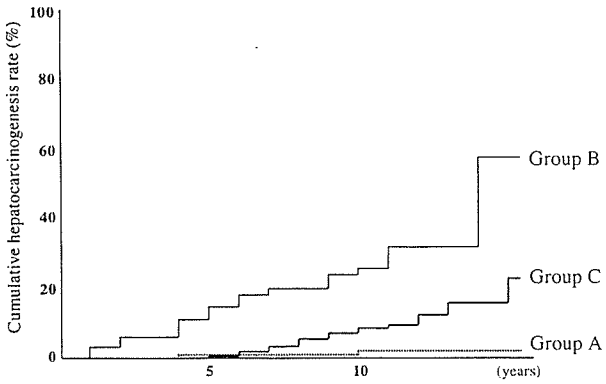

Figure 1. Cumulative hepatocarcinogenesis rates in the three study groups. The cumulative hepatocarcinogenesis rates were significantly different among the three groups ($p < 0.0001$; log-rank test). In particular, the rate in Group B was significantly higher than that in Group C ($p < 0.0001$; log-rank test) and that in Group A ($p < 0.0001$; log-rank test), and the rate in Group C was significantly higher than that in Group A ($p = 0.0030$; log-rank test). See text for the definition of the three groups.

related causes in Groups A, B, and C, respectively. All liver-related deaths were caused by liver cancer, and none died of hepatic failure without liver cancer. In Groups A, B, and C, the cumulative survival rates for liver-related deaths were 100, 96.6, and 100% at the end of 5 years; and 100, 83.7, and 97.8% at the end of 10 years, respectively (Figure 2). The rates were also significantly different among the three groups ($p < 0.0001$; log-rank test). In particular, the rate in Group B was significantly lower than that in Group C ($p = 0.0011$; log-rank test) and that in Group A ($p < 0.0001$; log-rank test), and the rate in Group C was significantly lower than that in Group A ($p = 0.0420$; log-rank test).

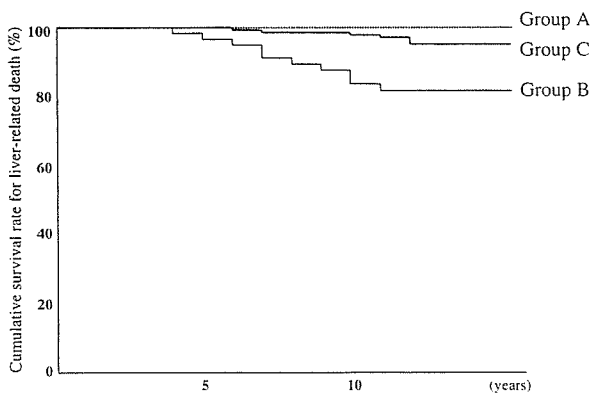

Figure 2. Cumulative survival rates for liver-related deaths. The cumulative survival rates for liver-related deaths were significantly different among the three groups ($p < 0.0001$; log-rank test). In particular, the rate in Group B was significantly lower than that in Group C ($p = 0.0011$; log-rank test) and that in Group A ($p < 0.0001$; log-rank test), and the rate in Group C was significantly lower than that in Group A ($p = 0.0420$; log-rank test).

Predictive factors associated with hepatocarcinogenesis by multivariate analysis

We then analyzed the data for the whole population sample to determine those factors that could predict hepatocarcinogenesis. Univariate analysis identified five parameters that tended to correlate, or significantly correlated with carcinogenesis. These included age ($p < 0.0001$), fibrosis stage ($p < 0.0001$), platelet count ($p < 0.0001$), total IFN dose ($p = 0.0691$), and Group ($p < 0.0001$). These factors were entered into a multivariate analysis, which then identified three parameters that independently influenced carcinogenesis; Group ($p = 0.008$), fibrosis stage ($p = 0.001$), and age ($p = 0.023$) (Table II).

Predictive factors associated with hepatocarcinogenesis in Group C by multivariate analysis

We also analyzed the data of 172 Group C patients to determine those factors that could predict hepatocarcinogenesis. Nine potential predictive factors associated with hepatocarcinogenesis were evaluated, excluding group of treatment. Three parameters were identified by univariate analysis that significantly correlated with hepatocarcinogenesis. These included age ($p = 0.0087$), fibrosis stage ($p < 0.0001$), and platelet count ($p = 0.0044$). All three factors were entered into a multivariate analysis, which in turn identified two parameters that independently influenced hepatocarcinogenesis, including fibrosis stage ($p < 0.0001$), and age ($p = 0.015$) (Table III).

Hepatocarcinogenesis rates according to fluctuation of ALAT levels at IFN-free period in Group C

In Group C, the hepatocarcinogenesis rates were also evaluated according to the mean ALAT levels at the IFN-free period. For this purpose, we selected 110 consecutive patients (64.0%) of Group C in whom ALAT levels were closely monitored. Thirty out of 110 patients (27.3%) achieved SR after multicourses of IFN monotherapy (SR group), and the

Table II. Factors associated with hepatocarcinogenesis in 454 patients, identified by multivariate analysis.

Factors	[Category]	Odds ratio (95% CI)	p-value
Fibrosis stage	1: F1, F2	1	
	2: F3	5.86 (2.18–15.7)	0.001
Group	1: A, C	1	
	2: B	3.17 (1.39–7.23)	0.008
Age (year)	1: <50	1	
	2: ≥50	2.63 (1.10–6.28)	0.023

Cox proportional hazards model.

Table III. Factors associated with hepatocarcinogenesis in 172 patients of Group C, identified by multivariate analysis.

Factors	[Category]	Odds ratio (95% CI)	*p*-value
Fibrosis stage	1: F1, F2	1	
	2: F3	10.9 (3.65–32.7)	<0.0001
Age (years)	1: <50	1	
	2: ≥50	3.87 (1.26–11.9)	0.015

Cox proportional hazards model.

remaining 80 patients (72.7%) could not attain an SR (NSR group).

Overall, the hepatocarcinogenesis rate in patients with ALAT levels below 1.5 × the upper limit of normal (<75 IU/l, normal for ALAT, 6–50 IU/l) was 0% (0 of 36 patients). The rates in those with ALAT from 1.5 × to 2 × (75–100 IU/l), from 2 × to 4 × (100–200 IU/l), and above 4 × (>200 IU/l) of the upper limit of normal were 23.8% (5 of 21 patients), 13.0% (6 of 46), and 28.6% (2 of 7), respectively (Table IV). In the NSR group, the hepatocarcinogenesis rate in patients with ALAT levels below 1.5 ×, from 1.5 × to 2 ×, from 2 × to 4 ×, and above 4 × were 0% (0 of 17 patients), 27.8% (5 of 18), 15.4% (6 of 39), and 33.3% (2 of 6), respectively. In the SR group, none of the patients developed HCC.

In conclusion, overall, the hepatocarcinogenesis rates in those patients with ALAT levels above 1.5 × (17.6%) the upper limit of normal were significantly higher than in those below 1.5 × (0%) of the upper limit of normal ($p = 0.005$). In the NSR group, the hepatocarcinogenesis rates in those patients with ALAT levels above 1.5 × (20.6%) tended to be higher than in those with below 1.5 × (0%) ($p = 0.059$).

Discussion

HCC is currently considered a very common malignancy and its incidence is increasing, in both Japan and the USA. Persistent HCV infection is a major risk factor for the development of HCC. About 80% of Japanese HCC patients are also diagnosed with HCV-associated cirrhosis or chronic hepatitis C [33]. Thus, HCV-related chronic liver disease is one of the major disorders affecting the national health of Japan, and prevention of HCC and improvement of survival remain the important issues in treatment for HCV-related chronic liver disease.

Several studies based on the single-course IFN monotherapy showed that IFN therapy reduced the risk of development of HCC and liver-related death in comparison with untreated patients and especially in responders to the treatment [1,8–12]. Furthermore, a recent report demonstrated that re-treatment with IFN at certain intervals also reduced the incidence of HCC in patients with chronic hepatitis C, even if eradication of HCV was not achieved by re-treatment [34]. Apart from defining the suppressive action of the multi-course IFN treatment on HCC, the same study, however, indicated that the long-term benefits of such treatment were still inadequately defined, probably because the survival rate and the incidence of hepatocarcinogenesis based on fluctuation of ALAT at the IFN-free period were not analyzed. Hence, in our study, we evaluated 454 consecutive naive cases with chronic hepatitis C, in whom more than 10 years had elapsed since the induction of IFN monotherapy at our hospital (i.e. a longer follow-up period than that in the previous report [34]), to examine the influence of multi-course IFN on hepatocarcinogenesis and survival.

Our group previously reported that the cumulative hepatocellular carcinogenesis rates in HCV patients of untreated and SR groups for IFN monotherapy were 4.8 and 1.4% at the end of 5 years, and 12.4% and 1.4% at the end of 10 years, respectively [10]. In this study, the hepatocarcinogenesis rates were low in the SR group who received a single-course IFN (Group A), which were 1.1 and 2.2% at the end of 5 and 10 years, respectively. However, the hepatocarcinogenesis rates in patients of Group B who received a single-course IFN but did not show SR (15.0 and 26.0% at the end of 5 and 10 years, respectively) were higher than those of untreated patients described in our previous study [10]. This discrepancy between our results and the previous report may be due to differences in patients' backgrounds, as patients of the present study had higher ALAT levels and more progressive stages of fibrosis, which are known risk factors for

Table IV. Hepatocarcinogenesis rates according to the fluctuation of ALAT levels at the IFN-free period in Group C.

	ALAT levels (IU/l)[a]			
	<75	75–100	100–200	>200
Hepatocarcinogenesis rate (%) (Number)	0% (0/36)	23.8% (5/21)	13.0% (6/46)	28.6% (2/7)

[a]Normal level of ALAT, 6–50 IU/l.

Abbreviations: ALAT = alanine aminotransferase; IFN = interferon.

HCC [11,33,35,36], than those of the previous study.

With regard to multi-course IFN, Hino et al. [34] recently reported that re-treatment reduced the incidence of HCC, although the follow-up duration of their population sample was less than 10 years. Our study, based on a longer follow-up period of 10 years or more, also indicated that hepatocarcinogenesis rate in the multi-course IFN (Group C) was lower than that in the non-SR group after a single course of IFN (Group B). Considered together, we conclude that the hepatocarcinogenesis rates in Groups A and C were significantly lower than those in Group B, indicating that HCV-RNA eradication at an early stage, or multi-course IFN irrespective of SR are important for the suppression of hepatocarcinogenesis. To our knowledge, our study is the first to report the hepatocarcinogenesis rates for a follow-up period of 10 years or more in multi-course IFN.

In a previous study from our laboratory, we reported that the cancer-suppressive activity of single-course IFN monotherapy in patients with HCV-RNA eradication was similar to that in patients with ALAT normalization without HCV-RNA elimination [10]. Other studies also indicated a higher incidence and more rapid development of HCC in HCV patients with high ALAT levels [35,36]. Collectively, these results suggest that the carcinogenic process in patients with chronic hepatitis C is enhanced by high levels and fluctuations of ALAT, and indicate a close relationship between suppression of inflammatory necrosis of hepatocytes and lower incidence of HCC in patients with HCV-associated chronic liver disease.

Our results of multi-course IFN therapy are the first to show a 0% hepatocarcinogenesis rate in patients with ALAT levels below 75 IU/l at the IFN-free periods, emphasizing the importance of keeping low ALAT levels at such periods with respect to suppression of hepatocarcinogenesis. Thus, patients who fail to achieve SR after single-course IFN should receive multi-course IFN at the time of ALAT relapse, at certain intervals. Based on our results and those of Okanoue et al. [37], who demonstrated an increased incidence of HCC in 5 years or more after IFN therapy in transient biochemical responders, it is important to normalize ALAT levels by multi-course IFN at certain intervals.

Previous studies have shown that gender, age, fibrosis stage, and IFN regimen are important pretreatment predictors of hepatocarcinogenesis [10,11,34]. In the present study, higher age and a more progressive fibrosis stage were associated with higher hepatocarcinogenesis rates in the whole population sample and in Group C. Furthermore, our analysis of IFN treatment-related factors showed that Group B (non-SR after single-course IFN) was also a risk factor for hepatocarcinogenesis. This result is almost similar to that of a previous study, which identified the number of courses of IFN as a risk factor for HCC [34].

Previous studies indicated that the liver-related overall mortality rates of HCV patients were 57–94%, emphasizing the need for improvement in survival of patients with chronic hepatitis C with or without cirrhosis [1,10,12,18–21,38]. Our results also indicated that the proportion of liver-related overall deaths was 69.6% (16 of 23 patients). Our study is the first to report the survival rates for overall death and liver-related death in multi-course IFN therapy (Group C). The results indicated that overall and liver-related deaths in Groups A and C were significantly lower than those in Group B, suggesting that HCV-RNA eradication at an early stage or multi-course IFN irrespective of SR is also important for reducing liver-related deaths, like the suppression of hepatocarcinogenesis.

The aims of IFN therapy for chronic hepatitis C include the reduction of the risk of development of HCC and liver-related death by viral clearance, and when viral clearance could not be achieved, then by ALAT normalization without viral clearance. The reported post-IFN monotherapy viral clearance rate for genotype 1b (the most major type in Japan) is as low as 10 to 20% [24,26,27]. The antiviral efficacy of IFN has improved recently following combination with ribavirin [39–41] and the use of pegylated IFN [42–45], but these modifications also could not achieve a sufficient SR (SR is still $<50\%$ in patients with genotype 1 with higher pretreatment viral loads). Furthermore, the combination therapy is sometimes associated with serious adverse effects such as hemolytic anemia [46]. Hence, the development of more effective and safe IFN regimens is needed.

In conclusion, our retrospective study indicates that multi-course IFN monotherapy reduces the risk of hepatocarcinogenesis and increases survival. Large-scale prospective studies should be conducted in the future to confirm this finding.

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Persistence of Acute Infection With Hepatitis B Virus Genotype A and Treatment in Japan

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Among the 97 adult patients with acute hepatitis B who were admitted to the Toranomon Hospital in Metropolitan Tokyo during 28 years from 1976 to 2003, 31 (32%) were infected with hepatitis B virus (HBV) genotype A, nine (9%) with genotype B, 44 (45%) with genotype C, one (1%) each with genotypes E and F. HBV in the remaining 11 (11%) patients were untypeable. All the 31 patients with acute hepatitis B caused by HBV genotype A infection were male with a median age of 31 years, and 16 (52%) contracted infection through extramarital sexual contacts. The baseline HBV DNA level was higher in the seven (23%) patients in whom infection with HBV genotype A persisted than the remaining 24 (77%) with spontaneous resolution (median: >8.7 vs. 6.0 log genome equivalents/ml, $P=0.004$). Persistent infection was more frequent in patients with maximum alanine aminotransferase <500 IU/L than ≥500 IU/L (83% [5/6] vs. 4% [1/25], $P=0.0001$). Of the six patients with persistent HBV genotype A infection who received interferon and/or lamivuidine for treatment of chronic active hepatitis, three (50%) responded with the loss of hepatitis B e antigen (HBeAg); hepatitis B surface antigen (HBsAg) was cleared from serum in one patient who received interferon and lamivudine in sequence. HBV genotype A persisted along with HBeAg in the remaining three patients given antiviral therapy as well as another who was not treated. In conclusion, infection with HBV genotype A prevails in patients with acute hepatitis B in Japan where genotypes B and C are common, is often contracted sexually (16/31 [52%]) and tends to persist (7/31 [23%]). Infection was cleared in only one of the six (17%) patients who received antiviral therapy. ***J. Med. Virol. 76:33–39, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; chronic hepatitis; genotypes; hepatitis B virus; interferon; lamivudine

INTRODUCTION

Approximately 350 million people are infected persistently with hepatitis B virus (HBV) throughout the world [Lee, 1997], and most reside in Asia and Africa. There are eight genotypes of HBV, defined by a sequence divergence in the entire genome exceeding 8%, and are designated by capital alphabet letters from A to H in the order of discovery [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Genotypes of HBV have distinct geographical distributions [Magnius and Norder, 1995; Lindh et al., 1997; Miyakawa and Mizokami, 2003], and are associated with the severity of liver disease and responses to antiviral treatment [Chu and Lok, 2002; Kao, 2002; Miyakawa and Mizokami, 2003].

In countries highly endemic for HBV, such as China and Africa, the carrier state is established mainly through horizontal transmission during infancy [Botha et al., 1984; Yao, 1996]. In Europe and the United States where the prevalence of HBV is low, by contrast, persistent infection occurs predominantly by infection in

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adults. Japan is exceptional in that perinatal transmission from infected mothers had been the main route of persistent HBV infection [Okada et al., 1976], before the national immunoprophylaxis program for babies born to carrier mothers was launched in 1986 [Noto et al., 2003].

In Japan, genotypes B and C prevail and together they account for more than 95% of persistent HBV infection [Orito et al., 2001; Kobayashi et al., 2002, 2003]. Acute infection with HBV genotype A keeps increasing, however, particularly in men with extramarital sexual contacts [Kobayashi et al., 2002, 2003, 2004; Ogawa et al., 2002]. Infection with HBV genotype A in the adult has a high propensity to become chronic, which would contribute to the persistent carrier state in Western countries where the perinatal or childhood transmission of HBV is rare [Heijtink et al., 1999; Lindh et al., 2000].

During 28 years from 1976 to 2003, 31 patients were diagnosed with acute hepatitis B who were infected with HBV genotype A at the Toranomon Hospital in the Metropolitan Tokyo. The infection persisted in seven (23%) patients accompanied by biopsy-proven chronic hepatitis B. Their clinical course were followed with a special reference to the response to antiviral treatment.

MATERIALS AND METHODS

Patients With Acute Hepatitis B

During 28 years from August 1976 through September 2003, 97 patients were diagnosed with acute hepatitis B at the Department of Gastroenterology at the Toranomon Hospital in Metropolitan Tokyo. Genotypes of HBV were A in 31 (32%) of them, B in nine (9%), C in 44 (45%), E and F in one (1%) each, while they were untypeable in the remaining 11 (11%). The 31 patients with acute hepatitis B caused by infection with HBV genotype A were followed clinically and examined virologically. Seven of them (23%) developed persistent HBV infection accompanied by chronic hepatitis B, and six received antiviral therapy and evaluated for the response. All the patients possessed IgM antibody to hepatitis B core (anti-HBc) in high titers, but they were negative for antibody to hepatitis delta virus, IgM antibody against hepatitis A virus or antibody to hepatitis C virus. A number of these patients have been reported previously with respect to clinical features [Kobayashi et al., 2003], perpetuation of acute infection [Kobayashi et al., 2002] and shifts of HBV genotypes with time [Kobayashi et al., 2004]. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committee of Toranomon Hospital. An informed consent for this study was obtained from each patient.

Serological Markers of HBV Infection

Hepatitis B surface antigen (HBsAg) was determined by hemagglutination (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan) and hepatitis B e antigen (HBeAg) by enzyme-linked immunosorbent assay (ELISA) with commercial kits (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). Anti-HBc of IgM class was determined by radioimmunoassay (HBc-antiM RIA, Dinabot, IL). HBV DNA was determined by transcription-mediated amplification and hybridization assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed as log genome equivalents (LGE) per milliliter of serum, over a detection range from 3.7 to 8.6 LGE/ml.

Genotypes of HBV

The six major genotypes (A–F) were determined by ELISA by the combination of epitopes on preS2-region products by monoclonal antibodies which is specific for each of them [Usuda et al., 1999, 2000] by commercial assay kits (HBV GENOTYPE EIA; Institute of Immunology, Co., Ltd., Tokyo, Japan). Genotype G was determined by the preS2 serotype for genotype D and HBsAg serotype adw [Kato et al., 2001]; the combination is specific for this genotype.

Genetic Subgroups of Genotype A

Subgroups of genotype A designated Ae prevalent in Europe and Aa frequent in Africa, as well as Asia [Sugauchi et al., 2004] that correspond to subgroup A′ reported originally by Bowyer et al. [1997], were determined by the nucleotide (nt) sequence in the S gene specific for each of them [Sugauchi et al., 2003]. Briefly, nucleic acids were extracted from serum and a sequence of the large S gene was amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was carried out with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2,757–2,776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1,050–1,079]) primers, and the second-round PCR with PLF5Bm (sense, 5′-TGT GGA TCC TGC ACC GAA CAT GGA GAA-3′ [nt 136–162]) and BR112 (antisense, 5′-TTC CGT CGA CAT ATC CCA TGA AGT TAA GGG A-3′ [nt 865–895]) as well as BGF5 (sense, 5′-TGC GGG TCA CCA TAT TCT TG-3′ [nt 2,811–2,830]) and BGR6 (antisense, 5′-AGA AGT CCA CCA CGA GTC TA-3′ [nt 249–268]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, CA). The they were purified by Qiquick PC purification kit (Qiagen, Hilden, Germany), and sequenced in AGI Prism 310 Genetic Analyzer (Applied Biosystems).

Antiviral and Other Treatment

Patients in whom HBV infection persisted and with chronic hepatitis diagnosed by liver biopsy, received interferon (IFN) or lamivudine, or both in sequence. Natural IFN-α (Smiferon; Sumitomo Pharmaceutical Co., Ltd., Tokyo, Japan) or IFN-β (Feron, Toray Co., Ltd., Tokyo, Japan) in a dose of 3 or 6 mega units (MU) was injected subcutaneously two or three times in week (tiw) for up to 1 year with or without induction by 6 MU

daily for 8 weeks. Lamivudine (Glaxo-Welcome, Greenford, UK) was given orally at a daily dose of 100 mg until HBeAg was lost from serum in responders, and continued indefinitely in non-responders who failed to achieve HBeAg seroconversion. The response to antiviral treatment was defined by the loss of HBeAg from serum accompanied by normalization of ALT levels and clearance of HBV DNA determined by the TMA method with the detection limit of 3.7 LGE/ml.

Some patients had received glycyrrhizin either intravenously (Stronger Neo-Minophagen C [SNMC]; Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) or orally (GLYCYRON Tab; Minophagen Pharmaceutical Co., Ltd.), with or without oral ursodeoxycholic acid (UDCA; Mitsubishi Welpharmar Co., Ltd., Tokyo, Japan), before they were referred to the Toranomon Hospital.

Statistical Analysis

Categorical variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorical variables by the Mann–Whitney U-test.

RESULTS

Patients With Acute Hepatitis B Infected With HBV Genotype A

Infection resolved spontaneously in 24 of the 31 patients with acute hepatitis B who were infected with HBV genotype A, while it persisted in the remaining seven (23%) patients none of whom carried human immunodeficiency virus type 1. The persistence of acute infection tended to be more frequent in patients infected with HBV genotype A than those with genotype B (1/9 [11%]), or C (3/42 [7%]); one each patient infected with genotypes E and F cleared infection.

Table I compares demographic, clinical, and virological characteristics between patients in whom HBV infection did and did not persist. All the seven patients with chronic HBV infection were men, and tested negative for HBsAg in serum before they developed acute hepatitis; HBsAg persisted in them during 6 months or longer after they first tested positive for it. Of the 31 patients with acute infection with HBV genotype A, 16 (52%) confided having had extramarital sexual contacts. Homosexual activities were experienced somewhat more frequently in patients with than without persistent HBV infection (4/7 [57%] vs. 5/24 [22%]).

The maximum median ALT level was significantly lower (234 vs. 1,836 IU/L, $P = 0.0001$), while median HBV DNA level was significantly higher (median: >8.7 vs. 6.0 LGE/ml, $P = 0.004$) in patients in whom HBV genotype A infection persisted than in those who cleared it (Table I). None of the eight patients with HBV DNA <5 LGE/ml developed persistent infection. Infection persisted significantly more often in patients with the maximum ALT <500 IU/L than ≥500 IU/L (5/6 [83%] vs. 1/25 [4%], $P = 0.0001$).

Genetic subgroup of genotype A was Ae (the original European type) in all the 20 patients for whom subgrouping was possible. None of them were infected with HBV of subgroup Aa (Asian/African type corresponding to A′ of Bowyer et al. [1997]). Of the seven patients in whom infection persisted, five were infected with HBV of subgroup Ae; subgrouping was not feasible in the remaining two (Cases 2 and 4 in Table II).

Clinical Courses of the Seven Patients in Whom Infection With HBV Genotype A Persisted

Of the seven patients in whom infection with HBV genotype A persisted, six received treatment with IFN and/or lamivudine after transfer to Tornomon Hospital (Table II). They all had chronic hepatitis in the first liver biopsy; it was undertaken before treatment in five (71%). Three of them (50%) responded to treatment with the clearance of HBeAg from serum, normalization of ALT levels and loss of HBV DNA determined by the TMA method with the detection limit of 3.7 LGE/ml. In the remaining four patients, including the single one (Case 7) who did not receive antiviral treatment due to the absence of active hepatitis, infection with HBV genotype A persisted along with HBeAg and fluctuating ALT levels in serum.

Figure 1 depicts clinical courses of the three patients who responded to antiviral treatments. Case 1 received

TABLE I. Baseline Characteristics of Patients With Acute Hepatitis Induced by HBV Genotype A in Whom Hepatitis Persisted or Resolved

Features	Persisted (n = 7)	Resolved (n = 24)	Differences
Male	7 (100%)	24 (100%)	NS[c]
Age (years)[a]	26 (21–54)	33 (25–56)	NS
Sexual transmission	5 (71%)	11 (48%)	NS
Homosexual	4 (57%)	5 (22%)	
Heterosexual	1 (13%)	6 (26%)	
Maximum ALT (IU/L)[a]	234 (143–774)	1,836 (46–3,300)	$P = 0.0001$
HBsAg titer (2^N)[b]	11 (11 to ≥13)	11 (8 to ≥13)	NS
HBeAg-positive	7 (100%)	23 (96%)	NS
HBV DNA (LGE/ml)	>8.7 (6.3 to >8.7)	6.0 (<2.6 to >8.7)	$P = 0.004$

[a]Median values are shown with the range in parentheses.
[b]Determined by the hemagglutination assay on serial twofold dilutions of serum.
[c]Not significant.

TABLE II. Routes of Infection, Liver Histology, and Treatment Outcomes of the Seven Patients in Whom Infection With HBV Genotype A Persisted

Case no.	Age/sex	Sexual contacts[a]	Liver histology[b]	Treatment[c]	HBeAg lost	HBsAg lost
1	54/M	None	F1/A2	Lam	Yes	Yes
2	43/M	Hetero	F2/A1	IFN	Yes	No
3	46/M	None	F4/A2	IFN	Yes	No
4	21/M	Homo	F1/A1	IFN/Lam	No	No
5	24/M	Homo	F1/A2	Lam	No	No
6	28/M	Homo	F1/A1	IFN/Lam	No	No
7	21/M	Homo	F1/A1	None	No	No

[a]Extramarital sexual contacts in which infection with HBV genotype A was implicated.
[b]Pathology of the liver in the first biopsy; F, fibrosis stage; A, activity grade.
[c]Treatment received after admission to the Department of Gastroenterology in Toranomon Hospital; IFN, interferon; Lam, lamivudine.

lamivudine 3 months after the admission to our hospital. He lost both HBeAg and HBsAg, respectively, within 83 and 90 days on lamivudine; it was withdrawn at the disappearance of serum HBsAg. ALT levels normalized after the loss of HBeAg and HBsAg from serum.

Cases 2 and 3 did not receive treatment during 4 and 2 years, respectively, after they visited the hospital. They both responded to IFN 3 MU three times in week and lost HBeAg from serum, along with the disappearance of HBV DNA and normalization of ALT levels. They did not, however, clear HBsAg from serum. Cases 1–3 had received oral (Glycyron) or intravenous (SNMC) glycyrrhizin with or without ulsodeoxicholic acid (UDCA) while they were admitted to other institutions before referral to the Toranomon Hospital.

Clinical courses of the three patients who did not respond to antiviral therapies (Cases 4–6) are illustrated in Figure 2, along with that of a single patient who did not receive treatment (Case 7). Case 6 had received intravenous glycyrrhizin (SNMC) before his

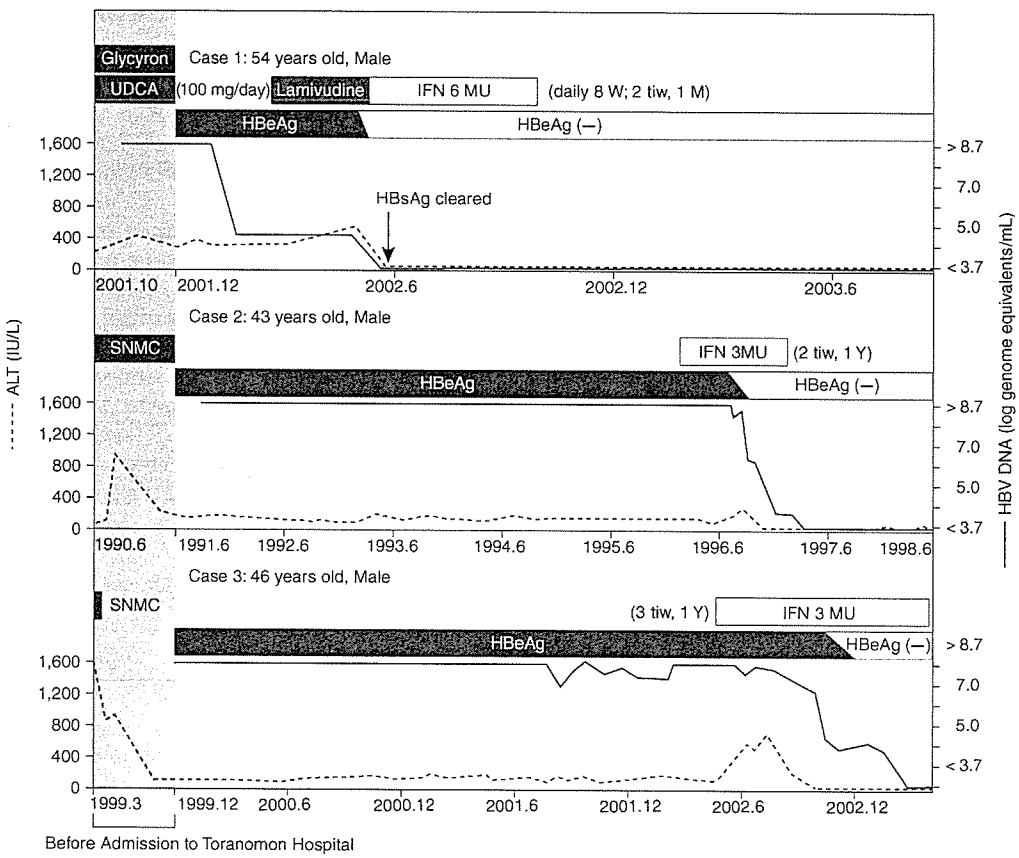

Fig. 1. Clinical courses of the three patients infected with HBV genotype A who responded to antiviral treatment with the loss of HBeAg. Courses and treatment they received before admission to Gastroenterology Department in Tornomon Hospital are shown in shaded areas on the **left**. The patient in Cases 1 lost HBsAg after treatment with lamivudine at time points indicated by arrows. IFN, interferon; SNMC, Stronger Neo-Minophagen C.

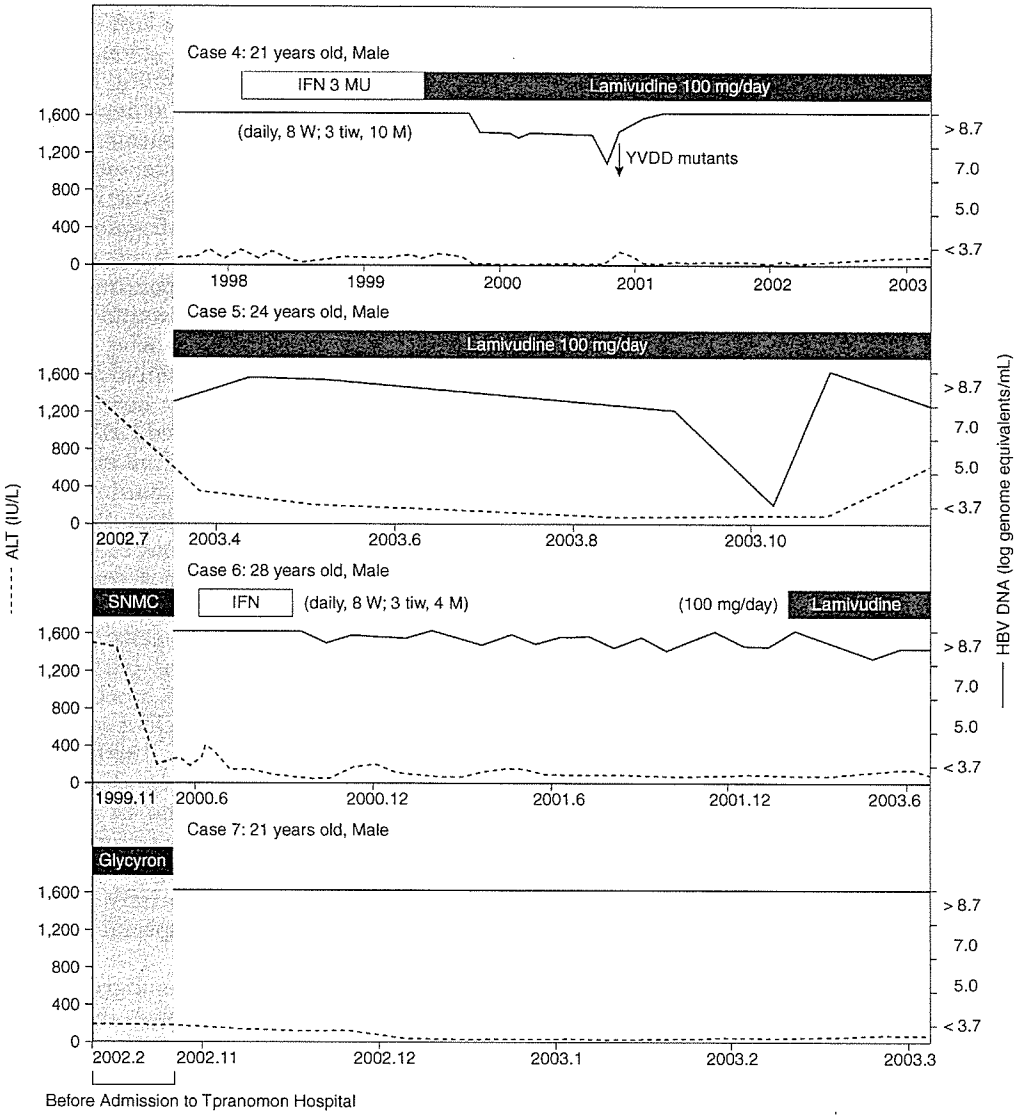

Fig. 2. Clinical courses of the four patients in whom HBeAg persisted after contracting infection with HBV genotype A despite antiviral treatment. The patient in Case 9 did not receive treatment after the admission. Courses and treatment they received before admission to Gastroenterology Department in Tornomon Hospital are shown in shaded areas on the **left**. IFN, interferon; SNMC, Stronger Neo-Minophagen C.

transfer to the Toranomon Hospital. Cases 4 and 6 did not respond to IFN or lamivudine that was commenced immediately after IFN or at an interval; lamivudine has been continued on them indefinitely. Case 5 was started on lamivudine soon after he was admitted to hospital and had been maintained on it for 1 year; he never responded to lamivudine. Variants with mutations in the YMDD motif of DNA polymerase/reverse-transcriptase developed in Case 4 while he was receiving lamivudine accompanied by a rise in ALT levels.

Although serum ALT returned to normal spontaneously (<50 IU/L) and then elevated only moderately in Case 7, high levels of HBV DNA (>8.7 LGE/ml) persisted through more than 1 year. Antiviral treatment was withheld because of the absence of active hepatitis.

DISCUSSION

There are marked geographical differences in the distribution of HBV genotypes [Magnius and Norder, 1995; Lindh et al., 1997; Miyakawa and Mizokami, 2003]. Of them, genotype A is not indigenous in Japan where genotypes B and C prevail and account for by far the majority of acute as well as chronic HBV infections [Orito et al., 2001; Kobayashi et al., 2002]. Some characteristics of HBV genotype A infection are increasingly coming to the fore in Japan and have aroused concerns in hepatologists at hospitals in urban areas with cosmopolitan populations. Ogawa et al. [2002] found that 14 of the 25 (56%) patients with acute hepatitis B in a downtown Tokyo (Shinjuku) were

infected with HBV genotype A. Moreover, the frequency of acute hepatitis induced by HBV genotype A in our hospital is higher after than before 1991 (2/22 [9%] vs. 26/46 [57%], $P < 0.0001$) [Kobayashi et al., 2004]. The present study sums up our experiences on acute infection with HBV genotype A at the Department of Gastroenterology in Toranomon Hospital situated in the Metropolitan Tokyo during the past 28 years, to supplement our previous reports with additional findings and new insights [Kobayashi et al., 2002, 2003, 2004].

First, infection with HBV genotype A spreads principally by extramarital sexual contact in the adulthood in Japan [Kobayashi et al., 2002; Ogawa et al., 2002]. All the 31 patients of acute hepatitis B infected with HBV genotype A in the present series were men, and 16 (52%) of them confided having had extramarital heterosexual or homosexual contacts. Only one mother of 32 patients with acute or chronic infection with HBV genotype A possessed HBV DNA in serum; her genotype was B [Kobayashi et al., 2003], thereby excluding perinatal transmission of genotype A. In a molecular epidemiological survey of HBV in Amsterdam, a cluster of genotype A related in men having sex with men has been recognized [van Steenbergen et al., 2002].

Secondly, acute infection with HBV genotype A tends to persist. Of the 31 patients with acute genotype A infection, seven (23%) failed to clear it within 6 months, in comparison with one of the nine (11%) with acute genotype B or three of the 42 (7%) with acute genotype C infection. In our previous report [Kobayashi et al., 2002], infection persisted in all three patients infected with genotype A, in contrasted to the clearance of HBsAg in all four with genotype B (one) or C (three).

Low maximum ATL levels (<500 IU/L [83%] vs. ≥500 IU/L [4%], $P = 0.0001$) and the high baseline HBV DNA levels (median: >8.7 vs. 6.0 LGE/ml, $P = 0.004$) were predictive of the perpetuation of acute HBV genotype A infection. Hence, compromised immune responses toward lower inflammation activity in the liver and higher viral replication may have a role in evolving HBV genotype A infection. Four of the seven (57%) patients who progressed to chronic were homosexuals. It is tempting to speculate that derangement in cytotocxic T cell response contributed to the failure in clearing acute HBV infection toward persistence [Handzel et al., 1984]. Immunomodulatory treatments to cope with severe acute hepatitis, given to five of the seven (71%) patients before referral to hospital (Figs. 1 and 2), may have promoted the persistence of infection with HBV genotype A. We have reported that acute prolonged HBV infection occurs more often in patients with than without immunomodulatory treatments during acute illness, regardless of genotypes (86% [6/7] vs. 2.4% [1/42], $P = 0.01$).

Thirdly, HBV genotype A infection persisting in patients with acute hepatitis B is not cleared often by antiviral therapy. HBV genotype A infection was terminated in only one of the six (17%) patients who received antiviral treatment. He was one of the three patients who seroconverted with the loss of HBeAg; interferon (IFN) and lamivudine was given to him early in the course of infection (Cases 1 in Fig. 1). Since most (~95%) patients with acute adulthood hepatitis B resolve infection in Japan, antiviral treatment is rarely used for them. As far as acute infection with HBV genotype A is concerned, however, therapeutic intervention needs to be considered in view of the frequent chronic outcomes. Since many (76% [24/31]) patients even with HBV genotype A can clear infection spontaneously, the timing of starting antiviral therapy would have to be contemplated. The single patient who cleared HBsAg was started on IFN and then lamivudine within 3 months after he was referred to our hospital. It is not certain whether he could have cleared HBV infection, should he never be placed on lamivudine early. HBsAg was not cleared, however, in the remaining two patients with HBeAg seroconversion in whom IFN was started 4 and 2 years, respectively, after they came to our care (Fig. 1).

Early antiviral treatment deserves consideration in patients who are infected with HBV genotype A, especially because of its propensity to become chronic. It is not certain how long patients should receive lamivudine after HBV DNA has disappeared from the circulation. Inasmuch as cccDNA continues to be present in the liver [Brechot et al., 1980; Yotsuyanagi et al., 1998], even after HBsAg is cleared from serum, a therapeutic option would be to continue lamivudine until anti-HBs is detected in serum as in Case 1. In view of the poor immune responses with low ALT levels, which might be inherent to HBV genotype A infection among homosexual, such a special care would have to be taken for its treatment.

There are two genetic subgroups of genotype A designated Ae which is common in Europe (the original genotype A) and Aa which is frequent in Africa as well as Asia [Sugauchi et al., 2003]; Aa is equivalent to subgroup A′ described by Bowyer et al. [1997]. It strikes as a surprise that of the 68 patients who were infected acutely or chronically with HBV genotype A and admitted to the Toranomon Hospital, 54 (79%) possessed HBV of subgroup Ae (European type); HBV of subgroup Aa (African/Asian type) was found in only four (6%) [Kobayashi et al., 2004]; they all were infected persistently. Since subgroup Ae was not found in any patients with acute hepatitis B in our series, it remains unclear whether or not the outcome of primary infection with HBV genotype A would be influenced by subgroup Aa and Ae.

Although acute HBV infection of genotype A tends to persist in comparison with those of the other genotypes, only a minority (7/31 [23%]) develops chronic infection. An efficient therapeutic strategy has to be found, however, since the infection with HBV genotype A was terminated in only one of the six (17%) patients who were treated. Recently, adefovir dipivoxil was found to be effective for the treatment of chronic hepatitis B [Marcellin et al., 2003], and it may offer a reasonable option for resolving persistent HBV genotype A infection.

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HEPATOLOGY

Virological differences between patients infected with subtypes Ba and Bj of hepatitis B virus genotype B

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Abstract

Background: Hepatitis B virus (HBV) genotype B is classified into subtype Ba with the recombination with genotype C in the precore region plus core gene and subtype Bj without recombination. Virological and clinical differences between infections with subtypes Ba and Bj, however, are yet to be determined.

Methods: During 1976 through 2001, 224 patients visited Toranomon Hospital in Tokyo, Japan who were infected with HBV genotype B. Subtypes of genotype B were determined by sequencing HBV-DNA recovered from sera for detecting recombination with genotype C.

Results: Subtype Ba was detected in 53 patients (24%) and Bj in 167 (75%); subtypes were not able to be determined in the remaining four (1%). The only virological difference was that detection of hepatitis B e antigen at the presentation was more frequent in the patients infected with subtype Ba than those with Bj (63% vs 33%, $P = 0.016$). There were no differences in the distribution of liver disease of various forms between the patients infected with subtypes Ba and Bj at presentation. No differences were noted, either, in the development of liver cirrhosis or hepatocellular carcinoma, or the loss of hepatitis B surface antigen from serum, between the patients infected with subtypes Ba and Bj during follow up of up to 26 years.

Conclusions: Although there were some virological differences between the patients infected with subtypes Ba and Bj of HBV genotype B, they do not seem to influence the long-term clinical outcome.

Key words: hepatitis B e antigen, hepatitis B surface antigen, hepatitis B virus, genotypes, subtypes.

INTRODUCTION

Hepatitis B virus (HBV) is classified into seven genotypes designated by the letters from A to G.[1–3] Recently, an eighth genotype, named H, has been proposed that is closely related to genotype F.[4] Genotypes of HBV have distinct geographic distribution and they influence the clinical course of hepatitis B. Because genotypes A and D frequently occur in Western countries, while genotypes B and C are common in Asia, clinical differences between genotypes A and D, as well as B and C, have been studied extensively.

It has been reported that genotype A induces chronic liver disease more frequently[5] and is associated with better response to interferon than genotype D.[6] Another recent study, however, has found that sustained biochemical remission and clearance of HBV-DNA, as well as the clearance of hepatitis B surface antigen (HBsAg), occurred at a higher rate in genotype A- than in genotype D-infected patients.[7] There have been increasing lines of evidence for more severe liver disease and longer duration of hepatitis B e antigen (HBeAg) in serum, accompanied by delayed seroconversion to antibody to HBeAg (anti-HBe), in infection with HBV genotype C than B.[8,9] Furthermore, hepatocellular carcinoma (HCC) develops more frequently in the patients infected with HBV genotype C than B.[10] Clinical courses may differ, however, even among the patients

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infected with the same genotypes of HBV. For instance, infection with HBV genotype B in Taiwan induces HCC much more frequently than that in Japan.[10,11]

Recently, two distinct subtypes of genotype B have been reported, designated Ba and Bj. Subtype Ba is ubiquitous in Asia, while Bj is restricted to Japan.[12] Notably, HBV isolates of subtype Ba possess the recombination with genotype C over the precore region plus core gene, while those of subtype Bj do not.[12] For the purpose of evaluating any clinical and virological differences, the 53 patients infected with subtypes Ba were compared with the 167 patients infected with subtype Bj at Toranomon Hospital in Tokyo, Japan.

METHODS

Patients infected with HBV

During 26 years from 1976 to 2001, 1674 patients infected with HBV visited Department of Gastroenterology, Toranomon Hospital in Tokyo Japan, and genotypes of HBV were determined in them. Genotype A was detected in 53 patients (3%), B in 224 (13%), C in 1332 (80%), D in four patients, E in one patient and F in three patients; genotypes were unidentifiable in the remaining 57 patients (3%). Subtypes of genotype B, in terms of Ba and Bj,[12] were determined in sera collected from the 224 patients infected with HBV genotype B at the presentation for evaluating any clinical and virological differences between infections with these two subtypes. Patients were considered to be in the asymptomatic carrier state when alanine aminotransferase (ALT) levels stayed normal (≤50 IU/L) throughout the observation period. Chronic hepatitis was diagnozed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy as well as ultrasonographic images and laparoscopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography and magnetic resonance imaging, and by liver biopsy if necessary. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the hospital. An informed consent was obtained from every patient.

Serum markers of HBV infection

The HBsAg was determined with commercial kits by hemagglutination (MyCell, Institute of Immunology, Tokyo, Japan) and radioimmunoassay (AUSRIA II-125, Dinabot, Tokyo, Japan), and antibody to HBV core of IgM class was tested for by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBc-antiM RIA; Dinabot). The HBeAg was determined by ELISA (ELISA, F-HBe; Kokusai Diagnostic, Kobe, Japan). The six major genotypes of HBV (A–F) were determined by ELISA with commercial kits (HBV genotype EIA, Institute of Immunology) after the method of Usuda *et al.*[13,14] It involves the expression of seven preS2 epitopes (*b, f, g, k, m, s and u*) detected by monoclonal antibodies, the combination of which is specific for each of the six HBV genotypes: *bsu* for genotype A; *bm* for B; *bks* for C; *bksu(g)* for D, *bksu* for E and bk for F. Genotype G, which was discovered recently,[3] was determined by the combination of preS2 serotype for genotype D and subtype adw of HBsAg; it is characteristic of this genotype.[15] Serotypes of HBsAg were determined by ELISA with commercial kits (HBs Antigen Subtype EIA, Institute of Immunology).

Determination of subtypes Ba and Bj of genotype B

Nucleic acids were extracted from serum (100 μL), which had been stored at −80°C, with a Smitest EX&R kit (Genome Science, Tokyo, Japan). The core gene of HBV-DNA in extracted nucleic acids were amplified by polymerase chain reaction (PCR) with nested primers. The first-round PCR was performed with BJF3 (sense, 5′-CCG ACC TTG AGG CAT ACT TC-3′ [nt 1690–1709]) and BJF4 (antisense, 5′-GGG TCC CAC AAA TTG CTT AC-3′ [nt 2580–2606]) primers, and the second-round PCR with FJF1 (sense, 5′-GCT GTG CCT TGG GTG GCT TTG-3′ [nt 1876–1897]) and BJR2 (antisense, 5′-GCG ACG CCG TGA TTG AGA CCT-3′ [nt 2398–2411]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, CA, USA). They were then purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced in the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The core-gene sequences from patients were analyzed phylogenetically along with reference Ba and Bj sequences by 6-parameter and neighbor-joining methods.[16,17]

Nucleotide sequences of the precore region and core promoter

For determination of the wild-type or mutants in the precore region, nucleic acids extracted from serum were amplified by PCR with nested primers. The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAC CAC CGT GAA C-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) for 35 cycles each (94°C, 1 min [5 min in the first cycle]; 53°C, 2 min; and 72°C, 3 min [7 min in the last cycle]). The amplification products were run on gel electrophoresis, purified and sequenced as described here. Mutations interfering with translation and transcription of HBeAg were sought in the precore region and core promoter, respectively. They included a G-to-A mutation at nucleotide 1896 (A1896) in the precore region and the double mutation in the core promoter converting the codon 1762 from A to T as well as codon 1764 from G to A (T1762/

A1764). Also examined was nt 1858 of T or C in HBV-DNA sequences.

Statistical analysis

Frequencies between groups were compared using the χ^2 test, Fisher's exact test and Mann–Whitney *U*-test. Data analysis was performed using SAS (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered significant. Differences in the progression rate of chronic hepatitis B and the frequency of HBsAg clearance were evaluated by Kaplan–Meier technique and log–rank test.

RESULTS

Clinical manifestations of the patients infected with subtype Ba or Bj of HBV genotype B

The HBV-DNA sequences were determined from nucleic acids extracted from 224 patents infected with HBV genotype B who visited Toranomon Hospital in Tokyo, Japan during 1976 through 2001, and who were subjected to phylogenetic analysis on the core gene. Subtype Ba having the recombination with genotype C was identified in 53 (24%) patients and subtype Bj without such recombination in 167 (75%); distinction between Ba and Bj was not possible in the remaining four patients (1%). Table 1 compares frequencies of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC, between 53 patients infected with HBV subtype Ba and 167 with Bj. There were no differences in the distribution of liver disease of various forms between the patients infected with subtypes Ba and Bj.

Demographic and virological characteristics of patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Demographic and virological features are compared between patients with chronic hepatitis B, 24 of whom were infected with subtype Ba and 82 with subtype Bj (Table 2). There were no differences in sex, age, duration of follow up and mothers persistently infected with HBV, between the patients infected with subtypes Ba and Bj. At presentation, however, the prevalence of HBeAg in serum was significantly higher in the patients infected with subtype Ba than Bj (63% vs 33%,

Table 1 Distribution of liver disease in patients infected with HBV subtype Ba or Bj

	Subtypes of genotype B		
Disease/condition	Ba ($n = 53$) *n* (%)	Bj ($n = 167$) *n* (%)	*P*
Acute hepatitis	0	5 (3)	NS
Asymptomatic carrier state	22 (42)	66 (40)	NS
Chronic hepatitis	24 (45)	82 (49)	NS
Liver cirrhosis or hepatocellular carcinoma	7 (13)	14 (8)	NS

NS, not significant.

Table 2 Comparison between patients with chronic hepatitis who were infected with HBV subtypes Ba or Bj

	Subtypes of genotype B		
Features	Ba ($n = 24$) *n* (%)	Bj ($n = 82$) *n* (%)	*P*
Male	19 (79)	73 (89)	NS
Age (years); median (range)	36 (23–62)	37 (21–83)	NS
Follow up (days); median (range)	3363 (50–11 642)	3475 (165–10 679)	NS
Mother with HBsAg	2 (8)	12 (15)	NS
Serotype of HBsAg			
adw	22 (92)	73 (89)	NS
adr	1 (4)	1 (1)	NS
adwr	1 (4)	0	NS
Untypeable	0	9 (11)	NS
HBeAg at presentation	15 (63)	27 (33)	$P = 0.016$
Clearance of HBeAg	10/15 (67)	21/27 (78)	NS
HBV-DNA at presentation (LEG/mL)†	7.5 ± 6.2	4.9 ± 3.6	NS

NS, not significant.

†Log equivalent genome (LEG)/mL by the transcription mediated assay.

$P = 0.016$). In contrast, the prevalence of HBeAg was no different between the asymptomatic carriers with Ba and Bj infections (3/22, 14% vs 6/66, 7%). Falling short of being significant, the mean titer of HBV-DNA was somewhat higher in the patients infected with subtype Ba than Bj.

Figure 1 depicts the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 20 years. There were no differences in the progression of liver disease between the patients infected with subtypes Ba and Bj. No differences were noted, either, in the loss of HBsAg from serum during follow up between them, although HBsAg tended to disappear earlier in patients infected with subtype Ba than Bj up to 15 years of follow up (Fig. 2).

Mutations in the core promoter and precore region, which increase with the duration of infection and influence the severity of liver disease, were examined in the patients with chronic hepatitis at the time of presentation. Table 3 compares mutations in the core promoter and precore region between the patients infected with subtypes Ba and Bj. There were no differences in the frequency of the stop-codon mutation in the precore region, or that of the double mutation in the core promoter, between the patients infected with the two different subtypes of genotype B.

Distributions of HBsAg serotypes were no different between the patients infected with subtypes Ba and Bj. The 1858th nucleotide of T or C that influences the precore mutation (A1896) was invariably T in all 18 patients infected with subtype Ba, and in all 70 with Bj who were examined.

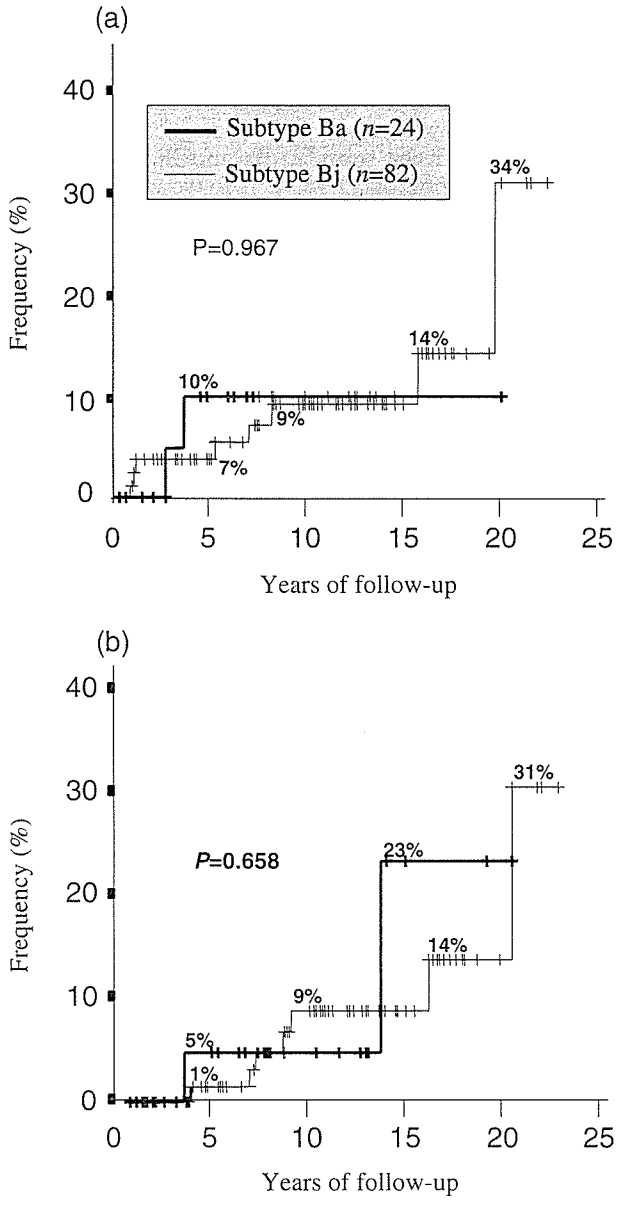

Figure 1 Evolution of chronic hepatitis in patients infected with subtype Ba or Bj of HBV genotype B. Development of liver cirrohosis (a) and hepatocellular carcinoma (b) were compared between the 24 patients infected with HBV genotype Ba and the 82 with Bj during follow up of 20 years or longer. There were no differences in the development of either liver cirrhosis of hepatocellular carcinoma by evalution of the results obtained by the Kaplan–Meier technique with the log–rank test.

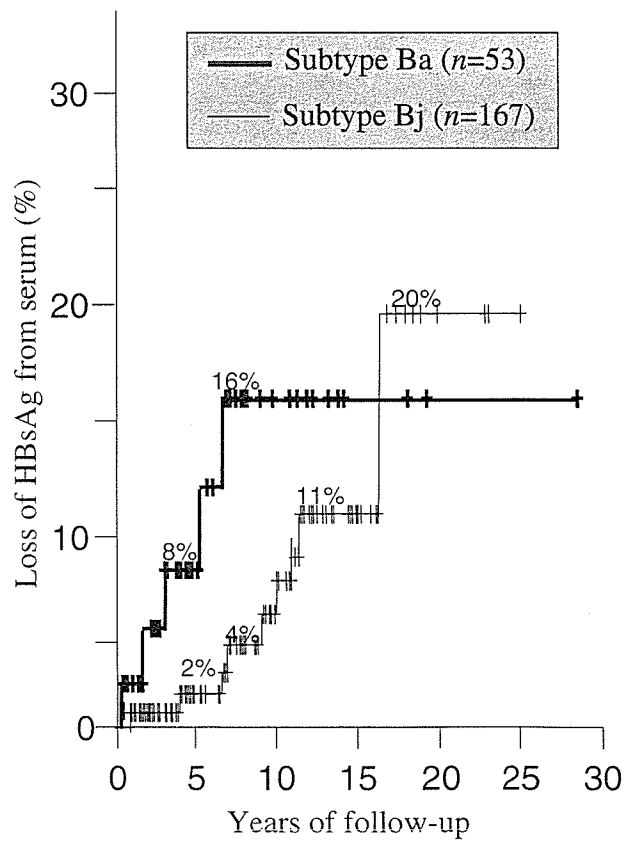

Figure 2 Loss of HBsAg from serum during long-term follow up. The 53 patients infected with subtype Ba and the 167 with subtype Bj of HBV genotype B, who presented with HBeAg in serum, were compared by the Kaplan–Meier technique, and differences were evaluated with the log–rank test.

Clinical and virological characteristics of patients with liver cirrhosis who were infected with subtype Ba or Bj

Table 4 lists demographic, histolgical and virological features of patients with liver cirrhosis, five of whom were infected with subtype Ba and 10 with subtype Bj. As for patients with chronic hepatitis B, the detection of serum HBeAg at presentation was significantly more frequent (60% vs 0%, $P = 0.022$), and the mean titer of HBV-DNA in serum tended to be higher, in the patients infected with subtype Ba than Bj.

DISCUSSION

Recombination between HBV isolates of distinct genotypes has been reported,[18,19] which may endow recombinants with a phenotype for virological characteristics or disease-inducing capacity distinct from those of parent genotypes. Because genotypes A and D are frequent in Western countries, A/D recombinants are reported there. Likewise, because genotypes B and C are common in Asia, B/C recombinants occur mostly in Asian countries. Not so many A/D or B/C recombinants have been reported, however, probably reflecting uncommon recombination events in the HBV infection.

Two subtypes of genotype B are reported, one of which has recombination with genotype C in the precore region and core gene, while the other does not.[12] It is surprising that essentially all HBV strains from Asian countries other than Japan are of the Ba subtype with the recombination (suffix 'a' representing Asia), in contrast to most of those from Japan that are of subtype Bj, without the recombination (suffix 'j' standing for Japan). Because Japan is unique in that both Ba and Bj subtypes occur in the genotype B infection, we set out to examine any demographic, virological and clinical differences between subtypes Ba and Bj. A study conducted at Toranomon Hospital in Tokyo was carried out to determine whether there would be any differences in subtypes Ba and Bj, in the same epidemiological and clinical setting in the patients of a single ethnic origin.

During 26 years from 1975 to 2001, 224 patients infected with HBV of genotype B presented to Department of Gastroenterolology, Toranomon Hospital located at the center of Tokyo, Japan. Subtypes of genotype B were determined by sequencing HBV-DNA, and Ba was found in 53 (24%) and Bj in 167 (75%); HBV isolates of genotype B from only four patients (1%) were untypeable into Ba or Bj. The 53 patients infected with subtype Ba and the 167 with subtype Bj were compared demographically, clinically and virologically.

The prevalence of subtype Ba (24%) in the patients who visited Toranomon Hospital in Tokyo was higher than that reported by Sugauchi *et al.* from Japan (7/97; 7%).[20] HBV subtype Ba is infrequent in Japan, in contrast to the other Asian countries, where subtype Ba accounts for all genotype B infections.[20] Because Toranomon Hospital is a tertiary referral hospital, selection may have occurred in favor of patients with severe disease or who were refractory to treatment. The frequency of HBsAg in mothers of patients tended to be higher in subtype Bj infection than Ba (15/167, 9% vs 2/53, 4%). Hence, the patients with subtype Ba would have had a higher chance of infection in later life than those with

Table 3 Mutations in the core promoter and precore region in the patients with chronic hepatitis who were infected with HBV subtype Ba or Bj

Mutation	Ba ($n = 18$) n (%)	Bj ($n = 69$) n (%)	P
Core promoter†			
Mutant	4 (22)	15 (22)	NS
Wild-type	14 (78)	54 (78)	
Precore region‡			
Mutant	9 (50)	36 (52)	NS
Wild-type	9 (50)	33 (48)	

NS, not significant.

Examination was possible in 18 of the 24 patients infected with subtype Ba and 69 of the 82 infected with subtype Bj of HBV genotype B.

†Double mutation for T1762/A1764; ‡A1896 mutation for a stop codon at amino acid 28.

Table 4 Comparison between patients with liver cirrhosis who were infected with HBV subtypes Ba or Bj

Features	Subtypes of HBV		
	Ba ($n = 5$) n (%)	Bj ($n = 10$) n (%)	P
Male	5 (100)	7 (70)	NS
Age (years); median (range)	44 (24–50)	37 (21–83)	NS
Follow up (days); median (range)	4505 (2001–8199)	1524 (487–5151)	NS
Mother with HBsAg	0	2 (20)	NS
HBeAg at presentation	3 (60)	0	0.022
Clearance of HBeAg	3/3 (100)	0/0	NS
HBV-DNA at presentation (LEG/mL)†	4.9 ± 4.1	4.1 ± 3.8	NS

NS, not significant.

†Log equivalent genome (LEG)/mL by the transcription mediated assay.

subtype Bj. The duration of HBV infection therefore may have been shorter in patients with subtype Ba than Bj, which needs to be taken into consideration when evaluating virological differences between them. The prevalence of HBeAg in sera is reported to be higher in patients of the same age who were infected with subtype Ba than Bj,[20] which has been confirmed in the preesnt study. These differences, however, would not readily be attributed to virological differences alone and need to be evaluated with reference to the duration of HBV infection.

Subtypes Ba and Bj did not seem to affect the severity of clinical disease. The distribution of acute hepatitis, asymptomatic carrier state, chronic hepatitis and liver cirrhosis with or without HCC was no different between the patients infected with Ba and Bj in the present series. Subtypes Ba and Bj, however, have been shown to influence resistance to lamivudine as well as virological and biochemical breakthroughs in our previous study.[21]

There was an important virological difference between Ba and Bj infection. The patients with chronic hepatitis or liver cirrhosis infected with subtype Ba possessed HBeAg in serum significantly more frequently than those infected with subtype Bj. Because HBeAg persists longer in patients infected with HBV genotype C than B,[8,9] this trait of genotype C would have borne out in HBV strains of subtype Ba that possess the recombination with genotype C over the precore region and core gene. The persistence of HBeAg over a longer period of time, before the seroconversion to anti-HBe takes place accompanied by hepatitis flares, would results in more severe disease in the patients infected with HBVgenotype C than B.[8]

Mutations in the core promoter and precore region that downregulate and abolish the synthesis of HBeAg, respectively, are under influence of HBV genotypes, and the double mutation in the core promoter (T1762/A1764) is detected more frequently in the patients infected with genotype C than B.[8,22] In so far as the core promoter region of subtype Ba is replaced by that of genotype C,[12] it would be more prone to the mutation for T1762/A1764 than that of subtype Bj. Because the T1762/A1764 mutation is implicated in hepatocarcinogenesis in patients infected with HBV,[23] a high frequency of this mutation in subtype Ba infection would be responsible, at least in part, for HCC in patients in Taiwan who develop this during youth.[10]

Very recently, Sugauchi *et al.* compared 80 patients infected with subtype Ba from Asian countries other than Japan, with 80 patients infected with subtype Bj from Japan while controlling for severity of liver disease.[20] They found a higher frequency of HBeAg in serum and the double mutation in the core promoter (T1762/A1764) in the patients infected with subtype Ba than Bj. Because the Sugauchi *et al.* study was case-controlled on patients with identical distribution of asymptomatic carrier state, chronic hepatitis, liver cirrhosis and HCC, the influence of genotype Ba and Bj on the clinical course of hepatitis B was not within the scope of the study.[20] In the present series of 53 Japanese patients infected with subtype Ba and the 157 infected with Bj, no influence of these subtypes was observed in terms of distribution of liver disease of distinct severity and the development of liver cirrhosis and HCC in patients with chronic hepatitis B during follow up of up to 26 years. These observations come as a surprise, in view of the response to lamivudine being poorer in the patients infected with subtype Ba than Bj.[21]

In conclusion, there is a significant virological difference between HBV infection of subtype Ba and Bj, which seem to be attributable to the recombination with genotype C in HBV isolates of subtype Ba. Persistence of HBeAg would influence the clinical course and response to antiviral therapies in the patients infected with subtype Ba, who would fare worse than those with subtype Bj in the long term. This would need to be confirmed in an extended series of patients who are infected with HBV of Ba or Bj in prospective studies, in view of the small number of studied patients with Ba infection in Japan, where Bj prevails.

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Usefulness of the Serum KL-6 Assay in Patients with Hepatitis C Virus

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Key Words
Chronic hepatitis C · Hepatocellular carcinoma · Idiopathic pulmonary fibrosis · KL-6

Abstract
Objective: The aim of this study is to evaluate the serum level of KL-6 in hepatitis C virus (HCV)-positive patients with chronic liver disease. ***Methods and Results:*** Subjects consisted of 502 HCV-positive patients. The serum samples of these patients stored at –80°C were measured by enzyme-linked immunosorbent assay for KL-6 at the same time. The cutoff point of the serum KL-6 level was defined as 500 U/ml. The serum KL-6 level of the 502 patients ranged between 71 and 2,295 (median, 223) U/ml. Thirty-two of the 502 (6.4%) patients showed an elevated KL-6 level of >500 U/ml. Three of the 32 (9.4%) patients with elevated KL-6 level >500 U/ml had idiopathic pulmonary fibrosis. Multivariate analysis showed that patients achieved elevated KL-6 when: (1) they had hepatocellular carcinoma (HCC; p = 0.0007), and (2) age was >60 years (p = 0.0085). The HCC rate was 37.5% (12/32) in the patients with elevated KL-6 and 8.3% (39/470) in the patients with normal KL-6 group. The median (range) age was 70 (56–77) years in the patients with elevated KL-6 group and 60 (12–92) years in the patients with normal KL-6. ***Conclusion:*** The patients with HCC aged >60 years had significantly elevated serum levels of KL-6.

Introduction

A variety of extrahepatic complications, such as essential mixed cryoglobulinemia, porphyria cutanea tarda, membranoproliferative glomerulonephritis, autoimmune thyroiditis, sialadenitis, cardiomyopathy, and idiopathic pulmonary fibrosis (IPF) have been reported in patients with chronic hepatitis C virus (HCV) infection [1–15]. Thus, it is necessary to predict these extrahepatic manifestations in the follow-up of patients with HCV. Various studies have been conducted on the diagnosis of IPF. Despite extensive research, IPF remains a disease of unknown etiology with a poor prognosis after acute exacerbation. It can progress rapidly after such exacerbation and often proves fatal, despite treatment with oral corticosteroids and intravenous high-dose corticosteroid therapy.

The serum level of KL-6 is a sensitive marker of disease activity in fibrosing lung diseases [16–19]. KL-6 is a high-molecular-weight glycoprotein and is classified as MUC1 mucin of lung tumor and differentiation antigens. The molecule consists of multiple heterogeneous submolecules. KL-6 can be detected by a murine monoclonal antibody, KL-6 antibody (IgG_1), which recognizes a sialylated sugar chain on the molecule. Although the presence of KL-6 in the serum has been reported to be a sensitive marker of disease activity in interstitial pneumonitis such as IPF, the serum KL-6 level in HCV-positive patients with hepatocellular carcinoma (HCC) sometimes increases.

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