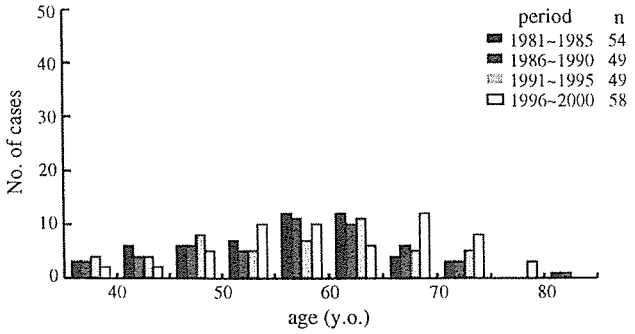

Fig. 1. Age distribution of the total number of deaths from hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. There was no change of number of patients and age distribution of patients who died from hepatitis B virus-associated hepatocellular carcinoma during the four time periods.

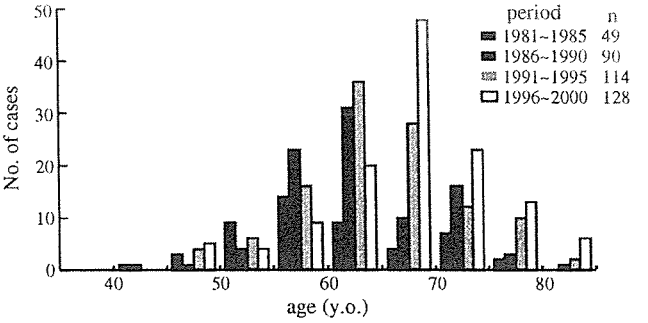

Fig. 2. Age distribution of the total number of deaths from hepatitis C virus-associated hepatocellular carcinoma from 1981 to 2000. The number of death from HCV associated HCC has increased 2.6 times during recent 20 years and this increase was provided by a close association with older shift of age distribution.

(1991–1995) and 128 (1996–2000). In addition, the mean age at death from HCV-associated HCC also increased over time. The mean age at death from 1996 to 2000 (67.0 ± 7.9 years old) was significantly higher than that from 1981 to 1985 (60.0 ± 8.1) ($p < 0.0001$), 1986 to 1990 (63.0 ± 7.0) ($p = 0.0016$) and 1991 to 1995 (64.1 ± 7.2) ($p = 0.0267$), respectively.

Fig. 1 shows the age distribution for deaths from HBV-associated HCC during the four 5-year periods. There was no change of number of patients and age distribution for deaths from HBV-associated HCC during these periods. In contrast, Fig. 2 shows the age distribution for deaths from HCV-associated HCC during the four 5-year periods. The number of patients with HCV-associated HCC aged more than 60 years in 1981–1985, 1986–1990, 1991–1995 and 1996–2000 were 22, 61, 88 and 110 patients, respectively. Fig. 2 indicated that the number of death from HCV associated HCC has increased during recent 20 years and this increase was provided by a close association with older shift of age distribution.

Table 3 shows the age distribution of HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The number of patients with HCV-associated HCC obviously had an increase in the ratio of patients aged more than 60 years ($p < 0.0001$): 18.6% (1981–1985), 37.9% (1986–1990), 51.2% (1991–1995) and 54.4% (1996–2000). There was a significant difference of age distribution in the patients with HCV-associated HCC between aged more than and less than 60 years old in each 5-year period ($p < 0.0001$). In contrast, there was no difference in the age distribution of patients with other types of during these periods.

Fig. 3 shows the ratio between HCV-associated deaths and HBV-associated HCC deaths in 5-year period (1981–1985, 1986–1990, 1991–1995 and 1996–2000). The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period: 0.9 (1981–1985), 1.8 (1986–1990), 2.3 (1991–1995) and 2.2 (1996–2000) (1981–1985 versus 1991–1995, $p = 0.0030$; 1981–1985 versus 1996–2000, $p = 0.0042$). Above all, the ratio of patients aged more than 60 years old increased during the observation period: 1.1 (1981–1985), 3.0 (1986–1990), 4.2 (1991–1995) and 3.8 (1996–2000) (1981–1985 versus

Table 3
Age distribution of HCC deaths in 5-year period

Age (y.o.)	1981–1985, no. (%)	1986–1990, no. (%)	1991–1995, no. (%)	1996–2000, no. (%)		p-Value
HBV						
<60	34 (28.8)	29 (18.0)	28 (16.3)	29 (14.4)	]	NS
>60	20 (17.0)	20 (12.5)	21 (12.2)	29 (14.4)		
HCV						
<60	27 (22.9)	29 (18.0)	26 (15.1)	18 (8.9)	]	<0.0001
>60	22 (18.6)	61 (37.9)	88 (51.2)	110 (54.4)		
Overlap						
<60	1 (0.9)	3 (1.9)	2 (1.2)	1 (0.5)	]	NS
>60	0	2 (1.2)	0	3 (1.5)		
Other						
<60	5 (4.2)	2 (1.2)	4 (2.3)	2 (1.0)	]	NS
>60	9 (7.6)	15 (9.3)	3 (1.7)	10 (4.9)		
Total	118 (100)	161 (100)	172 (100)	202 (100)		

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; overlap, both HBV and HCV positive; other, both HBV and HCV negative.

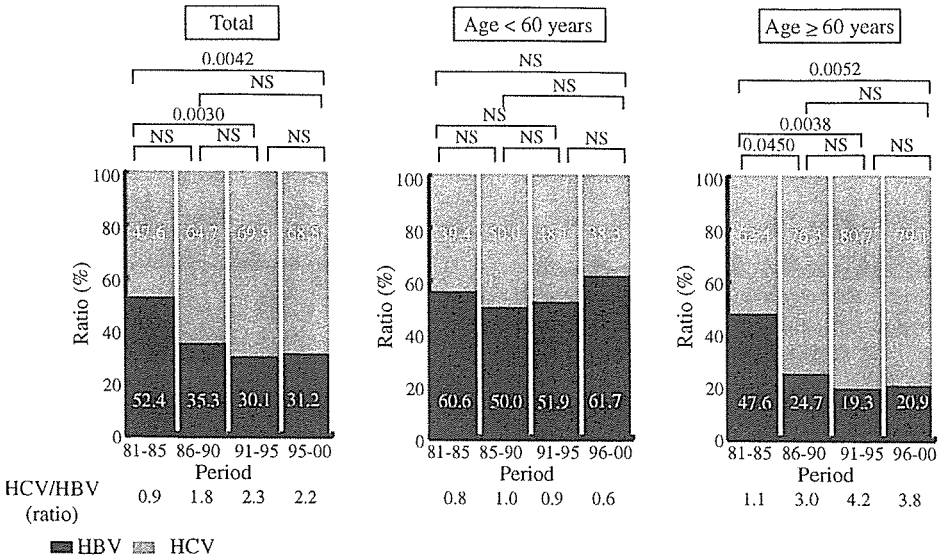

Fig. 3. Ratio between hepatitis C virus-associated hepatocellular carcinoma deaths and hepatitis B virus-associated hepatocellular carcinoma from 1981 to 2000. The ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau during the observation period.

1986–1990, $p=0.0450$; 1981–1985 versus 1991–1995, $p=0.0038$; 1981–1985 versus 1996–2000, $p=0.0052$). In contrast, there was no difference in the ratio of patients aged more than 60 years old of during these periods.

4. Discussion

HCC accounts for approximately 6% of all human cancers. It is estimated that half a million cases occur annually worldwide, making HCC the fifth most common malignancy in men and the ninth in women [1–7,9]. The age-adjusted mortality rate from HCC has increased over the past decades in Japan [16], and in the current study more than 90% of deaths from HCC were HBV- and/or HCV-related and the number of deaths from HCV-associated HCC apparently increased 2.6 times from 1981 to 2000, and the mean age of deaths from HCV-associated HCC also significantly rose. During the same period, the number and the age distribution of deaths from HBV-associated HCC remained unchanged. The increase in the number of deaths from HCV-associated HCC seemed to be closely associated with the shift of age distribution of HCV infected population between 1981 and 2000. Although our data had the limitations of applying the findings from two hospitals to a general population, Kiyosawa described that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age between 1982 and 2003. This also suggests that the average age of diagnosis of HBV-related HCC was similar in all three time periods. In contrast, the average age of patients with HCV-related HCC rose from 61.6 years in 1982 to 63.1 years in 1990 and 67.8 years in 2003 [11]. The research group for population-based cancer registration in Japan described that incidence of HCC in Japan have continued to increase and reached a plateau in males and female from 1975 to 1999. Above all, the age distribution incidence and incident rate of HCC reached a peak older than 65 years old in males and female [17]. And, this study suggested that the ratio between HCV-associated HCC and HBV-associated HCC increased and reached a plateau from 1981 to 2000, especially more than 60 years old. Where did these findings and difference of HCC development between HCV and HBV, which were considered to be both oncogenic virus after long-term persistent infection with inflammation and fibrotic change in the liver but popular hepatitis virus infections in Japan, come from?

The simple reason may be explained as follows. From 1981 to 2000, mortality from a variceal hemorrhage in cirrhotic patients has declined [9,18]. Long term nutritional supplementation with oral branched-chain amino acids has been useful in the prevention of progressive hepatic failure, and improvement of surrogate markers and perceived health status in advanced cirrhosis has occurred [19,20]. Additionally, many new treatments and techniques have been introduced for HCC, including transcatheter arterial embolization, percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation, systemic chemotherapy and advance surgical techniques. However, these advances of medical treatment cannot explain the difference between HBV-associated HCC and HCV-associated HCC.

Alternatively, well considered reasons of the recent rapid increase of the number of patients who died from HCV-associated HCC in Japan, were shown in the current two studies. First, Hamada et al. recently reported that the majority of HCC patients develop HCC when they are aged over 60 years old, regardless of the timing of HCV infection. This result was obtained by the long-term observation of the patients infected by post-transfused HCV infection [21]. This also suggests that HCC has increased among patients over 60 years old with HCV infection and such phenomenon has never been observed nor reported till now in patients with HBV infection.

Second, the chronically HCV-infected population is aging in Japan. Yoshizawa et al. reported that age-specific prevalence rates for the presence of anti-HCV antibody among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the age, reaching the highest rate of 7% in individuals who were more than 70 years old [11,22]. In a word, HCV infected people become older with years in Japan and they were regarded as a high risk for HCC. Then, the number of deaths from HCV-associated HCC has been increased recent 20 years in Japan.

El-Serag et al. reported that an increase in the number of cases of HCC affecting mainly younger age groups has occurred in the United States (U.S.) over the past two decades [23,24]. HCV infection accounts for most of the increase in the number of cases of primary liver cancer [4,6,7,9,25], while the rates of primary liver cancer associated with alcoholic cirrhosis and HBV infection have remained unchanged [4,6,9]. Tanaka et al. reported that HCV was introduced into the U.S. population around 100 years ago and was widely disseminated between 1954 and 1978 [26]. Most HCV-infected patients in the U.S. were born between 1940 and 1965 [27,28], and are therefore younger than HCV-infected Japanese patients. Hence, the burden of disease associated with HCV infection will probably increase in the U.S. during the next 10–20 years, as has occurred in Japan, as this cohort reaches an age at which complications of chronic liver disease typically occur [1–7,26]. The current study suggests that increased HCV-associated HCC will occur in the U.S. over the next two to three decades.

In conclusion, we found that the number of patients with HCV-associated HCC in Japan has increased, consistent with aging of the population, but the number of patients with HBV-associated HCC has remained unchanged over the last 20 years.

References

[1] El-Serag HB. Hepatocellular carcinoma and hepatitis C in the United States. Hepatology 2002;36:S74–83.

[2] El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 2002;35:S72–8.

[3] El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. Gastroenterology 2004;127:S27–34.

[4] El-Serag HB, Everhart JE. Diabetes increases the risk of acute hepatic failure. Gastroenterology 2002;122:1822–8.

[5] El-Serag HB, Hampel H, Yeh C, Rabeneck L. Extrahepatic manifestations of hepatitis C among United States male veterans. Hepatology 2002;36:1439–45.

[6] El-Serag HB, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med 2000;160:3227–30.

[7] Hassan MM, Frome A, Patt YZ, El-Serag HB. Rising prevalence of hepatitis C virus infection among patients recently diagnosed with hepatocellular carcinoma in the United States. J Clin Gastroenterol 2002;35:266–9.

[8] McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni Jr JF. International trends and patterns of primary liver cancer. Int J Cancer 2001;94:290–6.

[9] El-Serag HB, Everhart JE. Improved survival after variceal hemorrhage over an 11-year period in the Department of Veterans Affairs. Am J Gastroenterol 2000;95:3566–73.

[10] Kiyosawa K, Tanaka E. Characteristics of hepatocellular carcinoma in Japan. Oncology 2002;62:5–7.

[11] Kiyosawa K, Umemura T, Ichijo T, et al. Hepatocellular carcinoma: recent trends in Japan. Gastroenterology 2004;127:S17–26.

[12] Hamasaki K, Nakata K, Tsutsumi T, et al. Changes in the prevalence of hepatitis B and C infection in patients with hepatocellular carcinoma in the Nagasaki prefecture. Jpn J Med Virol 1993;40:146–9.

[13] Kato Y, Nakata K, Omagari K, et al. Risk of hepatocellular carcinoma in patients with cirrhosis in Japan. Analysis of infectious hepatitis viruses. Cancer 1994;74:2234–8.

[14] Shiratori Y, Shiina S, Imamura M, et al. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology 1995;22:1027–33.

[15] Shiratori Y, Shiina S, Zhang PY, et al. Does dual infection by hepatitis B and C viruses play an important role in the pathogenesis of hepatocellular carcinoma in Japan? Cancer 1997;80:2060–7.

[16] Wada I, Hara T, Kajihara S, et al. Population-based study of hepatitis C virus infection and hepatocellular carcinoma in western Japan. Hepatol Res 2002;23:18–24.

[17] Japan* T.R. G. f. P.-b. C. R. i. Cancer incidence and incidence rates in Japan in estimates based on. Jpn J Clin Oncol 2004;34:352–6.

[18] McCormick PA, O'Keefe C. Improving prognosis following a first variceal haemorrhage over four decades. Gut 2001;49:682–5.

[19] Marchesini G, Bianchi G, Rossi B, et al. Nutritional treatment with branched-chain amino acids in advanced liver cirrhosis. J Gastroenterol 2000;35:7–12.

[20] Marchesini G, Bianchi G, Merli M, et al. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. Gastroenterology 2003;124:1792–801.

[21] Hamada H, Yatsuhashi H, Yano K, et al. Impact of aging on the development of hepatocellular carcinoma in patients with posttransfusion chronic hepatitis C. Cancer 2002;95:331–9.

[22] Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.

[23] El-Serag HB, Anand B, Richardson P, Rabeneck L. Association between hepatitis C infection and other infectious diseases: a case for targeted screening? Am J Gastroenterol 2003;98:167–74.

[24] Rabeneck L, El-Serag HB, Davila JA, Sandler RS. Outcomes of colorectal cancer in the United States. No change in survival (1986–1997). Am J Gastroenterol 2003;98:471–7.

[25] Nair S, Shiv Kumar K, Thuluvath PJ, Shivakumar KS, Shiva Kumar K. Mortality from hepatocellular and biliary cancers: changing epidemiological trends. Am J Gastroenterol 2002;97:167–71.

[26] Tanaka Y, Hanada K, Mizokami M, et al. Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99:15584–9.

[27] Alter MJ, Kruszon-Moran D, Nainan OV, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med 1999;341:556–62.

[28] Armstrong GL, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. Hepatology 2000;31:777–82.

Influence of Genotypes and Precore Mutations on Fulminant or Chronic Outcome of Acute Hepatitis B Virus Infection

Atsushi Ozasa,[1,2] Yasuhito Tanaka,[1] Etsuro Orito,[2] Masaya Sugiyama,[1] Jong-Hon Kang,[3] Shuhei Hige,[4] Tomoyuki Kuramitsu,[5] Kazuyuki Suzuki,[6] Eiji Tanaka,[7] Shunichi Okada,[8] Hajime Tokita,[9] Yasuhiro Asahina,[10] Kazuaki Inoue,[11] Shinichi Kakumu,[12] Takeshi Okanoue,[13] Yoshikazu Murawaki,[14] Keisuke Hino,[15] Morikazu Onji,[16] Hiroshi Yatsuhashi,[17] Hiroshi Sakugawa,[18] Yuzo Miyakawa,[19] Ryuzo Ueda,[2] and Masashi Mizokami[1]

The outcome of acute hepatitis B virus (HBV) infection is variable, influenced by host and viral factors. From 1982 through 2004, 301 patients with acute HBV infection entered a multi-center cross-sectional study in Japan. Patients with fulminant hepatitis (n = 40) were older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P < .0017$), less predominantly male (43% vs. 71%, $P = .0005$), less positive for hepatitis B e antigen (HBeAg) (23% vs. 60%, $P < .0001$), less infected with subgenotype Ae (0% vs. 13%, $P < .05$), and more frequently with Bj (30% vs. 4%, $P < .0001$) than those with acute self-limited hepatitis (n = 261). Precore (G1896A) and core-promoter (A1762T/G1764A) mutations were more frequent in patients with fulminant than acute self-limited hepatitis (53% vs. 9% and 50% vs. 17%, $P < .0001$ for both). HBV infection persisted in only three (1%) patients, and they represented 2 of the 23 infected with Ae and 1 of the 187 with the other subgenotypes (9% vs. 0.5%, $P = .032$); none of them received antiviral therapy. In multivariate analysis, age 34 years or older, Bj, HBeAg-negative, total bilirubin 10.0 mg/dL or greater, and G1896A mutation were independently associated with the fulminant outcome. In *in vitro* transfection experiments, the replication of Bj clone was markedly enhanced by introducing either G1896A or A1762T/G1764A mutation. In conclusion, persistence of HBV was rare (1%) and associated with Ae, whereas fulminant hepatitis was frequent (13%) and associated with Bj and lack of HBeAg as well as high replication due to precore mutation in patients with acute HBV infection. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006; 44:326-334.)

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBc, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ALT, alanine aminotransferase.

From the [1]Department of Clinical Molecular Informative Medicine and the [2]Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3]Teinekeijinkai Hospital, Sapporo, Japan; the [4]Department of Internal Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [5]Akita City Hospital, Akita, Japan; the [6]First Department of Internal Medicine, Iwate Medical University, Morioka, Japan; [7]Shinshu University Graduate School of Medicine, Matsumoto, Japan; [8]University of Yamanashi, Yamanashi, Japan; [9]National Tokyo Hospital, Kiyose, Tokyo, Japan; [10]Musashino Red Cross Hospital, Musashino, Tokyo, Japan; [11]Showa University Fujigaoka Hospital, Yokohama, Japan; [12]Aichi Medical University School of Medicine, Aichi, Japan; [13]Kyoto Prefectural University of Medicine, Kyoto, Japan; [14]Tottori University, Tottori, Japan; [15]Yamaguchi University School of Medicine, Yamaguchi, Japan; [16]Ehime University School of Medicine, Matsuyama, Japan; [17]National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan; [18]University of the Ryukyus, Okinawa, Japan; and [19]Miyakawa Memorial Research Foundation, Tokyo, Japan.

Received February 8, 2006; accepted April 27, 2006.

Supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H16-kaken-3), Uehara Memorial Foundation, Toyoaki Foundation, and Miyakawa Memorial Research Foundation.

The nucleotide sequences of HBV DNA isolates used in this study have been deposited in the international DNA database under accession numbers AB249373-AB249636.

Address reprint requests to: Masashi Mizokami, M.D., Ph.D., Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. E-mail: mizokami@med.nagoya-cu.ac.jp; fax: (81) 52-842-0021.

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

DOI 10.1002/hep.21249

Potential conflict of interest: Nothing to report.

Approximately 3 billion people, one half of the world population, have been exposed to hepatitis B virus (HBV), of whom approximately 350 million are persistently infected with it.[1] Acute infection with HBV resolves in the great majority but can induce fulminant hepatitis or go on to become chronic. Host and viral factors may influence fulminant or chronic outcome of acute HBV infection, but they are not fully defined.

Eight genotypes have been detected by a sequence divergence greater than 8% in the entire HBV genome of approximately 3,200 nucleotides (nt), and designated by capital alphabet letters from A (HBV/A) to H in the order of documentation.[2-5] They have distinct geographical distributions associated with severity of liver disease as well as response to antiviral therapies.[6-8] Furthermore, subgenotypes have been reported for HBV/A, B, and C and named Aa/A1 (Asian/African type) and Ae/A2 (European type),[9] Bj/B1 (Japanese type) and Ba/B2 (Asian type),[10] as well as Cs/C1 (Southeast Asian type) and Ce/C2 (East Asian type).[11-13] Increasing lines of evidence indicate that subgenotypes of HBV/A and B influence the replication of HBV and bear clinical relevance.[14-16] Furthermore, genotypes affect mutations in precore region and core promoter, thereby influencing the expression of hepatitis B e antigen (HBeAg).[8,17]

During the 23 years from 1982 to 2004, a multi-center cross-sectional study was conducted throughout Japan on 301 patients with acute hepatitis B. We examined the influence of genotypes/subgenotypes on their fulminant or chronic outcome. Furthermore, the influence of G1896A or A1762T/G1764A on replication of HBV was evaluated in an *in vitro* replication model.

Patients and Methods

Patients With Acute Hepatitis B. During 1982 through 2004, 336 consecutive cases of acute hepatitis B were registered in 16 hospitals throughout Japan. These hospitals were from the following eight areas: Hokkaido (represented by J.-H. K. and S.H.), Tohoku (T.K. and K.S.), Kanto (H.T., Y.A. and K.I.), Koshin (E.T. and S.O), Tokai (A.O., Y.T., E.O., M.S., R.U., M.M., and S.K.), Kinki (T.O.), Honshu/Shikoku (Y.M., K.H., and M.O.), and Kyushu (H.Y. and H.S.). The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. Patients with initial high-titered anti-HBc (≥90% inhibition by a 1:200 diluted serum) were excluded; they were diagnosed as exacerbation of chronic hepatitis B. Patients with acute hepatitis A, hepatitis C, or human immunodeficiency virus co-infection, and drug- or alcohol-induced acute hepatitis also were excluded; hepatitis D virus infection was not examined because of its extreme rarity in Japan.[18] Most of them were followed for clinical outcomes until the disappearance of hepatitis B surface antigen (HBsAg) during 24 weeks or longer after the presentation. The criteria of fulminant hepatitis are based on the report by Trey et al.,[19] with a slight modification in 1981 (Inuyama symposium, Aichi, Japan): coma of grade II or higher and prothrombin time less than 40% developing within 8 weeks after the onset. Serum samples were collected at the presentation and had been stored at −80°C. HBV genotypes, HBV DNA, and HBeAg were determined, and clinical outcomes of acute hepatitis were analyzed. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committees of the institutions. Every patient gave an informed consent for this study.

Serological Markers of HBV Infection. HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme immunoassay (EIA) (Axsym; Abbott Japan, Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe; Kokusai Diagnostic, Kobe, Japan) or chemiluminescent EIA (Fujirebio Inc., Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan).

Genotypes and Subgenotypes of HBV. The six major HBV genotypes (A-F) were determined serologically by EIA using commercial kits (HBV GENOTYPE EIA; Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies, which is specific for each of them.[20] HBV/G was determined by a slight modification of the polymerase chain reaction (PCR) with specific primers.[21]

Subgenotypes of HBV/A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia,9 which corresponds to subgroup A′ originally reported by Bowyer et al.,[22] were determined by PCR restriction fragment length polymorphism (RFLP) involving nucleotide conversions in an immediate upstream of the precore region that are specific for each of them.[16,23] HBV/Bj (Japanese type) lacking the recombination with C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP based on specific nucleotide substitutions, after the methods described previously.[15,24]

Subgenotypes of HBV/C, Cs (Southeast Asian type) found only in Southeast Asia, including Vietnam, Myanmar, Thailand, Laos, Bangladesh, Hong Kong, and Southern China, and Ce (East Asian type), found in Far

East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.[12]

Quantification of HBV DNA and Sequencing. HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,[25] with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.[10] Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5′-CTC CAC AGT AGC TCC AAA TTC TTT A-3′). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA. Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100 μL serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html.

Cell Culture and DNA Transfection. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hours of culture, cells were transfected with 5 μg DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1 μg reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization. HBV DNA samples from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and noncategorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

Results

Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes. Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ($P = .0001$), Aa ($P < .01$), Bj or Cs ($P < .05$ for each) and Ce than Ba ($P < .05$). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ($P < .05$).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ($P < .005$), Ce ($P < .001$) or Ba ($P < .05$). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.[21] HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ($P < .005$) or Bj ($P < .05$) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ($P < .05$). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ($P < .05$); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D[a] (n = 5)	G[a,b] (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3[d]	41.5 ± 10.7[e]	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%)[f]	19 (86%)[g]	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%)[h]	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113[i]	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks)[c]	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7[j]	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08[k]	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

[a]Patients with HBV genotype D or G were not included in the analysis.
[b]All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.
[c]Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.
[d]$P = .0001$, Ae vs. Ba. $P < .01$, Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.
[e]$P < .05$, Ba vs. Ce.
[f]$P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.
[g]$P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[h]$P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.
[i]$P < .05$, Ae vs. Bj.
[j]$P < .01$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[k]$P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

Clinical Outcome of Patients With Acute Hepatitis B. Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% 2/23 vs. 0.5% 1/187, $P = .032$).

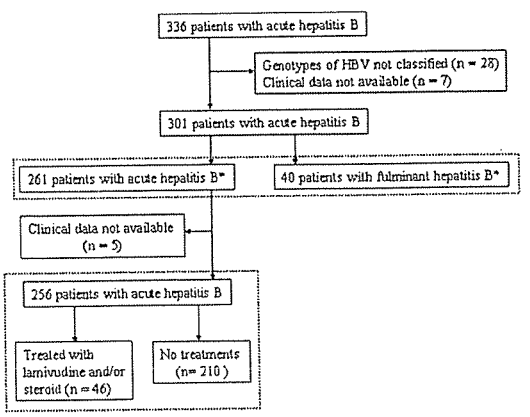

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8)[a]	Ae (n = 32)[a]	Ba (n = 21)[a]	Bj (n = 10)[a]	Cs (n = 10)[a]	Ce (n = 167)[a]	D (n = 3)[a]	G (n = 5)[a]
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%)c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36)[b]	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16)[b]	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%)c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations[a]			
nt 1753 and/or nt1754[b]	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	*P* Value
Age (yr)			
<34[a]	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200[a]	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0[a]	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00[a]	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (−)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (−)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754[b]			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1..013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

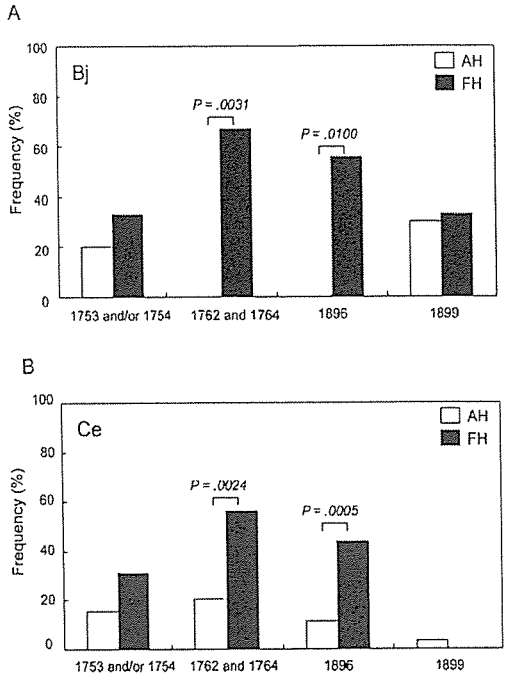

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares

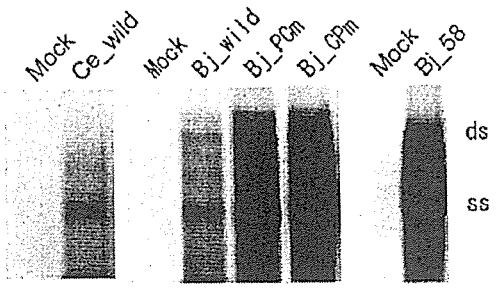

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

References

1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733-1745.
2. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.
3. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. 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 1992;73:1201-1208.
4. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69: 2575-2583.
5. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
6. Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. HEPATOLOGY 2002;35:1274-1276.
7. Kao JH. Hepatitis B viral genotypes: clinical relevance and molecular characteristics. J Gastroenterol Hepatol 2002;17:643-650.
8. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329-338.
9. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-820.
10. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985-5992.
11. Huy TT, Ushijima H, Quang VX, Win KM, Luengrojanakul P, Kikuchi K, et al. Genotype C of hepatitis B virus can be classified into at least two subgroups. J Gen Virol 2004;85:283-292.
12. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, Ito K, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. Hepatol Res 2005;33:216-224.
13. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepatol 2005;12:456-464.
14. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
15. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124: 925-932.

16. Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. HEPATOLOGY 2004;40:747-755.
17. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285-1293.
18. Mitamura K. Epidemiology of HDV infection in Japan. Prog Clin Biol Res 1991;364:81-87.
19. Trey C, Lipworth L, Chalmers TC, Davidson CS, Gottlieb LS, Popper H, et al. Fulminant hepatic failure. presumable contribution of halothane. N Engl J Med 1968;279:798-801.
20. Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81-89.
21. Kato H, Orito E, Gish RG, Bzowej N, Newsom M, Sugauchi F, et al. Hepatitis B e antigen in sera from individuals infected with hepatitis B virus of genotype G. HEPATOLOGY 2002;35:922-929.
22. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.
23. Hasegawa I, Tanaka Y, Kramvis A, Kato T, Sugauchi F, Acharya SK, et al. Novel hepatitis B virus genotype A subtyping assay that distinguishes subtype Aa from Ae and its application in epidemiological studies. J Virol 2004;78:7575-7581.
24. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, Watanabe H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis 2004;38:1222-1228.
25. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;38:2899-2903.
26. Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, et al. 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 2003;18:943-949.
27. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. HEPATOLOGY 2001;33:218-223.
28. Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshiba M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology 1991;100:1087-1094.
29. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med 1991;324:1699-1704.
30. Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. Ann Intern Med 1995;122:241-248.
31. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med 1991;324:1705-1709.
32. Karayiannis P, Alexopoulou A, Hadziyannis S, Thursz M, Watts R, Seito S, et al. Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. HEPATOLOGY 1995;22:1628-1634.
33. Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the precore region in patients with fulminant hepatitis B in the United States. Gastroenterology 1993;105:1173-1178.
34. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology 1995;109:1618-1623.
35. Liang TJ, Hasegawa K, Munoz SJ, Shapiro CN, Yoffe B, McMahon BJ, et al. Hepatitis B virus precore mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. J Clin Invest 1994;93:550-555.
36. Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. J Virol 2003;77:6601-6612.
37. Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut 2003;52:1630-1637.
38. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol 1996;70:5845-5851.
39. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. Virology 1993;194:263-276.
40. Bocharov G, Ludewig B, Bertoletti A, Klenerman P, Junt T, Krebs P, et al. Underwhelming the Immune Response: effect of slow virus growth on CD8+-T-lymphocyte responses. J Virol 2004;78:2247-2254.
41. Shimizu M, Ohyama M, Takahashi Y, Udo K, Kojima M, Kametani M, et al. Immunoglobulin M antibody against hepatitis B core antigen for the diagnosis of fulminant type B hepatitis. Gastroenterology 1983;84:604-610.
42. Rinker J, Galambos JT. Prospective study of hepatitis B in thirty-two inadvertently infected people. Gastroenterology 1981;81:686-691.
43. Kent GP, Brondum J, Keenlyside RA, LaFazia LM, Scott HD. A large outbreak of acupuncture-associated hepatitis B. Am J Epidemiol 1988;127:591-598.
44. Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology 1987;92:1844-1850.
45. Beasley RP, Hwang LY, Lin CC, Ko YC, Twu SJ. Incidence of hepatitis among students at a university in Taiwan. Am J Epidemiol 1983;117:213-222.
46. McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis 1985;151:599-603.
47. Schomerus H, Wiedmann KH, Dolle W, Peerenboom H, Strohmeyer G, Balzer K, et al. (+)-Cyanidanol-3 in the treatment of acute viral hepatitis: a randomized controlled trial. HEPATOLOGY 1984;4:331-335.
48. Sherlock S. The natural history of hepatitis B. Postgrad Med J 1987;63:S7-S11.
49. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002;68:522-528.
50. Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arfase Y, Akuta N, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. J Med Virol 2005;76:33-39.

Influence of Hepatitis B Virus Genotypes on the Intra- and Extracellular Expression of Viral DNA and Antigens

Masaya Sugiyama,[1] Yasuhito Tanaka,[1] Takanobu Kato,[1] Etsuro Orito,[2] Kiyoaki Ito,[2] Subrat K. Acharya,[3] Robert G. Gish,[4] Anna Kramvis,[5] Takashi Shimada,[6] Namiki Izumi,[7] Masahiko Kaito,[8] Yuzo Miyakawa,[9] and Masashi Mizokami[1]

Various genotypes of the hepatitis B virus (HBV) induce liver disease of distinct severity, but the underlying virological differences are not well defined. Huh7 cells were transfected with plasmids carrying 1.24-fold the HBV genome of different genotypes/subgenotypes (2 strains each for Aa/A1, Ae/A2, Ba/B2 and D; 3 each for Bj/B1 and C). HBV DNA levels in cell lysates, determined by Southern hybridization, were the highest for C followed by Bj/Ba and D/Ae ($P < .01$), and the lowest for Aa ($P < .01$), whereas in culture media, they were the highest for Bj, distantly followed by Ba/C/D and further by Ae/Aa ($P < .01$). The intracellular expression of core protein was more than 3-fold lower for Ae/Aa than the others. Hepatitis B e antigen (HBeAg) was excreted in a trend similar to that of HBV DNA with smaller differences. Secretion of hepatitis B surface antigen (HBsAg) was most abundant for Ae followed by Aa, Ba, Bj/C and remotely by D, which was consistent with mRNA levels. Cellular stress determined by the reporter assay for Grp78 promoter was higher for C and Ba than the other genotypes/subgenotypes ($P < .01$). Severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator (uPA/SCID), with the liver replaced for human hepatocytes, were inoculated with virions passed in mouse and recovered from culture supernatants. HBV DNA levels in their sera were higher for C than Ae by 2 logs during 4-7 weeks after inoculation. In conclusion, virological differences among HBV genotypes were demonstrated both *in vitro* and *in vivo*. These differences may influence HBV infections with distinct genotypes in clinical and epidemiological settings. *Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2006;44:915-924.)

Abbreviations: HBV, hepatitis B virus; PCR, polymerase chain reaction; uPA/SCID, severe combined immunodeficiency transgenic with urokinase-type plasminogen activator; ALT, alanine aminotransferase; SEAP, secreted alkaline phosphatase; ER, endoplasmic reticulum; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; Grp, glucose-regulated protein; RTD-PCR, real-time detection PCR.

From the [1]Department of Clinical Molecular Informative Medicine, and [2]Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; [3]Sub Bagian Hepatologi, Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India; [4]Hepatology and Gastroenterology, California Pacific Medical Center, CA; [5]MRC/CANSA/University Molecular Hepatology Research Unit, Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa; [6]PhoenixBio Co. Ltd., Higashi-Hiroshima, Japan; [7]Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan; [8]Division of Gastroenterology and Hepatology, Third Department of Internal Medicine, Mie University School of Medicine, Mie, Japan; [9]Miyakawa Memorial Research Foundation, Tokyo, Japan.

Received March 3, 2006; accepted July 18, 2006.

Supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H16-kanen-3), Uehara Memorial Foundation, and Toyoaki Foundation.

The nucleotide sequences of HBV DNA isolates used in this study have been deposited in the GenBank/DDBJ/EMBL databases under accession numbers AB246335–AB246348.

Address reprint requests to: Masashi Mizokami, M.D., Ph.D., Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. E-mail: mizokami@med.nagoya-cu.ac.jp; fax: (81) 52-842-0021.

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

DOI 10.1002/hep.21345

Potential conflict of interest: Nothing to report.

Worldwide there are 350 million people persistently infected with hepatitis B virus (HBV) of various genotypes.[1] Genotypes of HBV are defined by a sequence divergence >8% over the entire genome made of approximately 3,200 nucleotides (nt).[2] Presently, eight genotypes of HBV have been recognized, and they are designated A to H in the order of discovery.[3-5]

HBV genotypes have different geographic distributions.[6-8] Genotype D is ubiquitous and scattered worldwide, while genotype A is prevalent in sub-Saharan Africa, northern America as well as Europe, and genotypes B and C are common in Asia. Genotype E is restricted to Africa, and both genotypes F and H are localized to Central and South America. Because genotype G is infrequent, its epidemiology has not been determined.

Genotypes are further subdivided into subgenotypes. Subgenotype Aa/A1 was originally identified in HBV isolates from South Africa by phylogenetic analysis of preS2/S sequences,[9] and later confirmed by the analysis of complete genomes.[10,11] Sugauchi and colleagues[12] identified 2 subgenotypes of genotype B. One subgenotype (Bj/B1) is the authentic genotype B indigenous to Japan, whereas the other (Ba/B2) predominates in Asia and has a recombination with genotype C over the precore region and core gene.[12,13] Subgenotypes have also been recognized in genotypes C and D.[14,15]

Evidence for the influence of HBV genotypes/subgenotypes on liver disease in acute and chronic infections is increasing.[8,16-18] Because at most two genotypes prevail in a given country, comparison has been restricted between genotypes B and C in Asia as well as A and D in Europe and India,[19-21] except in multinational studies where more than two genotypes have been compared.[22,23] Host and environmental differences can confound the differences between genotypes making comparisons very difficult. Therefore a system that eliminates these factors and allows a direct comparison of the influence of HBV genotypes/subgenotypes on viral replication and expression would be ideal.

Since 1986, viral particles have been produced *in vitro* by transfection of cultured cells with a linear tandem dimer of HBV.[24,25] Virions thus produced are morphologically and virologically indistinguishable from the authentic virion,[26] and can infect chimpanzees.[27] The minimal length of replication-competent HBV DNA is 1.24-fold genome, which can be transcribed into overlength pregenomic and precore mRNAs.[28] Mice with severe combined immunodeficiency, carrying urokinase-type plasminogen activator transgenes controlled by an albumin promoter (uPA/SCID mice),[29] have been transplanted with human hepatocytes.[30] The graft hepatocytes have morphological and biochemical characteristics identical to human liver[31] and can be infected with HBV[31-34] and HCV,[35] thus providing an ideal small animal model for studying these viruses.

In the present study, by transfection of Huh7 cells and infection of uPA/SCID mice (hereafter referred to as chimeric mice), we evaluated genotype-dependent differences in the intracellular and extracellular expression of HBV DNA and antigens *in vitro* and *in vivo*, respectively.

Patients and Methods

Patients. Sera were obtained from 14 patients with chronic hepatitis. Demographic and clinical characteristics of the 14 patients and genotypes/subgenotypes of the HBV isolated from them are shown in Table 1. The sera had high HBV DNA levels and contained isolates with-

Table 1. Demographic, Biochemical, and Virological Characteristics of Patients From Whom HBV Isolates of Distinct Genotypes/Subgenotypes Were Recovered

Genotype/ subgenotype	Isolate	Country of origin	Age (years)	Sex	ALT (U/L)	HBeAg	HBV (LGE/mL)
Ae/A2	Ae_US	USA	41	Male	84	+	8.1
	Ae_JPN	Japan	30	Male	620	+	6.1
Aa/A1	Aa_IND	India	18	Male	10	+	5.7
	Aa_SAF	South Africa	22	Male	20	+	4.8
Ba/B2	Ba_JPN51	Japan	56	Male	120	+	7.7
	Ba_JPN58	Japan	50	Male	53	+	3.7
Bj/B1	Bj_JPN35	Japan	15	Male	57	+	8.7
	Bj_JPN56	Japan	55	Male	501	+	7.2
	Bj_JPN57	Japan	28	Male	57	+	3.4
C	C_JPN22	Japan	59	Male	49	+	8.3
	C_JPNAT	Japan	56	Female	180	+	8.5
	C_HK74	Hong Kong	22	Female	132	+	8.0
D	D_IND60	India	19	Male	19	+	6.8
	D_US68	USA	52	Male	37	+	3.8

Abbreviation: LGE, Log genome equivalents.

out the precore G1896A and core promoter A1762T/G1764A mutations. The study design conformed to the 1975 Declaration of Helsinki, and was approved by Ethic Committees of institutions. A written informed consent was obtained from each patient.

Plasmid Constructs of HBV DNA and Sequencing. HBV DNA was extracted from 100 μL of serum using QIAamp DNA blood kits (Qiagen, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV genome. Nested polymerase chain reaction (PCR) was carried out using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (95°C, 30 seconds; 57°C, 30 seconds; 72°C, 2 minutes) (see supplementary information). Amplified fragments were inserted into the pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (Toyobo Co. Ltd., Osaka, Japan). At least 5 clones of each fragment were sequenced and the consensus sequence determined. Among the 5 clones, those containing the consensus sequence were identified and used as templates for plasmid construction. To make up the 5′- and 3′-ends of replication-competent construct, fragment C [nt 1413-2815 (nucleotides numbered according to the prototype HBV/C clone with accession no. NC_003977)] was prepared by the fusion PCR technique involving forward primer introduced with an *Hind*III site and reverse primer with an *Eco*RI site. Amplified fragment C was cloned in the pGEM-T Easy Vector, and clones having the consensus sequence were selected. Clones for fragment D (nt 2815-1064) and E (nt 1064-2185) bearing the consensus sequence were selected. Fragment C was constructed into the pUC19 vector deprived of promoters (Invitrogen Corp., Carlsbad, CA) by digestion with *Hind*III and *Eco*RI. Fragments C, D and E were digested and ligated serially with *Eco*RI (fragments C and E), *EcoO65*I (fragments C and D) and *EcoT22*I (fragments D and E), resulting in 1.24-fold the HBV genome. Cloned HBV DNA sequences were confirmed by using ABI Prism BigDye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer.

Cell Culture and Transfection. After 16 hours of culture, Huh7 cells were transfected with 5 μg of DNA construct using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and estimating SEAP enzymatic activity in the culture supernatant. Triple experiments were conducted for each clone.

Determination of HBV Markers. HBsAg and HBeAg were determined by chemiluminescence with commercial assay kits (Fujirebio Inc., Tokyo, Japan). The product of the HBV core gene (core protein) was determined by enzyme immunoassay using the monoclonal antibody (HB50) that specifically recognizes SPRRR repeats in the arginine-rich domain of core protein.[36] The assay is capable of detecting core proteins in viral particles or complexed with antibodies, in addition to free core proteins, because the sample is pretreated to inactivate antibodies and dissociate antigens.

Detection of Extracellular HBV. The supernatant was collected from each 10-cm dish by centrifugation at 22,000*g* for 5 min, and a 100-μL portion was adjusted to 6 mM with $MgOAc_2$ and treated with 200 μg/mL DNase I and 100 μg/mL RNase A at 37°C for 3 hours. The reaction was terminated by EDTA at the final concentration of 10 mM, and the mixture was incubated at 65°C for 10 min. HBV DNA was extracted using microspin columns (QIAamp Blood kit, Qiagen K.K, Tokyo, Japan). For real-time detection PCR (RTD-PCR), 10 μL of eluted sample was amplified in a 50-μL mixture containing 2 × TaqMan Universal MasterMix (Applied Biosystems, Foster City, CA), forward primer (HBV-S190F), reverse primer (HBV-S703R) and TaqMan probe (HBSP2G) (details in supplementary information). To avoid the possibility for plasmid DNA remaining in supernatant, Huh7 cells were transfected with plasmid carrying 1.24-fold the HBV DNA with stop-codons in the polymerase gene or plasmid DNA in the absence of transfection reagent. Negative controls processed in parallel never created positive results.

Preparation of RNA. Transfected cells were lysed by Isogen (Nippon Gene Co. Ltd., Tokyo, Japan). The lysate was supplemented with chloroform, incubated for 15 minutes on ice, and centrifuged at 22,000*g* for 15 minutes. The aqueous phase was removed and precipitated with isopropanol. RNA was pelleted by centrifugation, washed with ethanol, and dissolved in 50 μL of water.

Southern and Northern Blot Hybridizations. Southern and Northern blot hybridizations were performed with a full-length probe of each genotype/subgenotype by previous methods.[37] No significant differences were observed in the detection between internal control HBV DNA and each probe for all genotypes/subgenotypes.

MTS and Luciferase Assay for Grp78 Promoter. The pGL3/glucose-regulated protein (Grp78)/−169 reporter plasmid, constructed by subcloning the rat Grp78 promoter subfragment (nt −169 to −29), was generously provided by Amy S. Lee (University of Southern California). Huh7 cells seeded in a 6-well plate at 2 × 10^5 cells/well were co-transfected with HBV plasmids (0.5 μg), SEAP vector (0.05 μg), and pGL3/Grp78/−169 (0.5

μg), and tested for MTS as well as luciferase 48 hours after transfection. To adjust the number of viable cells, MTS determination was performed by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) in accordance with the manufacturer's instructions. Thereafter in the same well, luciferase activity was determined by LUMAT LB9507 (EG&G Berthold, Bad Wildbad, Germany) and Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. In addition, the SEAP assay was performed for adjusting the transfection efficiency. Assays were performed at least in triplicate, and the results were expressed as the luciferase activity relative to that of a negative control (mock).

Chimeric Mice With the Liver Repopulated for Human Hepatocytes. Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene ($uPA^{+/+}/SCID^{+/+}$ mice) with the liver replaced with human hepatocytes[38] were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured by ELISA with commercial assay kits (Bethyl Laboratories Inc., Montgomery, TX). HBV DNA was determined by RTD-PCR as previously reported.[39]

Immunofluorescence. Freshly prepared liver tissues were snap-frozen in isopentane precooled in liquid nitrogen. Isolated epithelia of bovine cornea were embedded in OCT compound and frozen immediately. Specimens were cut at 5-6 μm by cryostat, mounted on glass slides, air-dried, and fixed in 100% acetone at room temperature for 10 min. Sections were blocked with Antibody Diluent (Dako, Tokyo, Japan), incubated with rabbit anti-HBc (Dako, Tokyo, Japan) at room temperature for 1 hour, washed in phosphate-buffered saline, and then incubated with goat anti-rabbit IgG conjugated with Cy3 (Chemicon International Inc., Temecula, CA) or goat anti-human albumin labeled with FITC (Bethyl Laboratories Inc., Montgomery, TX). Sections were washed with phosphate-buffered saline, and observed in a fluorescent microscope (Eclipse E800M; Nikon, Tokyo, Japan).

Statistical Analysis. Group means were compared by independent Student *t* test or one-way ANOVA test.

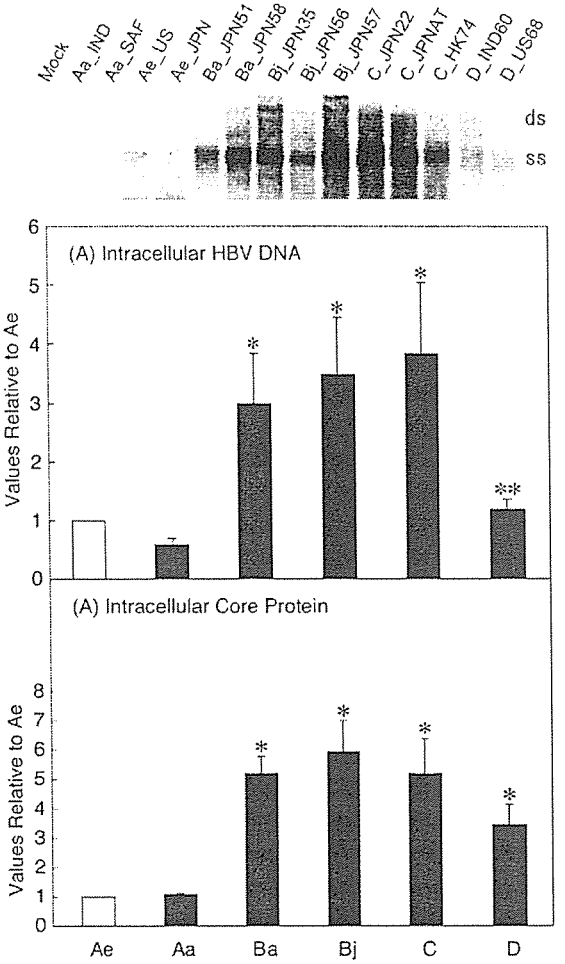

Fig. 1. Intracellular expression of HBV DNA and core protein 3 days after transfection. (A) The density of single-stranded DNA in Southern blot analysis of cell lysates of Huh7 cells transfected with plasmid constructs of various genotypes. The results of 14 clones of various genotypes [Aa (n = 2), Ba (n = 2), Bj (n = 3), C (n = 3), and D (n = 2)] in triplicate experiments are shown (same for the other figures). Values of two Ae clones were almost the same, and their mean served as a reference with the reading of 1 in the white bar; values of the other clones were expressed relative to this in black bars (same for the other Figures). An asterisk represents a statistical difference of $P < .01$ in comparison with Ae, Aa, and D, and double asterisks are that in comparison with Ae and Aa. Patterns of electrophoresis are shown on the top. (B) Intracellular expression of the product of the core gene (core protein). An asterisk represents a statistical difference of $P < .01$ in comparison with Ae and Aa. Each clone was tested at least three times.

Results

HBV Isolates for Transfection. HBV isolates of different genotypes/subgenotypes and characteristics of the 14 patients with chronic hepatitis B, from whom these isolates were recovered, are listed in Table 1. At least two isolates for each genotype/subgenotype were tested. None of these isolates possessed mutations for G1896A in the precore region or A1762T/G1764A in the basic core promoter, which may interfere with the expression of HBeAg and efficiency of pregenome encapsidation for replication. The genotypes/subgenotypes of the 14 HBV isolates were determined by the construction of phylogenetic tree, comparing their full genome sequences to 24 sequences from the DNA database, representative of genotypes A to D and subgenotypes of A and B. The 14 HBV isolates cluster with those of corresponding genotypes/subgenotypes (Supplementary Fig. 1).

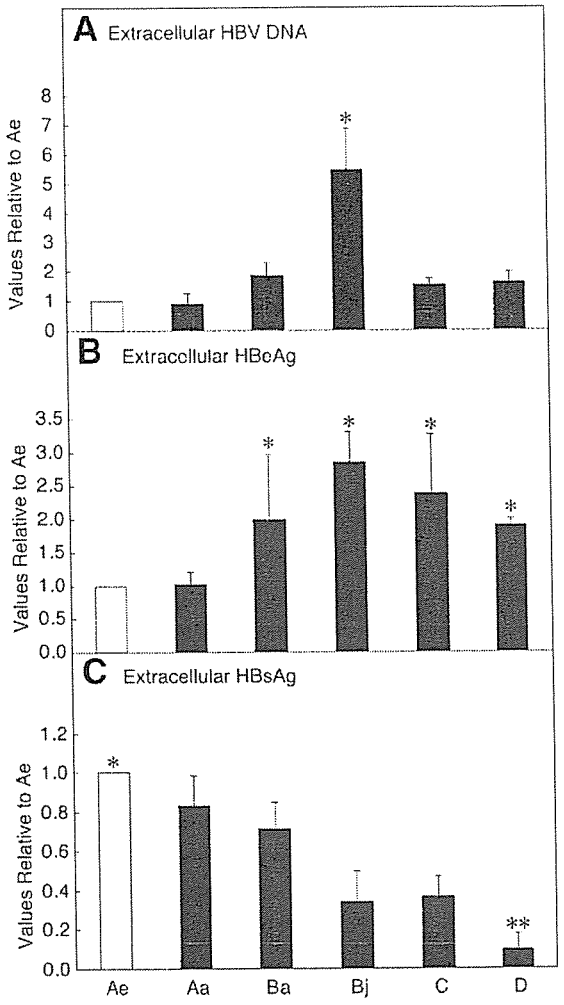

Fig. 2. Extracellular expression of HBV DNA and antigens. (A) Expression of HBV DNA. An asterisk represents a statistical difference of $P < .01$ in comparison with the other genotypes. (B) Expression of HBeAg. An asterisk represents difference of $P < .01$ in comparison with Ae and Aa. (C) Expression of HBsAg. An asterisk represent difference of $P < .05$ against Aa and that of $P < .01$ against the others. Double asterisks represent difference of $P < .01$ against the others.

Intracellular Expression of HBV DNA and Antigens. Huh7 cells were transfected with a pUC19 vector carrying 1.24-fold the HBV genome. Three days after transfection, they were harvested, lysed with NP-40 and tested for HBV DNA and antigens. The density of single-stranded HBV DNA was compared among different genotypes/subgenotypes by Southern blotting (Fig. 1A). Because the results for the two Ae clones (Ae_US and Ae_JPN) were similar, their mean was set at 1.0, and HBV DNA levels for the other genotypes/subgenotypes were expressed relative to this value. The expression of HBV DNA was the highest for genotype C, followed by two subgenotypes of B and further by A and D ($P < .01$); it was the lowest for subgenotype Aa.

Figure 1B compares the intracellular expression of core protein in cell lysates among different genotypes/subgenotypes. The expression was the highest for Bj followed by C and Ba. It was relatively lower for D than B and C, still about 3-fold higher for D than Ae and Aa ($P < .01$).

Extracellular Expression of HBV DNA and Antigens. After 3 days in culture, supernatants from cells transfected with the 14 HBV strains were compared for HBV DNA, HBeAg and HBsAg (Fig. 2A-C). As in evaluation of intracellular expression, the mean value for Ae was set at 1.0 and used as the reference.

Figure 2A compares HBV DNA levels in culture supernatants. The level of HBV DNA was the highest for Bj, followed by Ba, C and D by a margin of about 3-fold ($P < 0.01$), and further by Ae and Aa by that of approximately 5-fold ($P < .01$). These HBV DNA levels were in accord with those able to be immunoprecipitated by anti-HBs and exclusive of naked core particles (supporting Fig. 2). The expression of HBeAg showed a similar trend to HBV DNA with smaller yet significant differences (Fig. 2B).

The expression of HBsAg did not correspond with those of HBV DNA and HBeAg expression (Fig. 2C). HBsAg was expressed in the highest level for Ae, followed by Aa and Ba, and distantly by Bj as well as C ($P < .01$), and the least for D.

Intracellular Expression of Viral mRNA. In agreement with the results of HBsAg expression, Northern blot analyses revealed the highest expression levels of preS/S mRNA for Ae similar to those for Aa, followed by Ba, Bj and C, and distantly by D (Fig. 3). In contrast, pregenome/precore mRNA levels were the highest for Bj and C, followed closely by Ba. Genotypes A (Aa/Ae) and D expressed lower pregenome/precore mRNA levels, consistent with low HBV replication for A and D (Figs. 1A, 2A).

Cellular Stress. To compare the level of cellular stress induced by transfection with distinct HBV genotypes/subgenotypes, a marker for endoplasmic reticulum (ER) stress designated Grp78 was examined; it is one of the best characterized ER chaperon proteins.[40,41] Grp78 promoter

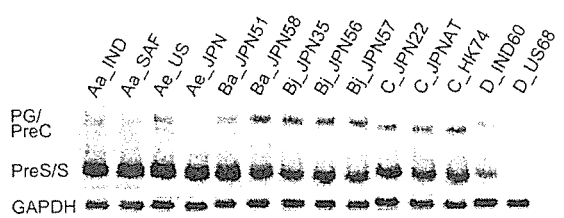

Fig. 3. Northern blot analysis of cell lysates of Huh7 cells transfected with HBV clones of distinct genotypes/subgenotypes. GAPDH was used as an internal control. Abbreviations: PG/PreC, pregenome/precore; S, surface.

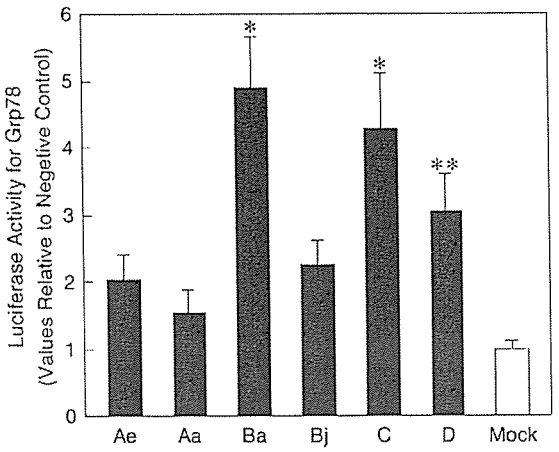

Fig. 4. Cellular stress in Huh7 cells transfected with HBV clones of distinct genotypes/subgenotypes. Relative activity of Grp78 promoter is shown. The mean value of negative controls (mock) served as a reference with the reading of 1, and values of the other clones were expressed relative to this.

activity was highest in cells transfected with HBV/Ba or C, followed by those with D ($P < .05$), Bj, Ae and Aa (Fig. 4), indicating that HBV/Ba and C could induce higher ER stress in Huh7 cells.

Viral Particles Secreted into Culture Media. Large Dane particles and small spherical HBsAg particles, precipitated in culture media with anti-HBs, were visualized by immune electron microscopy (details in supporting information). Figure 5 depicts viral particles isolated from

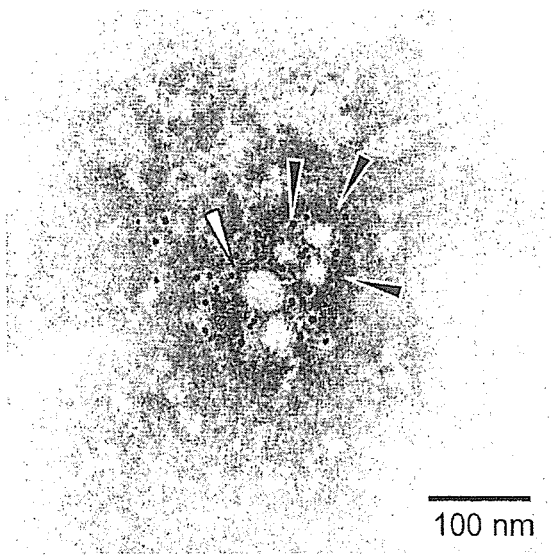

Fig. 5. Immune electron microscopy of the culture supernatant of Huh7 cells transfected with Ae_JPN clone. HBV particles were precipitated with anti-HBs, and overstained with goat anti-mouse IgG labeled with colloidal gold. The white arrow points to complete virions (Dane particles) and the black arrows indicate HBsAg particles.

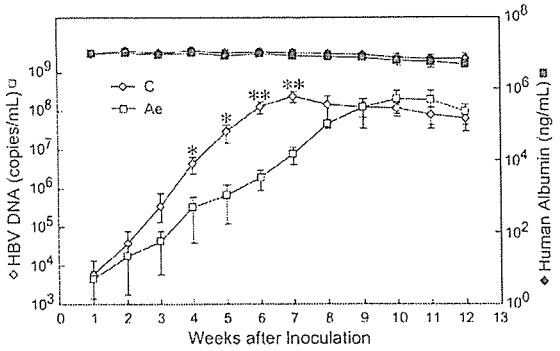

Fig. 6. Levels of HBV DNA and human albumin in sera from chimeric mice inoculated with subgenotype Ae or genotype C. For each genotype/subgenotype, 3 chimeric mice were inoculated. The upper graphs with the solid points show human albumin expression and the lower panel with the open points show the HBV DNA levels. An asterisk represents a statistical difference of $P < .01$, and double asterisks that of $P < .05$.

the medium of cell cultures transfected with plasmids carrying the Ae genome. Similar electron micrographs were visualized for virions recovered from culture media of cells transfected with the other HBV genotypes/subgenotypes. Using 5-nm colloidal gold as the internal standard, diameters of viral and subviral particles were estimated to be 42 and 22 nm, respectively.

Infection of Chimeric Mice With HBV from Culture Supernatants. Supernatants of Huh7 cultures harvested 3 days after transfection were condensed in the Amicon concentrator (Millipore SA, Molsheim, France) and injected intravenously into chimeric mice. Sera harvested 3 months after inoculation were diluted to 10^6 HBV DNA copies/mL. Chimeric mice were inoculated with 100 μL of mouse serum containing 10^5 HBV DNA copies of subgenotype Ae with the lowest (clone Ae_JPN) or genotype C with the highest (clone C_JPN22) replicative activity (Fig. 1A). Three mice were inoculated with each genotype, and followed weekly for expression of HBV DNA and human albumin in serum (Fig. 6). HBV DNA levels increased 2 weeks after inoculation and continued to rise until 7-8 weeks when they plateaued. HBV DNA titer was higher by 2 logs in the mice inoculated with genotype C than subgenotype Ae. Neither serum levels of human albumin nor the body weight differed between the mice inoculated with Ae and C. Control mice inoculated with plasmids constructed with 1.24-fold the HBV genome of Ae or C did not elicit HBV DNA in serum.

The liver from chimeric mice infected with HBV of subgenotype Ae was examined by immunofluorescent microscopy for hepatitis B core antigen (HBcAg) utilizing anti-HBc labeled with Cy3 (Fig. 7A). The staining for HBcAg was confined to areas where mouse liver had been replaced for human hepatocytes, and the same areas

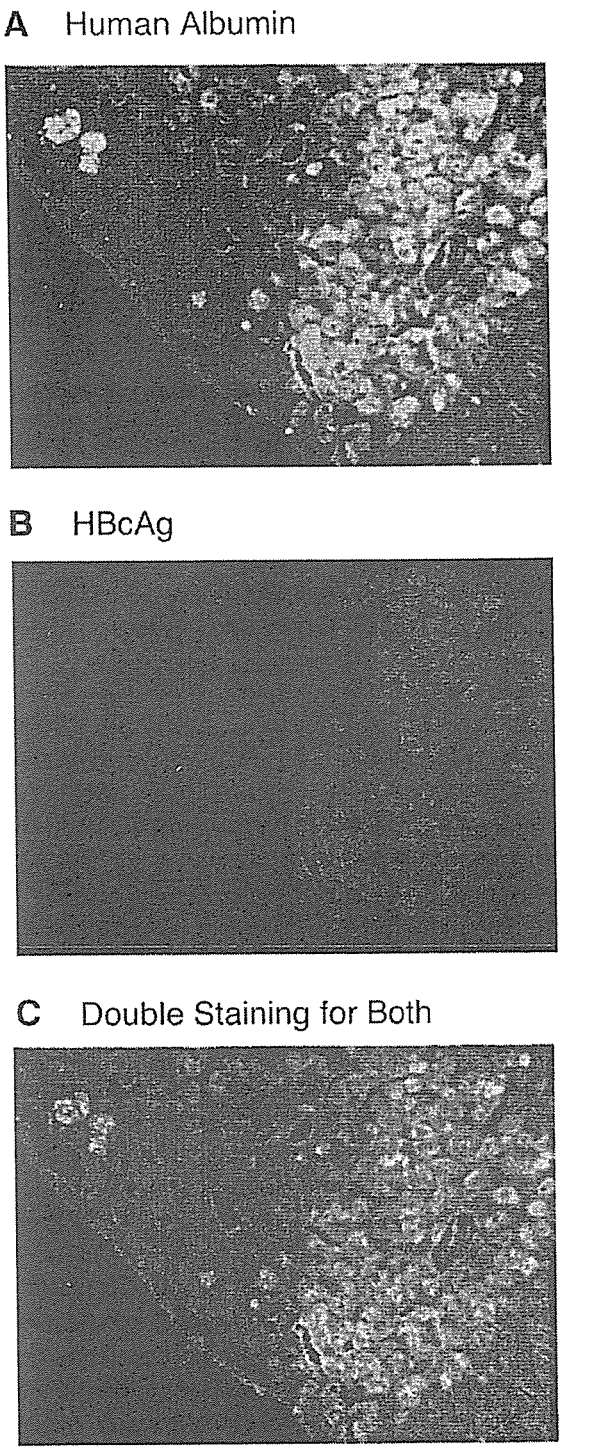

Fig. 7. Co-localization of HBcAg and human albumin in the liver of chimeric mice infected with a clone of subgenotype Ae (Ae_JPN) and examined 24 weeks later. Liver sections were stained for HBcAg (A), human albumin (B), or double-stained for both (C). Note that HBcAg was detected exclusively in cells expressing human albumin.

stained for human albumin (Fig. 7B). Co-localization of HBcAg and human hepatocytes was demonstrated by double staining for HBcAg and human albumin (Fig. 7C).

Nucleic acids were extracted from sera of mice infected with subgenotype Ae or genotype C at 12 and 24 weeks after inoculation. The full genome of the HBV isolate was amplified and sequenced. There were no differences in the sequence between isolates obtained from the mouse sera and plasmid constructs used for transfection.

Discussion

The influence of genotypes/subgenotypes on disease progression and clinical outcome of HBV infection is noted and documented. However, diverse geographical distributions of HBV genotypes/subgenotypes have made it difficult to evaluate these differences, which would be confounded by host and environmental variables. Therefore it would be beneficial to have *in vitro* and *in vivo* experimental systems that can control for such variables. In the present study, using *in vitro* transfection system in Huh7 cells and *in vivo* uPA/SCID mouse model carrying human hepatocytes, the intracellular and extracellular expression of HBV DNA and antigens was compared among different HBV genotypes/subgenotypes. In order to exclude differences in expression, as a result of variation within strains of the same genotypes/subgenotypes, at least two isolates for each genotype/subgenotype were employed to prepare plasmid constructs with 1.24-fold the HBV genome. HBV isolates containing mutations that affect the expression of HBeAg and viral replication, such as G1896A and A1762T/G1764A, were not used. Both *in vitro* transfection experiments in Huh7 cells and *in vivo* infection of uPA/SCID mice with human hepatocytes have demonstrated marked genotype-dependent differences in the expression of HBV DNA and antigens. These differences may contribute to understanding clinical differences among HBV infections with distinct genotypes.

The replication capacity of HBV in transfected Huh7 cells varied among subgenotypes of A (Aa/Ae) and B (Bj/Ba) as well as genotypes C and D (Fig. 1A), with genotype C having the highest replication capacity and subgenotype Aa the lowest. Genotype C is associated with more severe histological liver damage than genotype B.[20,42] It is possible that the intracellular accumulation of HBV DNA and antigens may play a role in inducing liver damage (Table 2). Indeed, the intracellular accumulation of core protein following transfection with genotype C or subgenotype Ba, which is a recombinant of genotype C on genotype B over the precore/core region,[12] was higher than those for the other genotypes with the exception of subgenotype Bj (Fig. 1B). Although intracellular HBV

Table 2. Summary for Comparison of Intra- and Extracellular Expression of HBV DNA and Antigens, as Well as ER Stress Among Various Genotypes/Subgenotypes

Expression and Stress	Genotypes/Subgenotypes					
	Ae	Aa	Ba	Bj	C	D
Intracellular						
HBV DNA	1.0	0.6	3.0*	3.5*	3.8*	1.2
Core protein	1.0	1.0	5.0*	6.0*	5.0*	3.5
Extracellular						
HBV DNA	1.0	1.0	2.0	5.5*	1.5	1.5
HBeAg	1.0	1.0	2.0*	2.8*	2.4*	2.0
HBsAg	1.0*	0.8	0.7	0.3	0.3	0.1
ER stress†	2.0	1.5	4.9*	2.2	4.3*	3.0

*Higher than genotypes/subgenotypes without asterisks.
†Values relative to negative controls; the other values are relative to that of Ae.

DNA levels for Bj were comparable with those for Ba or C, extracellular HBV DNA levels were much higher for subgenotype Bj than Ba or C. The intracellular virion retention was lower for Bj, in reflection of high extracellular HBV DNA expression for this subgenotype. Of note, the Grp78 promoter activity, which is one of the best ER stress markers,[40,41] was the highest for HBV/Ba and C. Increased ER stress (cellular stress) as well as virion retention, observed in Huh7 cells transfected with HBV of either genotype C or subgenotype Ba, could promote inflammation and lead to more severe liver disease than subgenotype Bj.[13,20,43] On the other hand, a strong tendency of Bj for extracellular virion secretion may endow a high infectious capacity to blood from individuals infected with this subgenotype; it would trigger strong immune responses in hosts. Indeed, such high replication and enhanced secretion of HBV/Bj may be associated with a greater incidence of fulminant hepatitis in individuals infected with subgenotype Bj than genotype C.[44] The data on ER stress, however, were obtained by assays of a single reporter gene and without looking into intracellular levels of Grp78 protein. It would be necessary to validate differences in ER stress, among infections with HBV of distinct genotypes, by determining promoter activities of the other genes responsive to ER stress.

Likewise, intracellular levels of HBV DNA and core protein were higher for genotype D than A, which may increase the activity of liver disease and explain an increased resistance to interferon in patients infected with genotype D.[19,45] Lower replication levels of genotype A than D might be due to imbalance between syntheses of HBsAg and core protein; it would be in favor of HBsAg for genotype A, and core protein for genotype D. The lowest replicative activity of genotype A may explain how HBV/A can evade the immune pressure against it and persist in up to 10% of the adulthood infection.[44,46-48] Higher levels of HBsAg secretion for subgenotypes Ae and Aa than the others may contradict their low replicative activity, but may be an immune escape mechanism. The mechanism needs to be sought for, by which genotype A can direct the synthesis of HBsAg in high levels out of proportion to viral DNA, core protein and HBeAg. High transcription efficiency of preS/S mRNA by subgenotypes Ae and Aa may account for this (Fig. 3). Enhanced hepatocarcinogenic potential of subgenotype Aa in young African adults[49] may be related to this high HBsAg expression, too. However, caution must be exercised when extrapolating the results of *in vitro* experimental models to patients, because the duration of infection or immune responses are not taken into account.

In the present study, chimeric mice were used to compare genotype C and subgenotype Ae. The mice were given an anti-human complement drug to increase the repopulation of mouse liver with human hepatocytes,[38] and this was reflected in high levels of human albumin in serum (Fig. 6). Human hepatocytes grafted were successfully infected with viral particles recovered either from culture media of Huh7 cells transfected with plasmid constructs or passages of these particles in mice. Chimeric mice have been infected with HBV recovered from serum[31-34] or HepG2 cells transfected with plasmid constructs carrying 1.4-times the HBV genome.[34] HBV DNA levels in mouse sera in this study were similar to those reported by Dandri et al.[32] and Tsuge et al.,[34] but lower than those by Meuleman et al.[31,33] Variables such as zygocities of the uPA gene, as well as sources of hepatocytes and HBV strains for inoculating mice, may make an accurate comparison of reported data difficult. However, by controlling for such variables in the present study, we were able to demonstrate serum levels of HBV DNA by 2 logs higher in mice inoculated with genotype C than subgenotype Ae (Fig. 6). This observation is consistent with the results of transfection studies in Huh7 cells. HBV DNA was expressed in higher levels, either intracellularly (Fig. 1A) or extracellularly (Fig. 2A), by cells transfected with genotype C than subgenotype Ae. Combined, *in vitro* and *in vivo* experiments point to a lower replicative activity of subgenotype Ae relative to genotype C.

No mutations were detected in HBV DNA sequences from mice 24 weeks after inoculation in comparison with those of inoculated strains. This is probably ascribable to the lack of immune pressure in severely immunodeficient mice and a low mutation rate of the HBV genome.[50] Mutations developing in minor strains may have been missed by the direct sequencing, however.

In conclusion, using both *in vitro* and *in vivo* experimental systems, we have been able to demonstrate differences in the expression of HBV markers among distinct genotypes/subgenotypes. These model systems allow