	A2_US	4	AB246337	Wild	+
	A2_JPN1	3	AB246338	Wild	+
	A2_JPN2	3	AB362931	Wild	+
C (C2/Ce)	C2_JPN22	4	AB246344	Wild	+
	C2_JPNAT	4	AB246345	Wild	+
	C2_JPN31	3	AB362932	Wild	+
B (B1/Bj_wild)	B1_JPN35w	4	AB246341	Wild	+
	B1_JPN56w	3	AB246342	Wild	+
	B1_JPN58w	4	AB362933	Wild	+
B (B1/Bj_PCm)*	B1_JPN35m	3	^a	Mutant	-
	B1_JPN56m	3	^a	Mutant	-
	B1_JPN58m	3	^a	Mutant	-

^aAccession numbers are not shown because these 3 clones identical to the above described HBV/B clones were constructed with G1896A point mutation.

of the host and environmental factors complicates evaluation of the veritable virologic differences between genetic variants of HBV in a clinical study. Therefore, a model that eliminates these factors and allows a direct comparison of early dynamics of HBV genotypes is essential for such investigation.

Recently engineered severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator received human hepatocyte transplants (hereafter referred to as *chimeric mice*)¹⁶⁻¹⁸ and are suitable for the experiments with hepatitis viruses *in vivo*,^{19,20} and offer a rare opportunity in modeling the early kinetics of the HBV replication.²¹

In the present study, infecting human hepatocytes in chimeric mice, we show that different HBV genotypes and even particular mutations within the same genotype have distinct virologic characteristics that may have contributed to the distinct histologic outcomes.

Materials and Methods

Inoculation of Chimeric Mice With the Liver Repopulated for Human Hepatocytes

The chimeric mice were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Human hepatocytes were imported from BD Biosciences (San Jose, CA). The human serum albumin was measured by enzyme-linked immunosorbent assay using commercial kits (Eiken Chemical Co Ltd, Tokyo, Japan). The serum levels of the human

albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HBV recovered from preinfected-mice sera or sera of patients as described in our previous report.²¹ Briefly, a mixture of immature virions can be present in supernatants of cell culture transfected with plasmids expressing HBV^{22,23}; therefore, to avoid direct use of the supernatants in experimental mice, the preinfected mice were infected instead, using the culture media, and then were used as a source of HBV inoculums for the experimental mice. Three clones for each HBV/A2, C2, B1_wild, or B1_PC mutant (precure stop-codon mutation [PCm]) were used in this study (Table 1), and each clone was inoculated to 3 or 4 mice.

Patients

Sera were obtained from 6 patients, 3 of whom had acute hepatitis B and the remaining 3 had fulminant hepatitis B. All sera were subjected to HBV extraction and direct sequencing, which determined genotype B (subgenotype Bj/B1) in all of them. HBV genome sequence analysis of the HBV clones isolated from 3 patients with fulminant hepatitis revealed both the presence of the PC mutation (G1896A) and the absence of any other featured mutations such as core promoter or tyrosine methionine aspartate mutations (Table 2). HBV strains isolated from the 3 acute hepatitis patients were wild type without core

Table 2. Characteristics of Patients From Whom HBV Isolates of Distinct Genotypes/Subgenotypes Were Recovered

Genotype/subgenotype	Isolates	Precore (1896)	Diseases	HBeAg	HBV (LGE ^a /mL)
B1/Bj_wild	B1_JPN1	Wild	AHB	+	6.8
	B1_JPN2	Wild	AHB	+	7.0
	B1_JPN3	Wild	AHB	+	6.7
B1/Bj_PCm	B1_JPN4	Mutant	FHB	-	8.7
	B1_JPN5	Mutant	FHB	-	8.0
	B1_JPN6	Mutant	FHB	-	8.6

AHB, acute hepatitis B; FHB, fulminant hepatitis B.

^aLog genome equivalents.

promoter, precore, and tyrosine methionine aspartate aspartate mutations. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committees of the participating institutions. Written informed consent was obtained from each patient.

Histopathologic Examination

Liver tissues were fixed in buffered formalin, embedded in paraffin, and stained with H&E, Masson's trichrome (MT), or orcein staining. To detect α -smooth muscle actin (α -SMA) and human nuclei, polyclonal antibodies against anti- α -SMA (Lab Vision Corp, Fremont, CA) and monoclonal antibody against anti-human nuclei (Chemicon International, Inc, Temecula, CA) were used as primary antibodies, respectively. The fibrosis stage was evaluated by an expert pathologist who was blinded to the nature of inocula (S.T.).

Dihydroethidium Labeling of Reactive Oxygen Species in Liver Tissue

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen, Carlsbad, CA) as previously reported with minor modification.²⁴ Briefly, in the presence of ROS, dihydroethidium is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA. The fluorescence was detected with laser scanning confocal microscopy. The relative stained area was quantified using National Institutes of Health image analysis for 5 randomly selected areas of digital images in each specimen.

Detection of 8-Hydroxydeoxyguanosine in Liver Tissue

Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHdG) was performed as previously reported with minor modification.²⁵ The detailed protocol is shown in the Supplementary Materials and Methods section (see Supplementary material online at www.gastrojournal.org).

Results

Differences of Replication Efficiency Among HBV Genotypes

The inoculums, each containing approximately 10^5 copies of any 1 of the 4 clones: HBV/A2, C2, B1_wild, and B1_PC mutant (PCm), were inoculated to 3 or 4 mice. HBV DNA was quantified in murine sera weekly. One week after inoculation, HBV DNA was detected in both the HBV/A2 and C2 groups. The titer increased approximately by 2 logs within the next 2 weeks, and continued to increase until 7–12 weeks before reaching a plateau. HBV-DNA levels were 2 logs higher in the mice inoculated with HBV/C2 than HBV/A2 at 6–8 weeks postinoculation ($P < .05$) (Figure 1A).

To assess the role of the PC mutation, 2 variants of HBV/B1 were included in the comparison between the genotypes: the HBV/B1_wild and HBV/B1_PCm. Differently from HBV/A2 and HBV/C2, both of the HBV/B1 variants had shown a so-called *window* period; characterized by the HBV-DNA levels remaining undetectable until weeks 4–5 after the inoculation. However, after the window period, the HBV-DNA level of the B1_PCm detected at week 5 had rapidly increased in titer, reaching the levels of HBV/C2 and A2 by week 11 (Figure 1A). Interestingly, HBV-DNA levels of B1_wild did not show this rapid increment during the whole follow-up period (until week 25). HBV-DNA titer was 3 logs lower in mice inoculated with HBV/B1_wild compared with those with the other genotypes ($P < .01$). To evaluate the replication dynamics of the different genotypes, the time required for a 10-fold increment of the viral load (*log time*) was estimated. When the window periods of HBV/B1_PCm were excluded from the comparison, the *log time* was similar between the HBV/C2 and B1_PCm, ranging from 7.3 to 8.4 days, whereas HBV/A2 had a longer index (12.9 days), suggesting slower replication. However, the lowest replication efficiency was observed for HBV/B1_wild, with a *log time* of 27.7 days.

Distinct Characteristics on Antigen Production Among HBV Genotypes

The expression of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) (depicted in Figure 1B) did not correspond with that of HBV DNA (Figure 1A) for HBV/B1_PCm, which had rapidly increased antigen expression in the early phase, and then decreased sharply. HBeAg of HBV/B1_PCm was undetectable as expected to confirm the function of the stop codon mutation. In contrast, dynamics of HBcrAg and HBeAg expression by HBV/C2 and HBV/A2 resembled those of HBV DNA. The HBcrAg levels of HBV/B1_PCm without HBeAg expression revealed lower levels than those of HBV/A2 or C2. To detect core protein alone without detecting HBeAg, only hepatitis B core antigen (HBcAg) was assessed in each mice group at the peak point of HBcrAg by enzyme-linked immunosorbent assay. The value of HBV/B1_PCm shown was equal to that of HBV/C2, and higher than that of HBV/A2 (data not shown). HBV antigens of HBV/B1_wild group were detectable, although they had extremely low levels, suggesting a very low replication level for this group. Core protein levels in liver tissue, with adjustment for human albumin levels, showed a similar trend to that of sera (data not shown).

Confirmation of HBV/B1_Wild Infectivity by Using Human Sera

Virus titer of the HBV/B1_wild group was very low and the *log time* was long in the present study. To further confirm these findings, we used 6 sera: 3 from

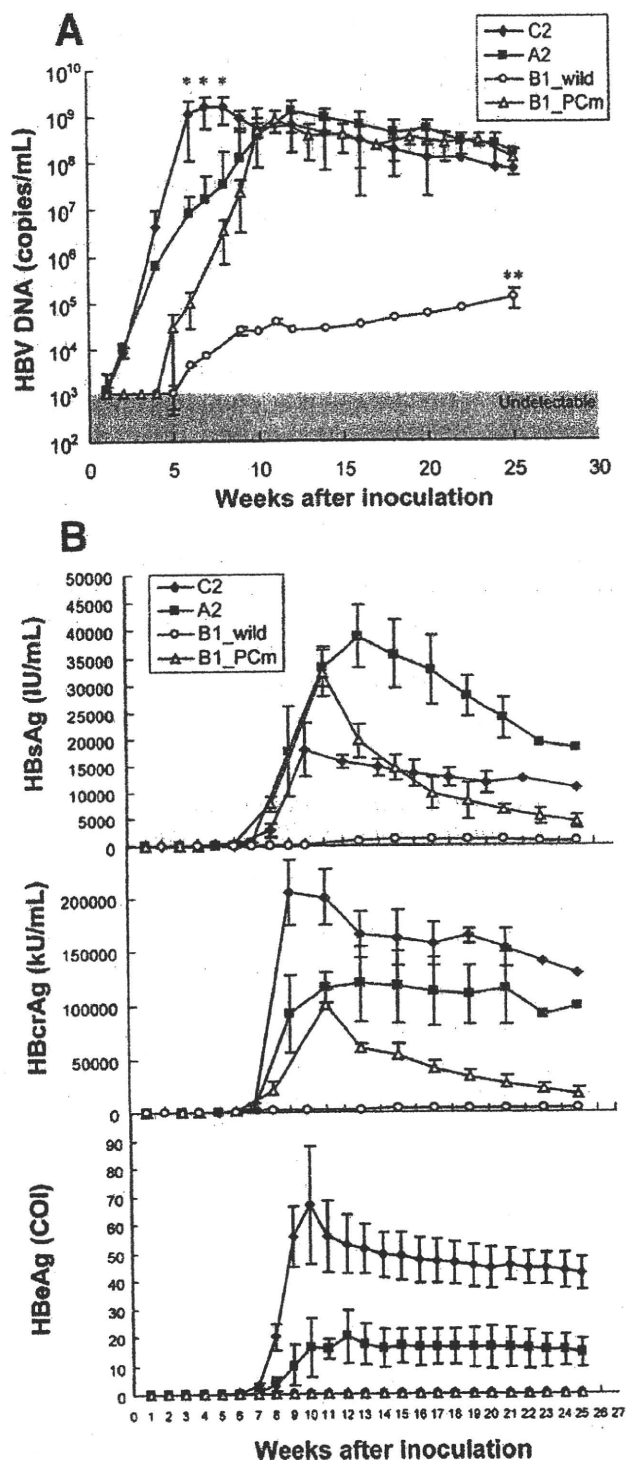


Figure 1. Comparative dynamic profile of HBV-DNA and antigen levels in sera of mice inoculated with preinfected-mice sera recovered from culture media transfecting HBV construct. (A) Levels of HBV DNA in sera of the chimeric mice inoculated with HBV/A2, C2, B1_wild, or B1_PCm. Shaded in gray is an area below the detection limit (<10³ copies/mL) of the real-time detection PCR assay. *Statistical differences with a P value of less than .05. **Statistical differences with a P value of less than .01. (B) Dynamic profiles of HBV antigen expression, as revealed by quantification of HBsAg, HBeAg, and HBcAg in sera of the chimeric mice (see Supplementary Materials and Methods section). For each group, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

acute hepatitis B patients harboring precore wild-type HBV/B1 and the other 3 from fulminant hepatitis B patients harboring precore nonsense-mutation B1 (B1_PCm) (Table 2). Three mice were inoculated with each one of the 6 serum specimens adjusted to contain approximately 10⁶ copies of HBV DNA (Figure 2A and B). Serum HBV-DNA levels increased immediately after inoculation of HBV/B1_PCm and continued to increase until they reached a plateau at week 6 (Figure 2A), showing extremely high replication efficiency. The window period was shortened to 2 weeks in the acute hepatitis B serum group with HBV/B1_wild; however, the peak of mean HBV-DNA levels still was low (5 × 10⁵ copies/mL), which was similar to the results by inoculation of preinfected-mice sera (Figure 1A). Neither serum levels of the human albumin nor the body weight differed among the mice groups. Based on direct sequencing, no mutations were detected in the HBV complete genomes from any mice 25 weeks after inoculation in comparison with those of inoculated strains.

HBV antigen expression levels of the groups inoculated with human serum samples were compared with those of the groups inoculated with the preinfected-mice sera (Figure 2B). HBV antigens of HBV/B1_PCm waxed and waned in profiles similar to that of the groups inoculated with the mice sera in the early phase.

Liver Pathology of Chimeric Mice Infected With Each Genotype

Figure 3 shows the histology of liver in representative chimeric mice infected with HBV/A2, C2, B1_wild, or B1_PCm during weeks 22–25. The immunofluorescence staining was performed using anti-HBcAg and anti-human albumin polyclonal antibody to confirm the location of HBV infection (Supplementary Figure 1; see Supplementary material online at www.gastrojournal.org). Colocalization of HBcAg and human hepatocytes was shown by double staining for HBcAg and human albumin. Almost all of the mice did not reveal apparent steatosis of hepatocytes with H&E stain. The majority of HBV/C2- or B1_PCm-infected human hepatocytes had a ground-glass appearance on H&E stain, fibrosis of stage 2 with MT stain, as well as neutrophil or monocyte invitation. In contrast, the mice infected with HBV/A2 or B1_wild had neither a ground-glass appearance nor fibrosis. To confirm the ground-glass appearance, these specimens were stained by orcein staining. The orcein staining clearly showed cytoplasmic positivity of human hepatocytes infected with HBV/B1_PCm or C2, but not the other group, including control mice.

Immunostaining Analysis on Expression of α-SMA

Active hepatic stellate cells (HSCs) express α-SMA in the early phase of fibrogenesis. To estimate the activation of stellate cells, we performed immunostaining

BASIC-LIVER, PANCREAS, AND BILIARY TRACT

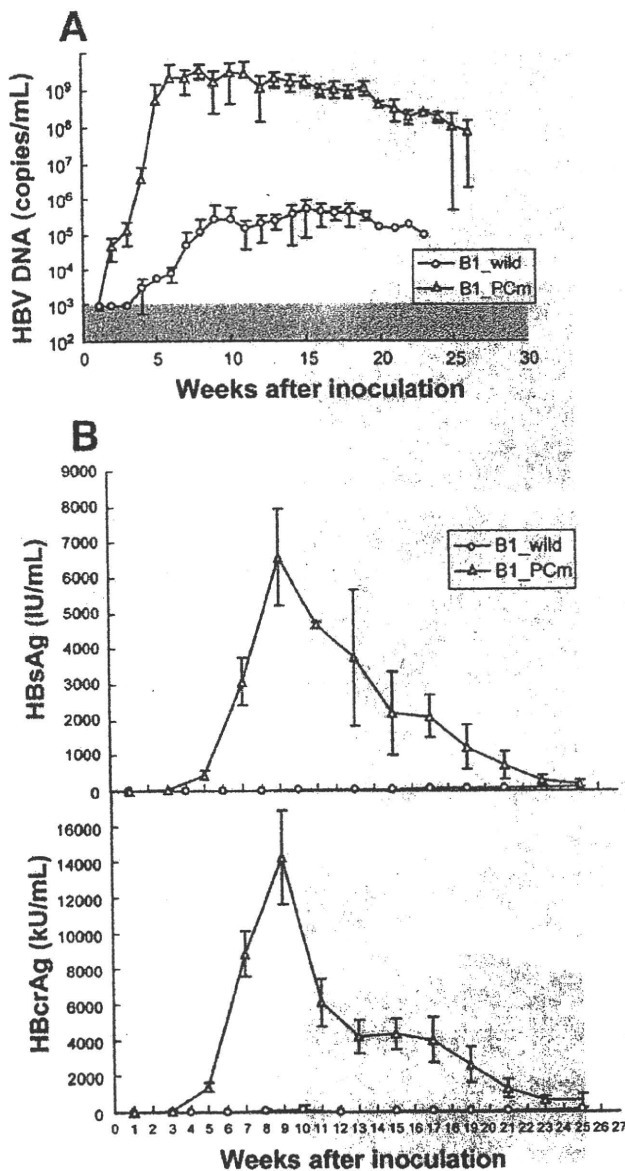


Figure 2. Comparative dynamic profile of HBV-DNA antigen levels in sera of mice inoculated with patient sera from HBV/B1_wild (PC wild-type) and HBV/B1_PCm (PC mutant). (A) Mice inoculated with sera from HBV/B1_wild-infected carriers developed acute hepatitis B or from HBV/B1_PCm-infected carriers developed fulminant hepatitis B and were assessed for levels of HBV DNA in mice sera with real-time detection PCR weekly. The area below the detection limit ($<10^3$ copies/mL) is shaded in gray. (B) Dynamic profiles of HBV antigens including HBsAg and HBcAg in mice corresponding to panel A. For each genotype, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

using anti- α -SMA antibody. Immunostaining analysis showed strong staining of α -SMA around fibrosis, which was found by MT staining (Figure 4A). These results indicated that liver fibrosis of HBV/C2 and B1_PCm occurred via profibrotic cytokines from the activated HSCs but not artifacts. The specimen was double-stained for human nuclei and α -SMA to distinguish between

human and mouse cells. As shown in Figure 4B, α -SMA and human nuclei did not stain in the same cells, suggesting that the active HSCs were of mouse origin.

Increased Oxidized State in Liver by HBV Infection

In the fibrosis process, current knowledge establishes that the production of ROS plays a critical role in HSC activation involving transforming growth factor- β 1 (TGF- β 1) signaling.²⁶ Because α -SMA expressed by HSCs was detected in chimeric mice liver, we next investigated ROS production in mice liver. The ROS production was confirmed by dihydroethidium staining (Figure 5A). The level of ROS production was increased statistically when mice were infected with HBV/B1_PC or C2 compared with HBV/A2 or B1_wild ($P < .01$) (Figure 5B). Figure 5C shows representative immunohistochemical staining for 8-OHdG, which is a marker of oxidative DNA damage, in liver; 8-OHdG-positive cells were recognized in both HBV/C2 and B1_PCm groups, whereas few 8-OHdG-positive cells were detected in the other groups. These data were consistent with those of ROS production.

Gene Expression of Fibrosis Markers in the Mice Liver

As for the change of factors associated with TGF- β 1 signaling in the mice, serum alanine aminotransferase (ALT) and TGF- β 1 levels were increased in the fibrosis group (B1_PC and C2) as compared with the nonfibrosis group (A2 and B1_wild) (Figure 6A and B). The TGF- β 1 levels in the fibrosis group showed significant difference ($P < .01$). To determine whether the representative fibrosis-related genes were of human or mouse origin, we established species-specific primer sets. Polymerase chain reaction using the species-specific primers gave bands of specific size showing reliable specificity (Figure 6C) and dissociation curves (data not shown) (the detailed protocol is provided in the Supplementary Materials and Methods section). Gene expression levels of tissue inhibitor of metalloproteinase 1, matrix metalloproteinase 2, and collagen type 1 α 2 were quantified by real-time detection reverse-transcription PCR analyses. Specifically, gene expression of human tissue inhibitor of metalloproteinase 1 and mouse collagen type 1 α 2 represented significantly higher expression in the fibrosis group than that of the nonfibrosis or control groups ($P < .001$). Matrix metalloproteinase 2 and collagen type 1 α 2 messenger RNA (mRNA) of human origin were undetectable because these genes are produced predominantly in mesenchymal cells.²⁷

Discussion

In the present study, the severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator mouse with human hepatocytes was applied to evaluate genotype-dependent differences in the

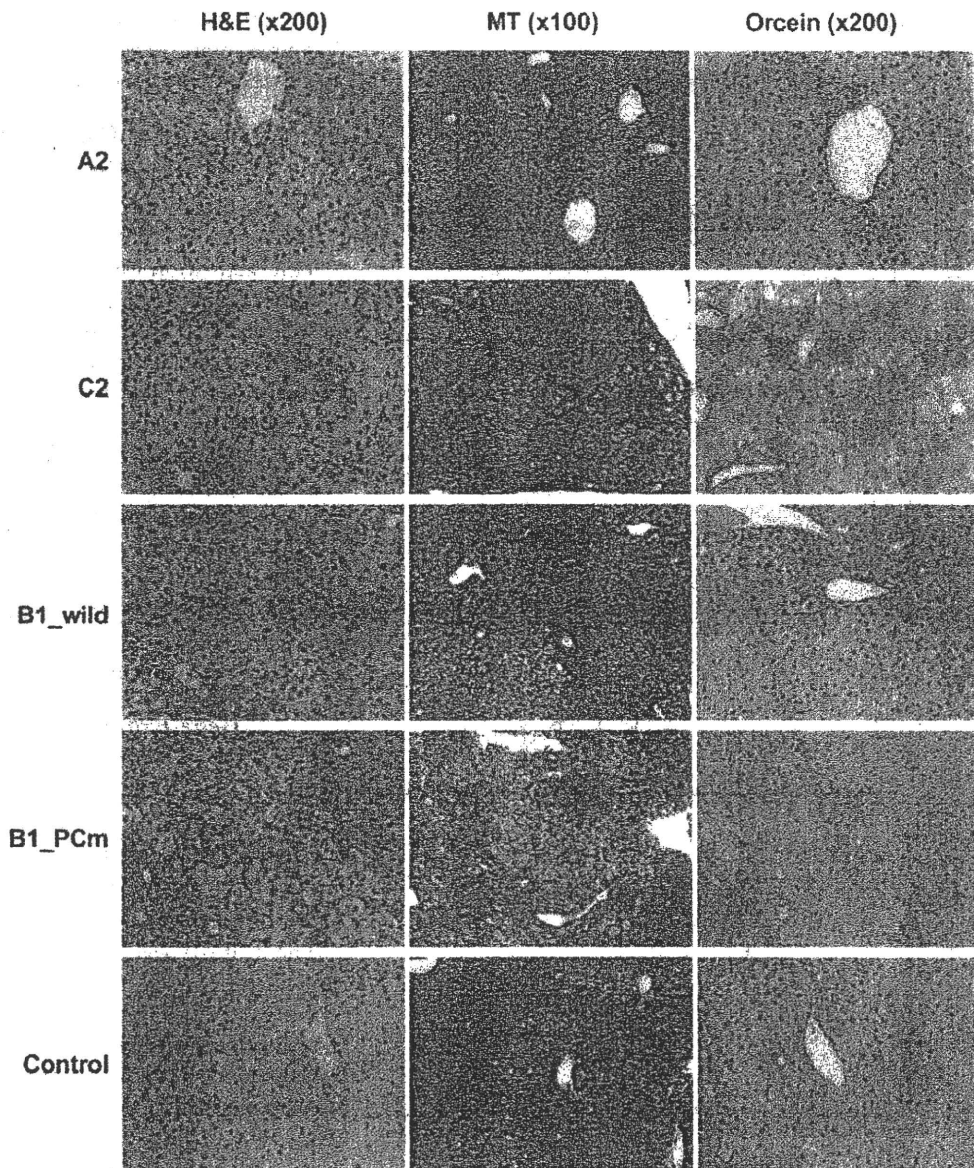


Figure 3. Immunohistochemical analysis of liver tissue. Comparison of liver histology in mice long-term (25 weeks) infected with HBV/A2, C2, B1_wild, B1_PCm, and noninfected control. Liver sections stained with H&E, MT, or orcein are shown. After deparaffinization, tissue slides were stained according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

expression of HBV DNA and antigens. This has allowed for an assessment of the direct cytopathic potential of different HBV genotypes (ie, particular subgenotypes) to be investigated without the host-related bias, under conditions of the absence of immune pressure. In addition, this may represent a novel mouse model for human liver fibrosis associated with ROS production leading to the activity of TGF- β by viral infection but not chemical trigger. The study thereby has shown that infection with HBV/C2 in contrast to HBV/A2 or B1_wild has induced an abundant ground-glass appearance of the human hepatocytes along with an increased fibrosis in the humanized liver of the chimeric mice in an immunosuppressive condition. A strong staining of α -SMA observed around areas of fibrosis indicated activation of HSCs in cases of HBV/C2 and B1_PCm, but not in A2 and B1_wild. In the chimeric mice, therefore, ROS produc-

tion could play a critical role in HSC activation. In connection with this study, we have evaluated the liver damages in chimeric mice killed at 3 months postinfection (early phase dynamics). The viral dynamics and ROS production of HBV/C2 or B1_PCm evaluated in the early phase indicated levels of alterations similar to those observed after long-term infection (Supplementary Figure 2; see Supplementary material online at www.gastrojournal.org). Fibrosis stage and orcein staining levels (ground-glass appearance), however, were expressed in lesser levels than in the long-term infected mice, suggesting that the liver damage can be detected even in the early stage of the infection, but its level correlates with the duration of exposure to oxidative stress.

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

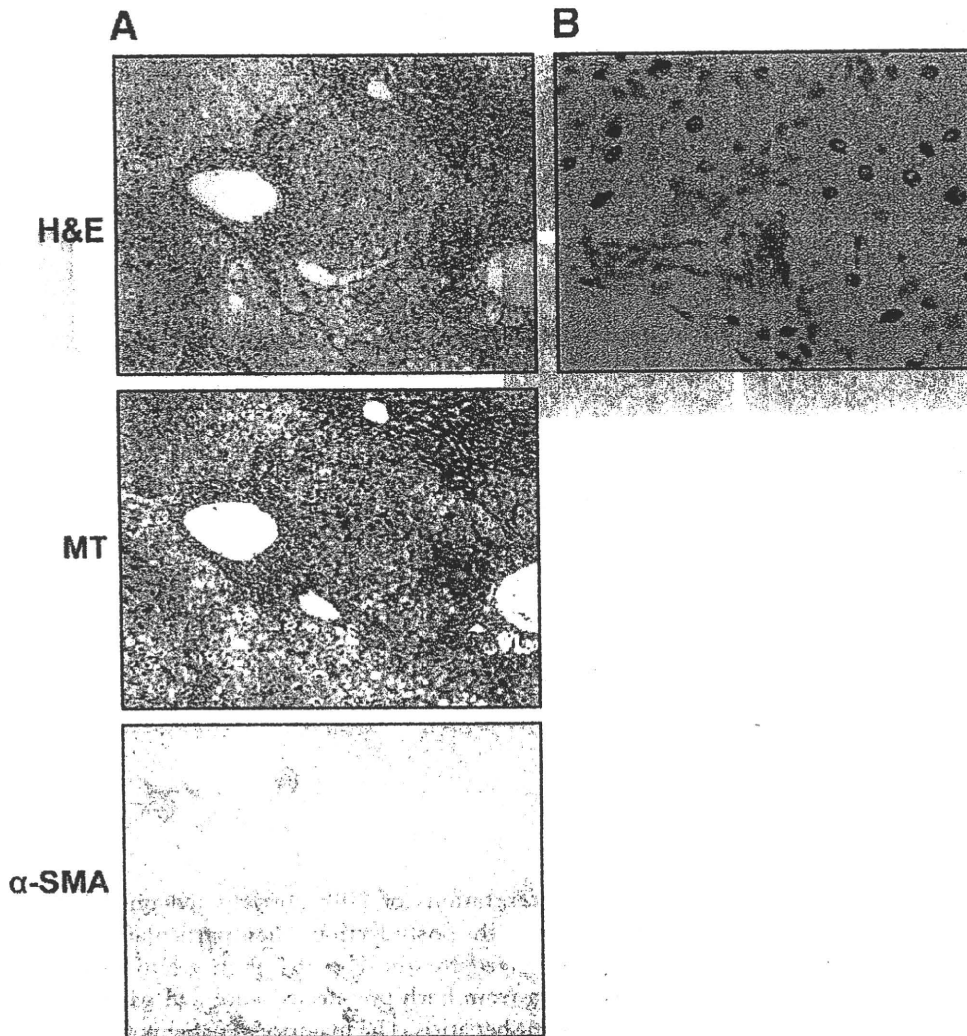


Figure 4. Confirmation of liver fibrosis by immunostaining using anti- α -SMA antibody. (A) Liver sections stained with H&E, MT, or immunostaining using anti- α -SMA antibody (as described in the Materials and Methods section). (B) Nuclei stained brown with the antibodies indicate human origin, and α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

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

The hepatic injury during acute and chronic HBV infection genuinely is considered to be caused by the host's immune response against the infected hepatocytes.³³ However, in some immunosuppressed chronic HBV patients, high viremia and liver fibrosis may oc-

cur.^{34,35} Previous reports have shown that HBV genotypes E or G cause intracellular changes and hepatocellular damage in human hepatocytes in severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator.^{2,3} We showed here that activation of oxidative stress led to TGF- β 1 production in chimeric mice as reported in previous studies.²⁶ Accumulation of oxidative damage, 8-OHdG, might enhance the possibility of carcinogenesis as observed in HCC patients. These findings suggest that hepatic injuries could arise in the absence of a mature immune system and the difference of genotype would affect the cytopathic potential of the virus.

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

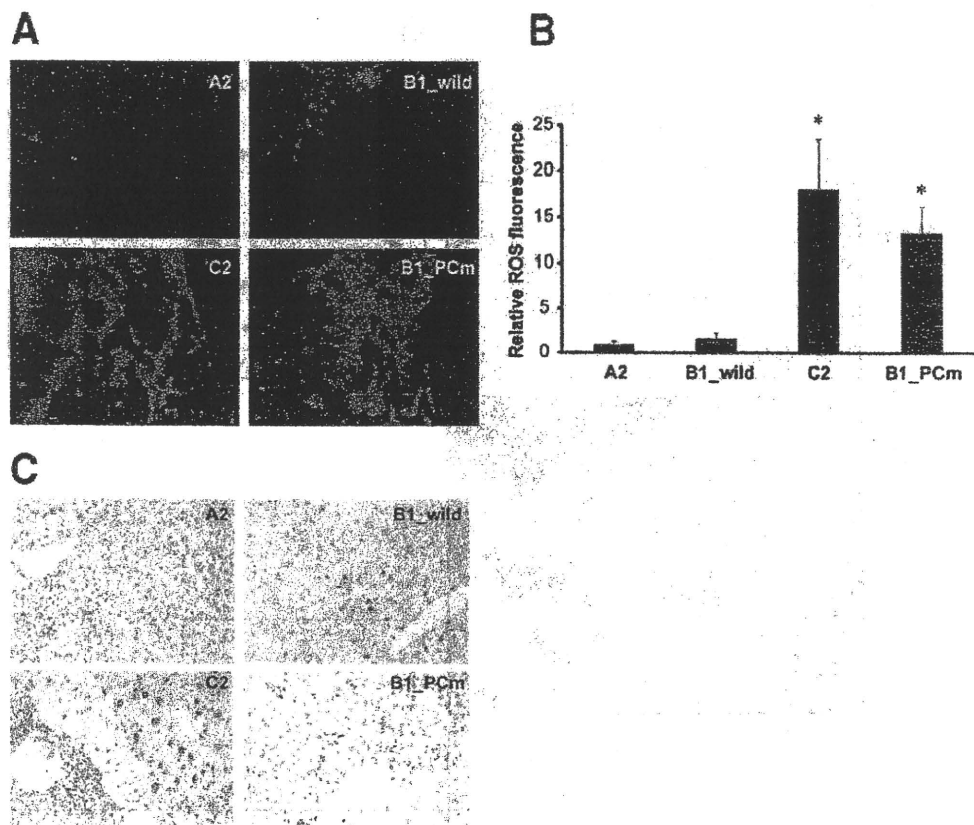


Figure 5. Differences in production of oxidative damage among HBV genotypes. (A) Frozen liver sections of mice inoculated with different HBV genotypes were stained by dihydroethidium. Fluorescence was detected with a laser scanning microscope. (B) Fluorescence intensities in randomly selected areas of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

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

Previous clinical observations on HBV/B1^{11,28} prompted a deeper investigation on the impact of the PC mutation on the virologic characteristics of the genotype. The unique characteristic of HBV/B1_wild stood out among genotypes harboring no major mutations. The HBV/B1_wild group revealed low replication efficiency with window periods and low antigen expression. The lower replicative activity and hepatic injuries of HBV/A2 and B1_wild may partially explain why carriers with either HBV/A2 or HBV/B1 often are asymptomatic in contrast to those with HBV/C infection.^{28,38,39} In our study, the PC mutation was the only difference between HBV/B1_PCm and HBV/B1_wild clone, and the former showed higher replication efficiency and severe damage in liver tissue. The antigen levels of the HBV/B1_PCm increased rapidly and decreased earlier than those of the HBV/A2 or C2 clone, whereas HBV/B1_wild showed that

the concentrations of HBV antigens remained low for several months postinfection. These particular characters were observed for the HBV/B1_PCm group inoculated with sera from both preinfected mice and patients with fulminant hepatitis. The majority of patients with fulminant hepatitis and fatal acute exacerbation have been found to have the G1896A mutation.^{11,40,41} A greater incidence of fulminant hepatitis might be associated with the high replication and protein production in the early phase, as was shown on the HBV/B1_PCm clone in this study. The defect of immunologic tolerance as a result of the absence of HBeAg may play an important role in the fulminant course of precore mutation in HBV infection.⁴² This would concur with a previous report by Bocharov et al which proposed that enhanced HBV replication would efficiently stimulate immune responses, represented by the cytotoxic T-lymphocyte response,⁴³ suggesting that enhanced replication by HBV/B1 with G1896A mutation might lead to an extremely high cytotoxic T-lymphocyte response, resulting in fulminant hepatitis. But in this study, HBV/B1_PCm showed similar responses to HBV/C2 infection because chimeric mice did not have an immune system that was strong enough to invite strong cytotoxic T-lymphocyte response against viral infection. To uncover these unique characteristics of PC mutant, further study would be needed by using the infection model but not gene transfer.