HBV persisted in only 5 of the 464 (1%) followed patients. Genotypes C accounted for more than 68% in northern as well as southern areas, contrasting with genotype A accounting for 34% in and around the Metropolitan areas. During 24 years from 1982 to 2005, genotype A increased from 5% to 33%, while genotype B gradually decreased from 26% to 8%. Fulminant hepatitis was significantly more frequent in infection with genotype Bj (41%) than those with the other genotypes ($p < 0.01$). The core-promoter double mutation (T1762/A1764) and precore stop-codon mutation (A1896) were more frequent in patients with fulminant than acute self-limited hepatitis (57% versus 15% and 58% versus 10%, respectively, $p < 0.01$ for both). In conclusion, genotype A distributes unevenly over Japan, prevails in younger patients through sexual transmission and has increased with years. Furthermore, fulminant outcome was more frequent in patients with genotype Bj than those with the other genotypes.

Keywords: Chronic hepatitis; Fulminant hepatitis; Hepatitis B e antigen; Hepatitis B surface antigen; Sexual transmission

1. Introduction

Mass vaccination with plasma-derived or recombinant vaccines has been effective in Asian countries hyperendemic with hepatitis B virus (HBV), as well as in the United States and France. In Japan, perinatal transmission of HBV from mothers with hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in serum used to be the principal route to establish the persistent carrier state [1]. Hence, passive and active immunoprophylaxis of babies born to carrier mothers with HBeAg by combined hepatitis B immunoglobulin and vaccine was mandated since 1986, and extended to carrier mothers without HBeAg in 1995. As the results, HBsAg has become rare in the Japanese born after 1986, and is detected in merely 0.3% of the first-time blood donors younger than 19 years at present [2].

There is an increasing trend, however, for acute HBV infection to occur preferentially in young men through promiscuous sexual contacts [3–7]. Foreign sexual workers from neighboring Asian countries are suspected as significant sources of de novo HBV infection in them [4,8]. Thus, patients with acute hepatitis visiting hospitals are increasing all over Japan. Since the majority of acute HBV infections ran subclinical courses, hospitalized cases of acute hepatitis B would represent the tip of an iceberg.

Eight genotypes have been detected by the sequence divergence >8% in the entire HBV genome composed of approximately 3200 nucleotides (nt), and designated by capital alphabet letters from A to H in the order of documentation [9–12]. They have distinct geographical distribution and are associated with severity of liver disease as well as response to antiviral therapies [13–15]. Furthermore, subgenotypes have been reported for HBV/A, B and C, and named Aa (Asian/African type) and Ae (European type) [16], Bj (Japanese type) and Ba (Asian type) [17], as well as Ce (east Asian type) and Cs (southeast Asian type) [18]. There have been increasing lines of evidence that Aa and Ae, as well as Ba and Bj, influence the replication of HBV and bear clinical relevance [19–21].

Over 24 years from 1982 to 2005, a multicenter study was conducted throughout Japan on 547 patients with acute hepatitis B. Genotypes were determined on viral isolates recovered from them, and analyzed for distributions changing with time and in place. The results highlighted foreign HBV genotypes represented by HBV/A which have increased through sexual contacts, and HBV/Bj prevalent in patients with fulminant hepatitis.

2. Patients and methods

2.1. Patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals throughout Japan, of them, 147 and 336 cases are overlapping with previous report from Yotsuyanagi et al. [7] and Ozasa et al. [22], respectively, and 64 cases were newly registered in this study. These hospitals were grouped into the following eight areas: Hokkaido, Tohoku, Kanto, Koshin, Tokai, Kinki, Chugoku/Shikoku and Kyushu/Okinawa. 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. The great majority of them were followed for clinical outcomes until the disappearance of HBsAg through 24 weeks or longer after the presentation. HBV genotypes were determined in sera stored at −40 °C, and their geographical distributions and chronological changes were analyzed. Further, they were correlated with sources of infection and clinical outcomes of acute hepatitis. The study protocol conformed to the 1975 declaration of Helsinki, and was approved by Ethics Committees of institutions. Every patient or his/her next of kin gave an informed consent on the purpose of this study.

2.2. Serological markers of HBV infection

HBsAg was determined by hemagglutination (MyCell, Institute of Immunology Co. Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (AxSYM, Abbott Japan, Tokyo, Japan), and HBeAg by ELISA (ELISA, F-HBe, Kokusai Diagnostic, Kobe, Japan). Anti-HBc of IgM class was determined by ELISA (HBc-antiM RIA, Dainabot, Tokyo, Japan).

2.3. Genotypes and subgenotypes of HBV

The six major HBV genotypes (A–F) were determined serologically by ELISA using commercial kits (HBV GENO-

TYPE EIA, Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies that is specific for each of them [23,24]. Genotypes were confirmed by restriction fragment length polymporphisim (RFLP) when required [25].

HBV/Bj (Japanese type) without the recombination with genotype 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 involving on specific nucleotide substitutions, by the method described previously [26].

2.4. Point mutations in the precore resion and basic core-promoter (BCP)

Mutations in the precore region for A1896 and BCP for T1762/A1764 were detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostic, Tokyo, Japan) according to the manufacturer's instructions, or by sequencing the precore region and BCP using the method described previously [27]. The results were recorded as "the wild-type" or "the mutant-type" expressed dominantly by HBV isolates.

2.5. Statistical analysis

Categorial variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorial variables by the Mann–Whitney's U-test. A p-value less than 0.05 was considered significant.

3. Results

3.1. Clinical profiles of patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals from all over Japan. Genotypes of HBV were unclassifiable in 40 (7%) and sufficient clinical data not available in 22 (4%) of them. Exclusive of these 62 patients, 485 (89%) were left for the evaluation of geographic distribution of HBV genotypes, as well as their changes with time, transmission routes and relevance with clinical outcomes.

The 485 patients with acute hepatitis B had the mean ± S.D. age of 35.7 ± 13.7 years, and included 338 (70%) men. Their peak alanine aminotransferase (ALT) averaged 2576 ± 1673 IU/L and peak total bilirubin 9.5 ± 9.5 mg/dL. They all possessed anti-HBc of IgM class in high titers, and HBeAg was detected in sera from 338 (70%) of them at the presentation. Fulminant hepatitis with coma of grade >II and prothrombin time <40% developed within 8 weeks after the onset in 45 (9%) of them.

Fulminant hepatitis led to death in 18 (5%) patients, and 3 (1%) received liver transplantation. Exclusive of these 21 patients, HBV persisted in only 5 of the 464 (1%) studied patients with acute hepatitis. They represented 3 of the 92 (3%) infected with HBV/A, 1 of the 58 (2%) with B and 1 of the 330 (0.3%) with C. None of the five patients with chronic outcome had received antiviral or steroid treatment during their acute phase of illness.

3.2. Geographic distribution of HBV genotypes in patients with acute hepatitis B

Overall, HBV/A was detected in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Distribution of HBV genotypes over Japan is illustrated in Fig. 1. They dispersed unevenly in place. HBV/C accounted for more than 68% in both northern and southern areas, contrasting with HBV/A accounting for 34% in and around the Metropolitan area. Among HBV/B infections, the proportion of HBV/Ba was higher in Koshin, Kinki and Kyushu/Okinawa (14/17, 1/1 and 2/3), while that of HBV/Bj was higher in Hokkaido, Tohoku, Kanto and Tokai (4/4, 6/7, 13/21 and 5/5).

3.3. Demographic and clinical differences of patients infected with various genotypes

Table 1 compares demographic and clinical characteristics of patients with different HBV genotypes. Patients with HBV/D were excluded from the analysis due to their small numbers. The mean age was lower in patients with HBV/A (31.8 ± 10.9 years) than HBV/Ba (40.7 ± 10.9, $p < 0.01$), HBV/Bj (41.2 ± 17.0, $p = 0.01$) and HBV/C (35.8 ± 13.9, $p < 0.03$); it was higher in patients with HBV/Ba than HBV/C (40.7 ± 10.9 versus 35.8 ± 13.9, $p = 0.02$). The proportion of patients aged <30 years was significantly greater in HBV/A (58%) than HBV/Ba (12%, $p < 0.01$), HBV/Bj (38%, $p < 0.04$) or HBV/C infection (42%, $p < 0.01$). Men predominated ($p < 0.01$) in infections with HBV/A and HBV/Ba (92% and 88%, respectively) than those with HBV/Bj and HBV/C (56% and 64%, respectively). The peak ALT level was higher in HBV/Bj (3371 ± 2342 IU/L) than HBV/A (2051 ± 1009, $p = 0.04$) or HBV/C (2650 ± 1747, $p < 0.03$) infection. HBeAg was detected in 84% of patients with HBV/A at the frequency much higher than that in those with HBV/Ba (54%, $p < 0.01$), HBV/Bj (59%, $p < 0.01$) or HBV/C (60%, $p < 0.01$).

The routes of transmission were sexual contacts in 216 (45%) patients, followed by medical accidents in 14 (8%), blood transfusion in 4 (1%) and drug in 1 (0.2%); transmission routes were not identified in the remaining 245 (51%) patients. Sexual transmission was the most frequent cause of infection in 57% of HBV/A, 73% of HBV/Ba, 34% of HBV/Bj and 40% of HBV/C infections.

Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (41%) than the other genotypes ($p < 0.01$); it occurred in 2 of the 5 (40%) patients with HBV/D, also. In reflection of severe clinical courses, the peak ALT level tended to be high in patients with HBV/Bj.

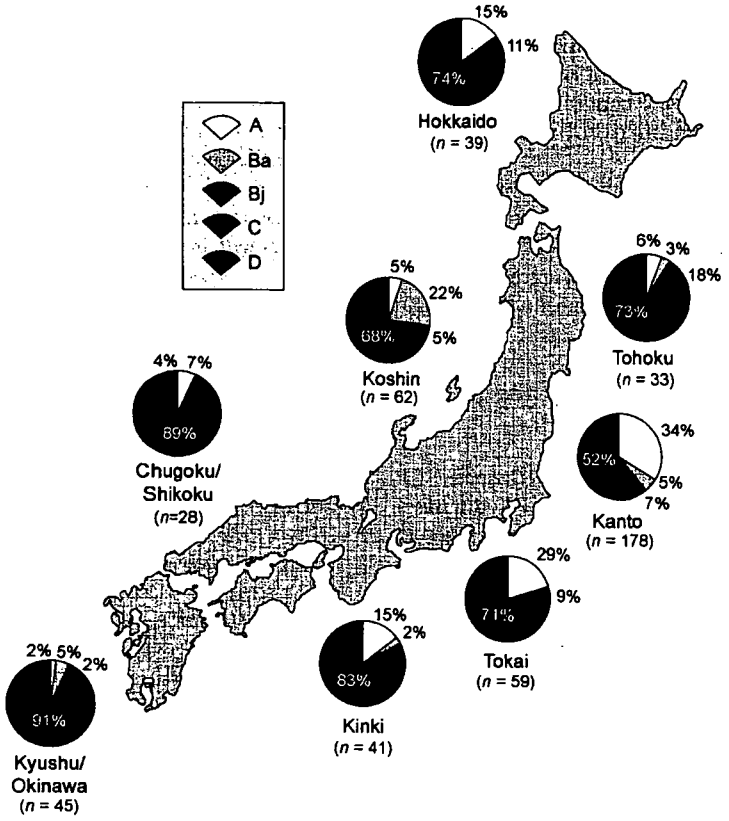

Fig. 1. Geographic distribution of HBV genotypes among 485 patients with acute hepatitis in Japan during 1982–2005.

The core-promoter double mutation (A1762T/G1764A) was more frequent in patients with fulminant than acute self-limited hepatitis infected either with HBV/Bj (70% versus 0%, $p<0.01$) or HBV/C (50% versus 18%, $p<0.01$). Also, precore stop-codon mutation (G1896A) was more offen in patients with fulminant than acute self-limited hepatitis who were infected with HBV/Ba (100% versus 5%, $p=0.01$), HBV/Bj (60% versus 0%, $p<0.01$) or HBV/C (50% versus 14%, $p<0.01$).

3.4. Changes in the distribution of HBV genotype with the age and time

Fig. 2 depicts the distribution of HBV genotypes stratified by the age. Prevalence of HBV/A decreased with the age, and was higher in the patients aged <30 years (26%) than in the 40s (14%, $p=0.03$) or aged ≥50 years (8%, $p<0.01$). Prevalence of HBV/B increased with the age, in converse, and was lower in the patients aged <30 years (7%) than in the 40s (18%, $p=0.01$) or aged ≥50 years (17%, $p=0.02$). No significant differences were observed in the distribution of HBV/Ba or HBV/Bj among all age groups.

Fig. 3 illustrates changes in HBV genotypes through the four time spans covering 24 years. HBV/A accounted for 5% (2/42) in 1982–1990, 5% (4/78) in 1991–1995 and 14% (18/133) in 1996–2000, and thereafter increased to 33% (58/176) in 2001–2005. There was significant difference between 1982–1995 and 1996–2005 ($p<0.01$). HBV/B accounted for 26% (11/42) in 1982–1990, 17% (13/78) in

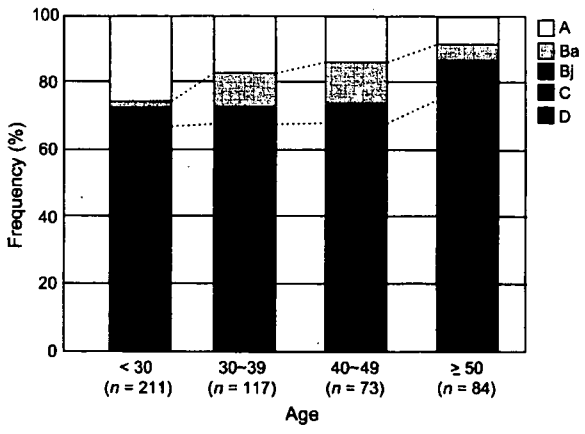

Fig. 2. Age-specific prevalence of HBV genotypes among 485 patients with acute hepatitis.

Table 1
Demographic and clinical differences among patients infected with HBV of distinct genotypes

	Genotypes			
	A ($n=92$)	Ba ($n=26$)	Bj ($n=32$)	C ($n=330$)
Mean age (years)	31.8 ± 10.9[a]	40.7 ± 10.9[b]	41.2 ± 17.0	35.8 ± 13.9
Age <30 years	54 (58%)[c]	3 (12%)[d]	12 (38%)	140 (42%)
Male	85 (92%)[e]	23 (88%)[f]	18 (56%)	210 (64%)
Peak ALT (IU/L)	2051 ± 1009[g]	2536 ± 1104	3371 ± 2342[h]	2650 ± 1747
Peak total				
Bilirubin (mg/dL)	10.3 ± 10.4	8.9 ± 5.8	10.5 ± 8.1	9.3 ± 9.7
HBeAg positive	78 (84%)[i]	14 (54%)	19 (59%)	199 (60%)
Transmission routes				
Sexual	52 (57%)	19 (73%)	11 (34%)	134 (40%)
Medical accident	0	0	3 (9%)	11 (3%)
Drug/tattoo	0	0	0	1 (1%)
Blood transfusion	0	0	1 (4%)	3 (1%)
Unknown	40 (43%)	7 (23%)	17 (53%)	181 (55%)
Metropolitan areas	60 (65%)[j]	8 (31%)	13 (41%)	93 (28%)
Fulminant hepatitis	1 (1%)	2 (8%)	13 (41%)[j]	29 (9%)
Mutations in HBV DNA				
BCP (1762T/1764A)				
Acute self-limited	2/67 (3%)	4/22 (18%)	0/13 (0%)[k]	41/223 (18%)[k]
Fulminant	0/1 (0%)	0/2 (0%)	7/10 (70%)	10/20 (50%)
Precore (1896A)				
Acute self-limited	1/67 (1%)	1/22 (5%)[k]	0/13 (0%)[l]	31/223 (14%)[k]
Fulminant	1/1 (100%)	2/2 (100%)	6/10 (60%)	10/20 (50%)

$p<0.01$, acute vs. fulminant.
[a] $p<0.01$, A vs. Ba. $p=0.01$, A vs. Bj. $p=0.03$, A vs. C.
[b] $p=0.02$, Ba vs. C.
[c] $p<0.01$, A vs. Ba. $p<0.04$, A vs. Bj. $p<0.01$, A vs. C.
[d] $p<0.01$, Ba vs. C. $p<0.04$, A vs. Bj. $p<0.01$, A vs. C.
[e] $p<0.001$, A vs. Bj. $p<0.01$, A vs. C.
[f] $p<0.01$, Ba vs. Bj. $p<0.01$, Ba vs. C.
[g] $p=0.04$, A vs. Ba. $p<0.01$, A vs. B1. $p<0.01$, A vs. C.
[h] $p=0.03$, Bj vs. C.
[i] $p<0.01$, A vs. Ba, Bj or C.
[j] $p<0.01$, Bj vs. A, Ba or C.
[k] $p<0.01$, acute vs. fulminant.
[l] $p=0.01$, acute vs. fulminant.

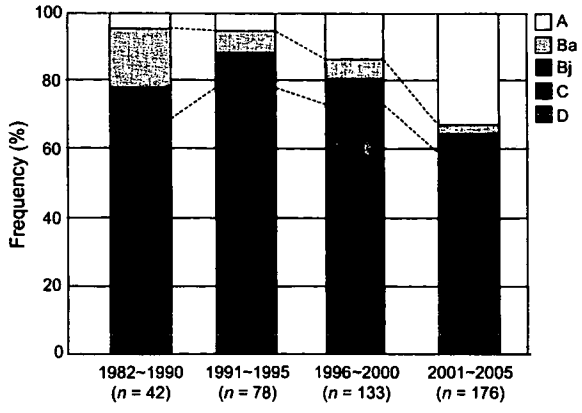

Fig. 3. Chronological changes in the distribution of HBV genotypes among 429 patients with acute hepatitis.

1991–1995 and 14% (18/133) in 1996–2000, and thereafter decreased to 8% (14/176) in 2001–2005; there was significant difference between 1982–1990 and 2001–2005 ($p<0.01$). However, no significant differences were observed in the distribution of HBV/Ba or HBV/Bj among the four time spans.

4. Discussion

As in most Asian countries, the persistent HBV carrier state had been established mainly through perinatal transmission and horizontal infection during the infancy in Japan, until 1986 when combined active and passive immunoprophylaxis was started in the national program. There still are approximately million HBV carriers estimated by the prevalence of HBsAg in the first-time blood donors [28]; they had been infected with HBV before the prophylaxis started. The age-specific prevalence is high in the elderly, and some of them develop chronic liver disease culminating in cirrhosis and hepatocellular carcinoma. By far the majority of genotypes in

patients with persistent HBV infection are HBV/B (approximately 30%) and HBV/C (about 70%) [4,29]. In patients with acute hepatitis in hospitals in the Metropolitan Tokyo, however, HBV/A was detected in 23% with HBV/Ae prevailing [5], in contrast to HBV/B in 14% and HBV/C in 44% [4]. It is not certain if a high prevalence of HBV/A in acute HBV infection in Tokyo is extrapolated to the other areas in Japan.

The present study was performed on 547 patients with acute hepatitis B visiting 25 hospitals in Japan during 24 years from 1982 to 2005. They include the 147 (27%) patients reported by Yotsunangi et al. [7] and the 336 (61%) by Ozasa et al. [22]; the remaining 64 (12%) patients were recruited anew. Yotsuyanagi et al. [7] dealt with a peculiar distribution of HBV/A clustering in and around Metropolitan areas and clinical manifestation dependent on genotypes, while Ozasa et al. [22] focused on the influence of HBV genotypes on clinical outcomes, in terms of fulminant hepatitis and viral persistence, as well as the occurrence of precore stop-codon mutation. The assortment of these cohorts of patients have given us the power to precisely picture the epidemiology of genotypes in acute HBV infection all over Japan and shift thereof during the past quarter of century. In addition, it confirmed the results of previous studies on the precore mutations, and extended them to the core-promoter double mutation that can make differences in fulminant or self-limited outcome of acute HBV infection under the influence of HBV genotypes. In the present multicenter study on patients with acute hepatitis B, HBV/C was the most frequent and HBV/A was the second most common in central areas in Japan harboring big cities (Fig. 1). Along with HBV/A, the other foreign genotypes (Ba and D) accounted for 123 of the 485 (25%) acute HBV infections. Furthermore, foreign origins of acute HBV infections would increase further, if overseas origins of HBV/C (Ce and Cs) are examined in the future.

Clinical relevance of HBV genotypes, in terms of severity of liver disease and response to antiviral treatment, has been evaluated mostly in patients with chronic liver disease [27,30,31]. Due to uneven distributions of HBV genotypes over the world, however, comparison is largely restricted to only two genotypes prevailing in each country. All in all, it would be reasonable to state that patients with HBV/A or HBV/B fare better than those with HBV/D or HBV/C [21,27,30–32]. Influence of HBV genotypes on acute hepatitis B is also reported [33]. In the present study, in which patients with acute hepatitis infected with HBV/A, HBV/Ba, HBV/Bj or HBV/C were compared, there were significant differences in age, gender, peak ALT levels, HBeAg positivity and outcome (Table 1). These results indicate that HBV genotypes influence the clinical profile in not only chronic, but also acute hepatitis B.

Fulminant hepatitis developed rather frequently in 45 of the 485 (9%) patients, although it would be overrepresented in them who visited hospitals due to overt liver disease. Remarkably, chances for patients infected with HBV/Bj to develop fulminant hepatitis were significantly higher than those with HBVA, HBV/Ba or HBV/C (41% versus 1%, 8% or 9%, $p < 0.01$). These results were consistent with those in the previous study [22]. In patients with acute hepatitis B from Chiba in Japan [34], HBV/B was more frequent in those with fulminant than self-limited hepatitis (63% versus 31%, $p = 0.027$); however, some cases of acute exacerbation of persistent HBV infection were included in their study.

The precore stop-codon mutation (G1896A) was detected significantly more frequently in patients with fulminant than acute self-limited hepatitis, in confirmation of our previous report [22]. Furthermore, the BCP double mutation (A1762T/G1764A) was detected more often in fluminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of BCP mutation was much higher in the patients with fulminant (70%) than that reported in those with chronic hepatitis (16%) [27]. Precore and BCP mutations are very frequent in patients with fulminant hepatitis in Asia [35–37] and the Middle East [38], but rare in Western countries [39–41]. These differences may be explained by distinct geographical distributions of HBV genotypes.

An extremely wide range (0–12%) has been reported in the rate of persistence after acute HBV infection in the adulthood [42–49]. Why such a big difference arises needs to be clarified, because the HBV genotype is implicated in a high chronicity rate in European countries where HBV/A is predominant. In accordance with this view, HBV/A was more frequent than HBV/D in 32 patients with chronic active hepatitis (80% versus 11%), while the reverse was the case in acute hepatitis (10% versus 80%) in Switzerland [33]. In Japan, also, infection with HBV/A tends to prolong longer than that with HBV/B or HBV/C in the adulthood (23% [3/13] versus 13% [1/8] or 12% [3/25]) [4]. In the present series on 464 patients with acute hepatitis B in Japan, for whom genotyping was feasible, HBV infection persisted in only 5 (1%) representing 3 of the 92 (3%) with HBV/A, 1 of the 58 (2%) with HBV/B and 1 of the 330 (0.3%) with HBV/C. Hence the infection with HBV/A would persist longer than those with the other genotypes.

The present study highlighted the recent rapid increase in cases of acute hepatitis B in Japan, which gained about five-fold from 4.7 per year before 1990 to 33.1 after 2000 in participant hospitals. Primary HBV infection in the adulthood can occur by homo/heterosexual contacts, intravenous drug use, medical accidents and blood transfusion [50–54]. Acute HBV infection in the 485 patients studied was transmitted by sexual contacts in 216 (45%), medical accidents in 14 (3% (none since 2001)) and blood transfusion in 4 (1% (none since 2001)). The route is unknown for the remaining 245 (51%) patients; some of them might have been infected by undeclared sexual contacts and/or intravenous drug use, although recreational drugs are still uncommon in Japan. It comes as a surprise that the overall proportion of foreign genotypes (A, Ba and D) in patients with acute hepatitis B was high at 25%. Of these foreign genotypes, Asian genotypes such as HBV/Ba have decreased gradually, while HBV/A has increased in converse (Fig. 3). Furthermore,

some of "domestic" HBV/C infections that accounted for the majority (68%) may well have been imported by sexual workers from foreign countries where this genotype is prevalent. Combined, a substantial part of acute HBV infection in Japan does seem to have been imported.

In conclusion, the distribution of HBV genotypes in patients with acute hepatitis B is different geographically, and has changed with time in Japan. Recently, acute HBV infection is increasing among the Japanese adults, although it rarely becomes chronic. A significant part of it is transmitted sexually with foreign HBV genotypes. Furthermore, a fulminant outcome is frequent after infection with HBV/Bj having precore and/or BCP mutations. These facts should be made open to the public for making them aware of the risk and taking measures to prevent it.

Acknowledgments

This work was supported in part by a grant-in-aid from the Miyakawa Memorial Foundation and the Ministry of Health, Labour and Welfare of Japan.

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

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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.

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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.

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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, $P = .032$).

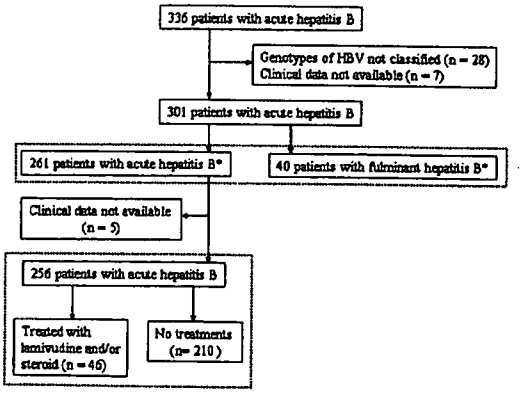

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 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 = 187)[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.

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.

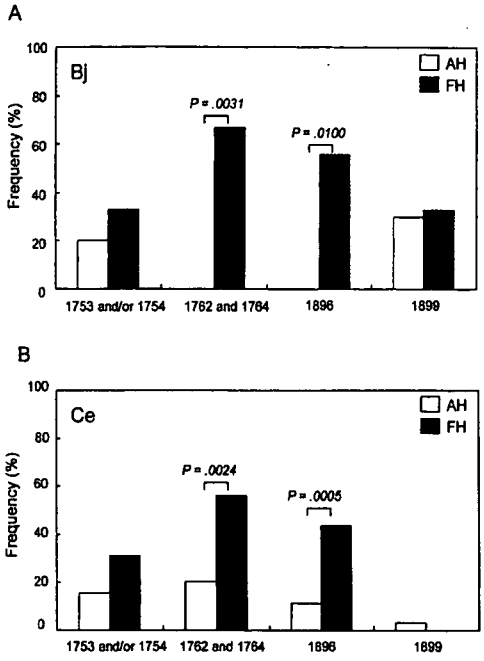

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

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

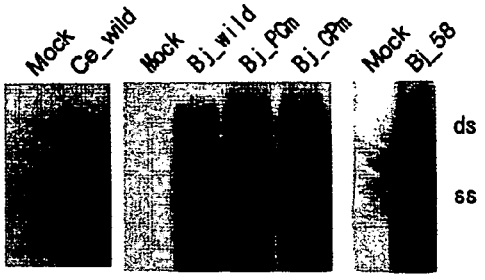

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

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.

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Liver International 2006: 26: 90–96

Liver International
DOI: 10.1111/j.1478-3231.2005.01200.x

Clinical Studies

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

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

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

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

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Received 19 June 2005,
accepted 8 August 2005

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

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

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

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

Serological markers for HBV

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

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

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

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

Statistical analysis

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

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

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

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

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

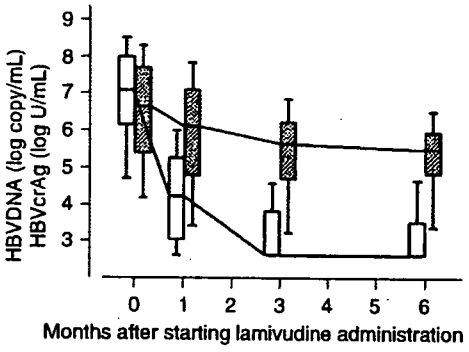

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The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

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

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

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

Discussion

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

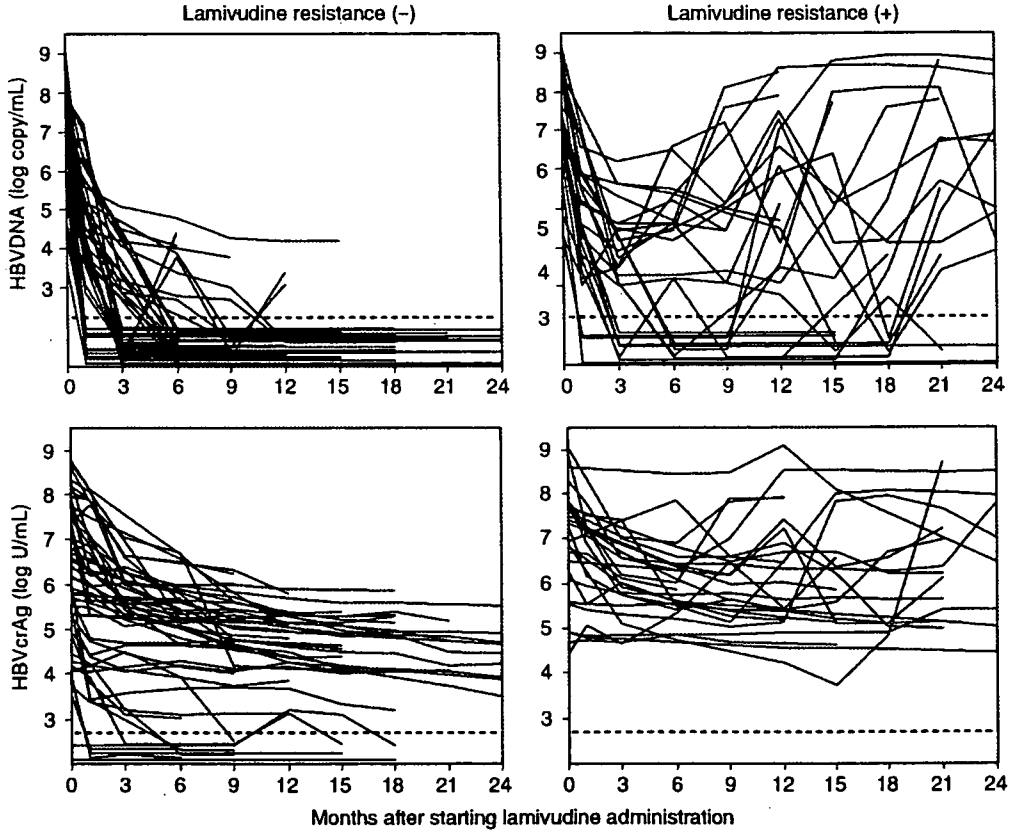

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.