

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 B_j/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 ($\geq 90\%$ 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/B_j (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 μ L 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 μ g 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 μ g 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 non-categorical 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 |

^aPatients with HBV genotype D or G were not included in the analysis.

^bAll patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.

^cExclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.

^dP = .0001, Ae vs. Ba. P < .01, Ae vs. Aa. P < .05, Ae vs. Bj or Cs.

^eP < .05, Ba vs. Ce.

^fP = .0001, Ae vs. Bj. P < .005, Ae vs. Ce.

^gP < .005, Ba vs. Bj. P < .05, Ba vs. Ce.

^hP < .005, Ae vs. Bj. P < .01, Ae vs. Ce. P < .05, Ae vs. Ba.

ⁱP < .05, Ae vs. Bj.

^jP < .01, Ba vs. Bj. P < .05, Ba vs. Ce.

^kP < .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% $\frac{2}{23}$ vs. 0.5% $\frac{1}{187}$, P = .032).

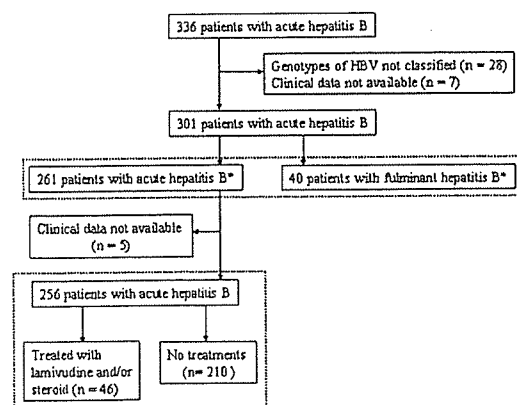


Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including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

^aExclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.

^bSix 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 unde-

etectable 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 |

^aExclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.

^bT1753C/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 |

^aMedian values.^bT1753C/A/G or T1754C/A/G.

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], $P = .019$)

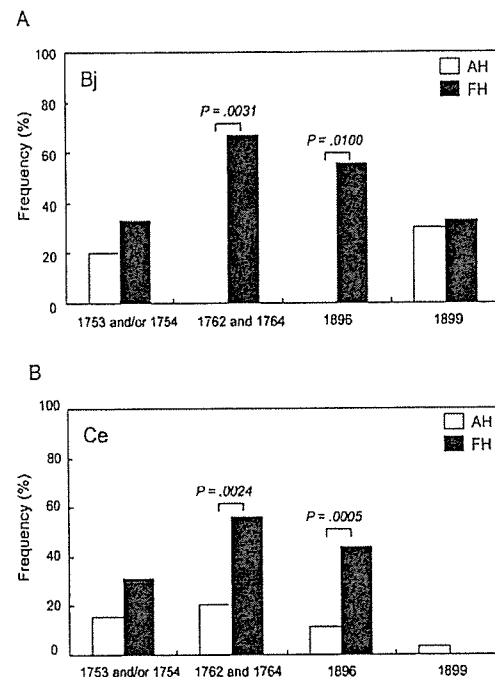


Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

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 Pre-core 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

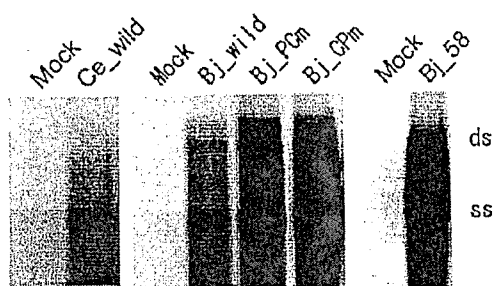


Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

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 Asia²⁸⁻³⁰ and the Middle East.³¹ The failure in detecting these mutations in Western countries³²⁻³⁵ 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 Greece⁴⁴ through 2.7% (1/37) in university students in Taiwan⁴⁵ to 10.4% (5/8) in Alaskan Eskimos⁴⁶ 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% $\frac{3}{13}$ vs. 13% $\frac{1}{8}$ or 12% $\frac{3}{25}$).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.

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

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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; HBeAg, hepatitis B core antigen; Grp, glucose-regulated protein; RTD-PCR, real-time detection PCR.

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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 colleagues¹² 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 over-length 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 liver³¹ and can be infected with HBV³¹⁻³⁴ 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 μ L 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), *Eco*O65I (fragments C and D) and *Eco*T22I (fragments D and E), resulting in 1.24-fold the HBV genome. Cloned HBV DNA sequences were confirmed by using ABI Prism Big-Dye (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 μ g 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 μ g 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 22,000g for 5 min, and a 100- μ L portion was adjusted to 6 mM with MgOAc₂ and treated with 200 μ g/mL DNase I and 100 μ g/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 μ L of eluted sample was amplified in a 50- μ L mixture containing 2 \times 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 22,000g for 15 minutes. The aqueous phase was removed and precipitated with isopropanol. RNA was pelleted by centrifugation, washed with ethanol, and dissolved in 50 μ L 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 \times 10⁵ cells/well were co-transfected with HBV plasmids (0.5 μ g), SEAP vector (0.05 μ g), 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 hepatocytes³⁸ 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.

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 pro-

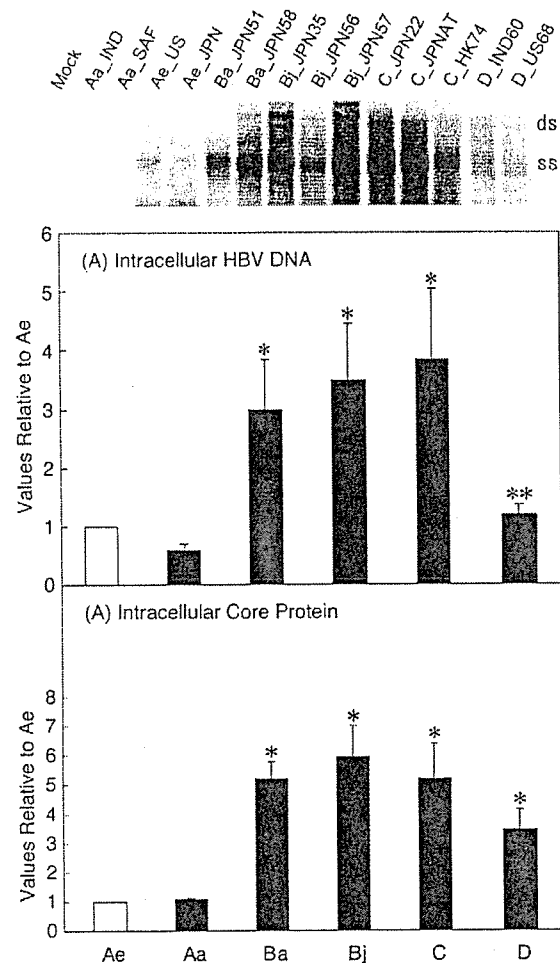


Fig. 1. Intracellular expression of HBV DNA and core protein 3 days after transfection. (A) The density of single-stranded DNA in Southern blot analysis of cell lysates of Huh7 cells transfected with plasmid constructs of various genotypes. The results of 14 clones of various genotypes [Aa (n = 2), Ba (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.

tein, 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).

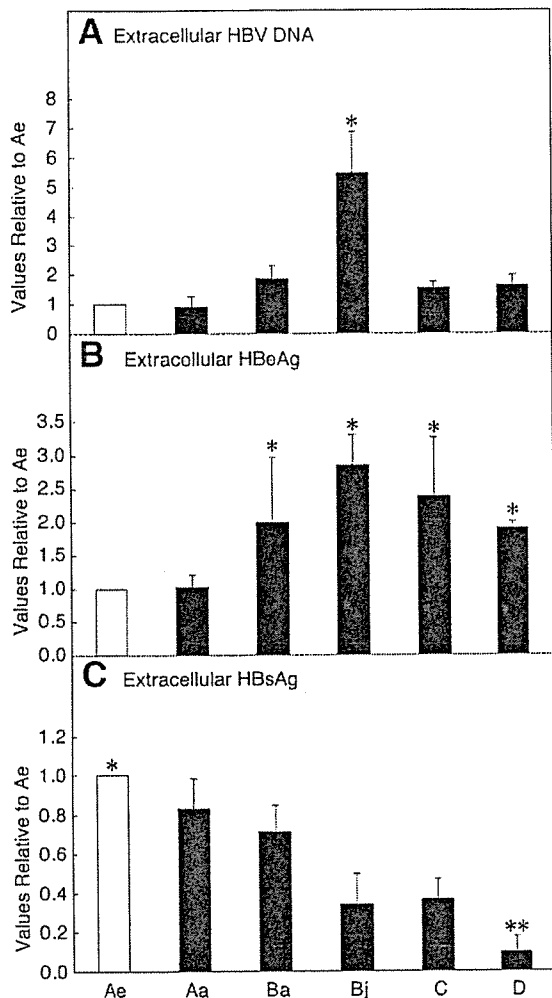


Fig. 2. Extracellular expression of HBV DNA and antigens. (A) Expression of HBV DNA. An asterisk represents a statistical difference of $P < .01$ in comparison with the other genotypes. (B) Expression of HBeAg. An asterisk represents difference of $P < .01$ in comparison with Ae and Aa. (C) Expression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

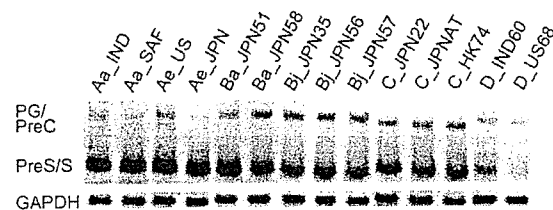


Fig. 3. Northern blot analysis of cell lysates of Huh7 cells transfected with HBV clones of distinct genotypes/subgenotypes. GAPDH was used as an internal control. Abbreviations: PG/PreC, pregenome/precore; S, surface.

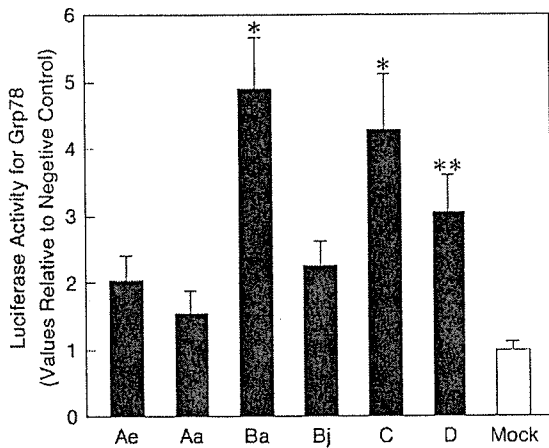


Fig. 4. Cellular stress in Huh7 cells transfected with HBV clones of distinct genotypes/subgenotypes. Relative activity of Grp78 promoter is shown. The mean value of negative controls (mock) served as a reference with the reading of 1, and values of the other clones were expressed relative to this.

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

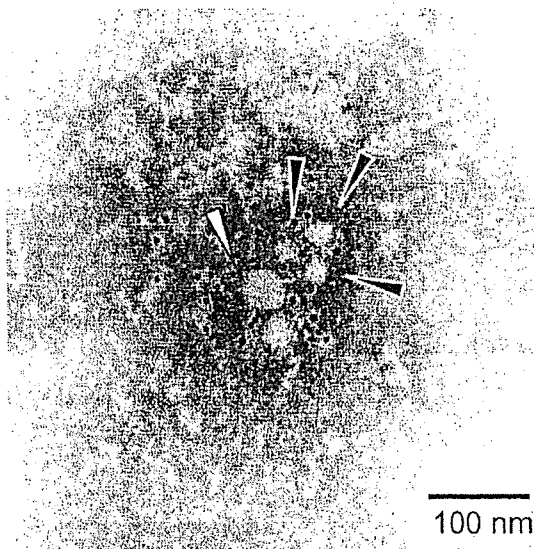


Fig. 5. Immune electron microscopy of the culture supernatant of Huh7 cells transfected with Ae_JPN clone. HBV particles were precipitated with anti-HBs, and overstained with goat anti-mouse IgG labeled with colloidal gold. The white arrow points to complete virions (Dane particles) and the black arrows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

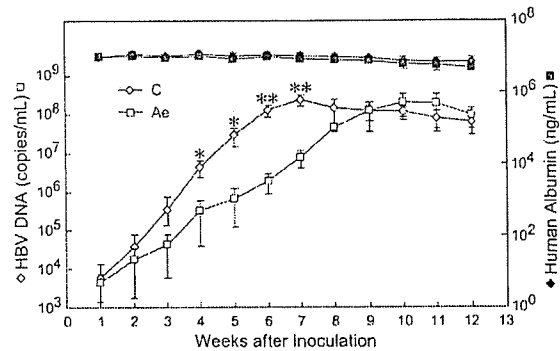


Fig. 6. Levels of HBV DNA and human albumin in sera from chimeric mice inoculated with subgenotype Ae or genotype C. For each genotype/subgenotype, 3 chimeric mice were inoculated. The upper graphs with the solid points show human albumin expression and the lower panel with the open points show the HBV 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 μ L 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

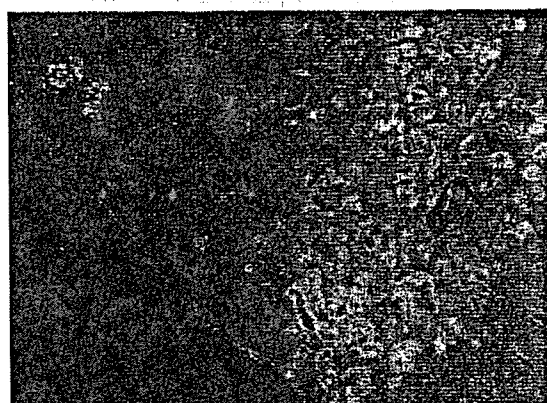
A Human Albumin**B HBcAg****C Double Staining for Both**

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 adults⁴⁹ 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 serum³¹⁻³⁴ 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

comparison among HBV genotypes/subgenotypes free of confounding variables such as host and environmental factors. Furthermore, the lack of immune responses in chimeric mice enables analysis of sheer virological differences. These experimental models may contribute to understanding the influence of genotypes/subgenotypes on clinical outcomes of HBV infection and response to various antiviral treatments.

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T1653 Mutation in the Box α Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Genotype C Infection

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Background. Most patients with chronic hepatitis B virus infection become carriers of inactive virus after hepatitis B e antigen seroconversion; however, a subgroup of patients have persistent abnormal transaminase levels and develop hepatocellular carcinoma after seroconversion.

Methods. In an age-matched case-control study, 40 carriers of inactive virus (mean age \pm standard deviation [SD], 50.9 \pm 11.1 years), 40 patients with chronic hepatitis (mean age \pm SD, 50.2 \pm 8.9 years), and 40 patients with hepatocellular carcinoma (mean age \pm SD, 50.7 \pm 9.4 years) who were infected with hepatitis B virus genotype C and had test results positive for antibody to hepatitis B e antigen were analyzed.

Results. The prevalence of T1653 in the box α was significantly higher among patients with hepatocellular carcinoma than among carriers of inactive virus who did not have hepatocellular carcinoma (70% vs. 25%; $P < .0001$) or chronic hepatitis (70% vs. 35%; $P = .003$). Mutations in the basic core promoter region (T1762/A1764) were frequently found in all groups, regardless of clinical status (in 77.5% of carriers of inactive virus, 77.5% of patients with chronic hepatitis, and 90% of patients with hepatocellular carcinoma). In the multivariate analysis, the presence of T1653, an alanine aminotransferase level of ≥ 37 U/L, and a platelet count of $< 18 \times 10^4$ platelets/mm³ were independent predictive values for hepatocellular carcinoma (odds ratio [95% confidence interval], 5.05 [1.56–16.35], 12.56 [3.05–51.77], and 11.5 [3.47–38.21], respectively). High α -fetoprotein level was the only independent predictive value for T1653 in patients with hepatocellular carcinoma (odds ratio, 12.67; 95% confidence interval, 1.19–134.17). Among patients with test results positive for antibody to hepatitis B e antigen who had hepatocellular carcinoma and were infected with different genotypes of hepatitis B virus, the prevalence of T1653 was 40%, 15%, 25%, 25%, 67%, and 23% in patients infected with hepatitis B virus genotypes Aa, Ae, Ba, Bj, C, and D, respectively ($P < .05$ for genotype C vs. genotypes Ae, Ba, Bj, or D).

Conclusions. Our data indicate that the addition of T1653 mutation in the box α to the basic core promoter mutation increases the risk of hepatocellular carcinoma in patients with hepatitis B virus genotype C.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year [1]. It is now

accepted that hepatitis B virus (HBV) has a carcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between these molecular alterations and the development of HCC in patients with HBV infection.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is a common genotype in East

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Asian countries) by perinatal transmission become carriers of inactive virus after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe-positive phase. Many of these patients have active viral replication and are infected with several mutant viruses. The association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined.

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore (preC) region at nt 1896 (A1896) are associated with seroconversion and persistent viral replication. It is noteworthy that both BCP and preC mutations are often found in patients with advanced liver disease, (e.g., HCC) [2–8]. The T1762/A1764 mutation alters HBeAg production at the transcription level, and the A1896 in the preC region terminates translation of the precursor protein, abrogates HBeAg production, and results in seroconversion. A1896 was also reported previously to be associated with severe forms of chronic liver disease [7,8].

HBV has been classified into 8 major genotypes with use of the complete nucleotide sequence of the viral genome [10]. HBV genotypes not only have distinct geographical distributions [7, 11, 12] but also have different clinical manifestations and responses to therapy (e.g., IFN therapy). Furthermore, HBeAg positivity and levels of HBV DNA, which are controlled by specific mutations, differ between HBV genotypes (e.g., the BCP double mutation is more prevalent among strains of HBV genotype C, followed by HBV genotype A, and the A1896 mutation is frequently found in HBV genotypes B and D) [13–16].

There have been many studies involving viral mutations associated with clinical features, but most previous studies have ignored age, sex, HBeAg status, and HBV genotypes. In Japan, most patients with HCC experience seroconversion (i.e., they are anti-HBe positive) and have HBV genotype C; therefore, we performed an age-matched case-control study among anti-HBe-positive patients infected with HBV genotype C (including carriers of inactive virus, patients with chronic hepatitis, and patients with HCC) to determine the specific HBV genome mutations associated with disease progression.

PATIENTS AND METHODS

Serum samples. Serum samples were obtained from 211 patients from different regional areas worldwide. A total of 120 patients from Japan who were infected with HBV genotype C (40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC) were matched with control subjects according to age and HBe status. Control serum samples were obtained from patients with HCC who were positive for anti-HBe and who were infected with HBV genotype Aa (10 subjects), Ae (13), Ba (20), Bj (20), C (15), and D (13). Control subjects

were from Hong Kong (19 subjects), Japan (36), and the United States (36). The majority of patients infected with HBV genotypes Aa, Ba, Bj, and C were Asian, and the majority of patients infected with HBV genotypes Ae and D were white and black. None of the subjects had serological test results positive for markers of infection with hepatitis C virus or HIV-1.

The study protocol was approved by ethics committees of the participating institutions in accordance with the 1975 Helsinki declaration. Informed consent was obtained from each patient.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by chemiluminescent EIA (Lumipulse f, Fujirebio). HBV genotypes were determined by the restriction fragment-length polymorphism method on the S gene sequence amplified by PCR [29] and ELISA with monoclonal antibodies directed to distinct epitopes on the preS2 region products [18], with use of commercial kits (HBV genotype EIA; Institute of Immunology). The genotypes were also confirmed with use of a phylogenetic tree analysis. α -Fetoprotein and serum protein induced by the absence of vitamin K (antagonist II) were examined with use of chemiluminescent EIA.

Amplification and sequencing of the core promoter and the precore region plus core gene. HBV DNA sequences bearing the core promoter and preC or core regions were amplified by PCR with heminested primers by the method described elsewhere [19]. Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems) in the ABI 3100 DNA automated sequencer (Applied Biosystems). Accession numbers for all strains are AB236515–AB236634.

Case-control study. A carrier of inactive virus was defined as an HBsAg-positive individual with normal alanine aminotransferase (ALT) levels for a 2-year period (with at least 4 evaluations at 3-month intervals) and without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels ($> 1.5 \times$ upper limit of normal [35 U/L]) during a 6-month period (with at least 3 evaluations at 2-month intervals) without a decrease in platelet count or albumin level, and hypersplenism (splenomegaly on ultrasonographic examination). Twenty-one patients were confirmed to have chronic hepatitis by means of a fine-needle biopsy of the liver. Staging and grading (expressed as mean value \pm SD [95% CI]) were 1.24 ± 0.64 (0.99–1.58) and 1.36 ± 0.58 (1.07–1.59), respectively, as previously described [30]. None had received antiviral treatment during the follow-up period. Of 40 patients with HCC, 23 patients received a diagnosis of HCC on the basis of a pathologic examination, and 17 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, CT, or MRI, as well as an elevated serum α -fetoprotein level (≥ 400 ng/mL).

Statistical evaluation. Data were expressed as mean \pm