distribution, however, only two HBV genotypes prevail in most countries; the United States is the only exception with seven (A–G) genotypes [Chu et al., 2003; Westland et al., 2003]. Thus, genotypes A and D are common in Europe and India, while genotypes B and C are frequent in Asia [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Therefore, comparison has been restricted between patients infected with genotypes A and D, as well as those with B and C [Zhang et al., 1996; Mayerat et al., 1999; Kao et al., 2000; Orito et al., 2001; Chu et al., 2002; Thakur et al., 2002].

During 31 years from 1973 to 2003, 4,121 patients visited Toranomon Hospital in the Metropolitan Tokyo, and HBV genotypes were determined in them. There were 128 patients with genotype A, of whom 87 were chronically infected with HBV at the presentation. They were followed along with the 413 patients chronically infected with genotype B and the 3,389 with genotype C for seroclearance of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). Furthermore, patients with genotype A were grouped by the presence or absence of HBeAg at the presentation, as well as seroconversion during the follow-up, and they were compared for virological and clinical outcomes.

MATERIALS AND METHODS

Patients Chronically Infected With HBV

During 31 years from April 1973 to December 2003, genotypes of HBV DNA were determined in 4,121 patients with HBsAg in the Department of Gastroenterology at Toranomon Hospital in the Metropolitan Tokyo. Genotypes were A in 128 (3.11%) patients, B in 431 (10.46%), C in 3,434 (83.32%), D in 4 (0.97%), E in 1 (0.02%), and F in 3 (0.07%); they were not classifiable in the remaining 120 (2.91%) patients.

Of the 128 patients infected with HBV genotype A, 41 (32%) presented with acute hepatitis B as diagnosed by high-titered IgM antibody to hepatitis B core antigen. The remaining 87 (68%) patients were chronically infected with HBV genotype A when they visited our hospital. Their diagnoses were asymptomatic carriers with persistently normal ALT levels in 38 (44%) and chronic hepatitis in 39 (45%). In addition, nine (10%) patients presented with cirrhosis and one (1%) with hepatocellular carcinoma. Chronic hepatitis was diagnosed by liver biopsies performed under laparoscopy, and liver cirrhosis by liver biopsy and/or ultrasonographic images plus laparocopic findings. Hepatocellular carcinoma was diagnosed by imaging modalities, such as ultrasonography, computed tomography, and magnetic resonance imaging, and by liver biopsy if necessary.

The 87 patients infected chronically with HBV genotype A had the median age of 34 years (range: 11–67 years), included 72 (83%) men and were followed for the median of 5.0 years (0.1–22 years). Only two (2%) had a history of blood transfusion, and three (3%) were co-infected with hepatitis C virus. They had the median serum HBV DNA level at 4.2 log copies/ml, and HBeAg was detected in sera from 32 (37%). Subgenotypes of A [Bowyer et al., 1997; Sugauchi et al., 2004] were Aa (Asian or African type) in 5 (6%) and Ae (European type) in 65 (75%); they were not classifiable in the remaining 17 (19%).

Serological Markers of HBV Infection

HBsAg was determined by hemagglutination (MyCell; Institute of Immunology Co., Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (ELISA, F-HBsAg; Sysmex, Kobe, Japan), and HBeAg by ELISA (ELISA, F-HBe; Sysmex). HBV DNA was determined by quantitative polymerase chain reaction (PCR) (Amplicor HBV Monitor Test; Roche Molecular Systems, Inc., New Jersey) and the results were expressed in log copies/ml within a detection range from 2.6 to 7.6.

Genotypes of HBV

The six major genotypes (A–F) were determined serologically by ELISA (HBV GENOTYPE EIA; Institute of Immunology). The method utilizes the combination of epitopes on preS2-region products that is specific for each genotype [Usuda et al., 1999, 2000]. Genotype G was determined by preS2 serotype for genotype D and HBsAg subtype adw, and H was recognized by serotype for genotype C and subtype adw, respectively; these combinations were specific for genotypes G and H, respectively [Kato et al., 2001, 2004].

Subgenotypes of A designated Ae prevalent in Europe and Aa frequent in Africa as well as Asia [Sugauchi et al., 2004] (corresponding to A′ originally reported by Bowyer et al. [1997]), were determined by the nucleotide sequence in the *S* gene [Sugauchi et al., 2004]. Briefly, nucleic acids were extracted from serum and a sequence of the large *S* gene was amplified by PCR with nested primers. The first-round PCR was performed with BGF1 (sense, 5′-CTG TGG AAG GCT GGC ATT CT-3′ [nt 2757–2776]) and BGR2 (antisense, 5′-GGC AGG ATA GCC GCA TTG TG-3′ [nt 1050–1079]) 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 2811–2830]) 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]). Amplification products were run on gel electrophoresis and stained with BIG Dye (Applied Biosystems, California), purified by Qiquick PC purification kit (Qiagen, Hilden, Germany) and then sequenced in AGI Prism 310 Genetic Analyzer (Applied Biosystems). The large S-gene sequences were analyzed phylogenetically along with reference Aa and Ae sequences by six-parameter and neighbor-joining methods [Gojobori et al., 1982; Saitou and Nei, 1987].

Determination of Hyaluronic Acid in Serum

Hyaluronic acid was determined by the agglutination of microparticles coated with proteins that specifically bind with it (Elpia-Ace HA, Fujirepio, Tokyo, Japan).

Statistical Analysis

Frequencies were compared between groups by the Mann–Whitney *U*-test and Fisher's exact test, and means by the Wilcoxon signed rank test. Loss of HBeAg or HBsAg was compared in the Kaplan–Meier life table, and differences were evaluated by log-rank test after the production limit method. A *P*-value less than 0.05 was considered significant.

RESULTS

Patients Infected Chronically With HBV Genotype A

There were 45 patients who were infected chronically with HBV genotype A and had been followed for 3 years or longer. Of them, 19 had persistently normal ALT levels (asymptomatic carriers), while the remaining 26 with elevated ALT levels possessed biopsy-proven chronic hepatitis. Table I compares demographic and virological characteristics at the baseline between the 19 asymptomatic carriers and 26 patients with chronic hepatitis. HBeAg was more frequent and the median HBV DNA level higher in patients with chronic hepatitis than asymptomatic carriers. The majority of asymptomatic carriers (79% [15/19]) and patients with chronic hepatitis (73% [19/26]) were infected with subgenotype Ae. There were three (12%) patients infected with subgenotype Aa and two of them had chronic hepatitis. Subgenotypes were not classifiable in the remaining four (21%) asymptomatic carriers and four (15%) patients with chronic hepatitis. Liver disease worsened in a single patient with chronic hepatitis. He was 47 years old at the presentation and infected with subgenotype Ae. Cirrhosis developed followed by hepatocellular carcinoma in him.

HBsAg and HBeAg in Patients With Chronic Hepatitis Infected With HBV Genotype A

Of the 26 patients infected with HBV genotype A, 4 (15%) lost HBsAg during follow-up, in comparison with 16 of the 116 (14%) patients with genotype B and 68 of the 862 (8%) with genotype C. Figure 1 compares seroclearanace of HBsAg among patients with genotype A, B, or C. The loss of HBsAg at 5 years was significantly more frequent in patients with genotype A than B or C (12% vs. 2% or 3%, $P = 0.0395$).

Of the 26 hepatitis patients with genotype A, 19 (75%) possessed HBeAg at the presentation. HBeAg was cleared from serum in 14 (74%) of them during follow-up, in comparison with the seroclearance in 36 of the 41 (88%) patients with genotype B and in 347 of the 562 (62%) with genotype C. Figure 2 compares seroclearance of HBeAg among patients with genotype A, B, or C. At 5 years of follow-up, HBeAg was cleared more frequently in patients with genotype B than in those with genotype A or C (78% vs. 58% or 45%, $P = 0.00001$).

Development of Cirrhosis and Hepatocellular Carcinoma in Patients Infected With HBV of Various Genotypes

Figure 3 compares the development of cirrhosis in patients infected with genotype A, B, or C. Of the patients with genotype A, cirrhosis developed in only one at 5 years, but not any more during follow-up for 20 years. In contrast, cirrhosis increased steadily in patients with genotype B or C; it developed twice more often in patients with genotype C than B (30% vs. 14%).

Hepatocellular carcinoma developed in the single cirrhotic patient with genotype A, but did not in any others with genotype A during follow up for 20 years (Fig. 4). It increased with time, however, in patients with genotype B or C. Hepatocellular carcinoma tended to develop more frequently in patients with genotype C than B at 20 years (15% vs. 11%).

Changes in HBV DNA Levels and Hyaluronic Acid in the Patients Infected With HBV Genotype A

Of the 26 patients with genotype A, 14 (54%) seroconverted for the loss of HBeAg, while 5 (19%) kept it throughout follow-up longer than 3 years; the remaining 7 (27%) patients were without HBeAg at the presentation and thereafter. Table II compares demographic and virological characteristics of the three

TABLE I. Baseline Characteristics of the 45 Patients Infected With HBV Genotype A Who Were Followed for Longer Than 3 Years

Feature	Asymptomatic carriers (n = 19)	Chronic Hepatitis (n = 26)	Differences
Age (years)[a]	29 (11–48)	32 (13–59)	NS[c]
Male	15 (79%)	24 (92%)	NS
Follow-up (years)[a]	6.5 (3.4–17.7)	6.8 (3.5–18.6)	NS
History of transfusion	0 (0%)	1 (4%)	NS
Anti-HCV	0 (0%)	1 (4%)	NS
HBeAg positive	3 (16%)	19 (75%)	$P = 0.0002$
HBV DNA (log copies/ml)	<2.6 (<2.6–5.9)	>7.6 (<2.6–>7.6)	$P = 0.001$
Subgroups (Aa/Ae/ND[b])	0%/79%/21%	12%/73%/15%	NS

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

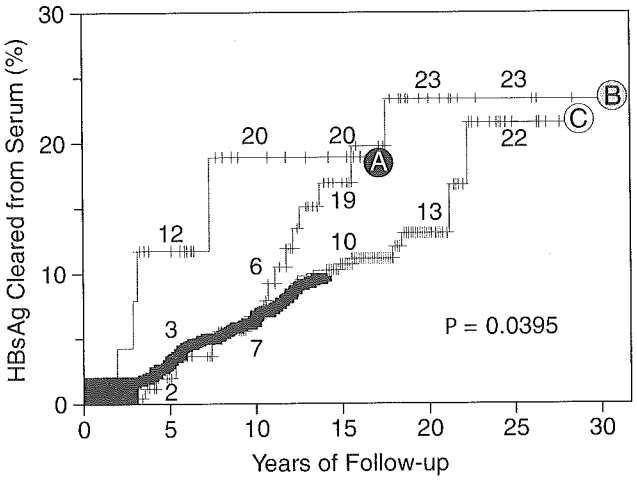

Fig. 1. Seroclearance of HBsAg during follow-up. Clearance rates of HBsAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant between genotype A and genotypes B and C at 5 and 10 years, as well as between genotypes B and C at 20 years by the log-rank test. Seroclearance of HBsAg did not spontaneously occurr in all of them.

groups of patients at the baseline. Levels of HBV DNA were significantly lower in the patients without HBeAg than in those whom HBeAg persisted or who seroconverted within 3 years ($P = 0.03$).

Figure 5 compares changes in HBV DNA levels among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained negative for HBeAg. HBV DNA levels >7.6 log copies/ml continued for longer than 3 years in four of the five (80%) patients with persistent HBeAg. HBV DNA levels decreased in 13 of the 14 (93%) patients with seroconversion; they slightly changed from 6.7 to 7 log copies/ml in the remaining one patient. HBV DNA decreased to

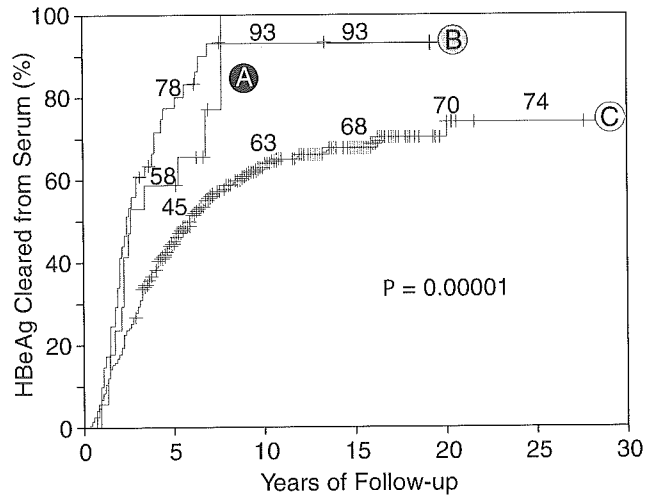

Fig. 2. Seroclearance of HBeAg during follow-up. Clearance rates of HBeAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant among genotypes A–C at 5 years as well as between genotypes B and C since 10 years or later by the log-rank test. Seroclearance of HBeAg did not spontaneously occurr in all of them.

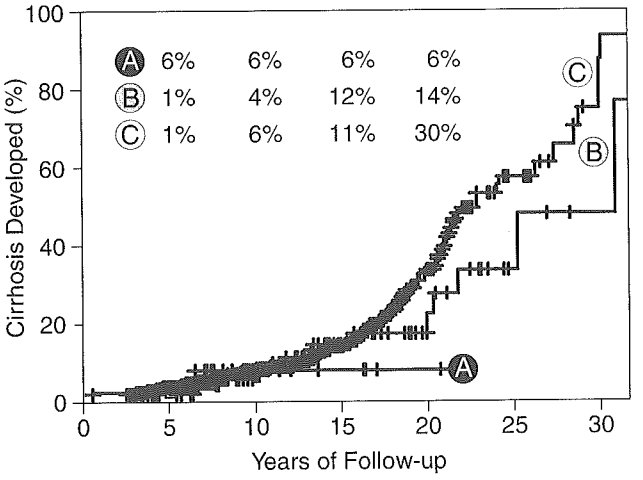

Fig. 3. Development of cirrhosis in patients infected with HBV genotype A, B, or C.

levels below the detection limit in 4 of the 14 (29%) patients with seroconversion and 1 of the 7 (14%) without HBeAg at the baseline. Of the 7 patients without HBeAg, 4 (57%) kept HBV DNA in detectable levels, comparable to 9 of the 14 (64%) patients with seroconversion. Decreases in HBV DNA during follow-up for 3 years or longer were significantly more frequent in the patients with seroconversion and those without HBeAg than in those with persistent HBeAg (93% [13/14] and 86% [6/7] vs. 20% [1/5], $P = 0.0095$ by the Fisher's exact test).

Figure 6 compares serum levels of hyaluronic acid among patients infected with genotype A in whom HBeAg persisted, who seroconverted and who had remained HBeAg-negative. Hyaluronic acid increased in four of the five (80%) patients in whom HBeAg persisted in contrast to only one of the seven (14%) patients without HBeAg. Increases in serum levels of hyaluronic acid ≥10 ng/ml was more frequent in the

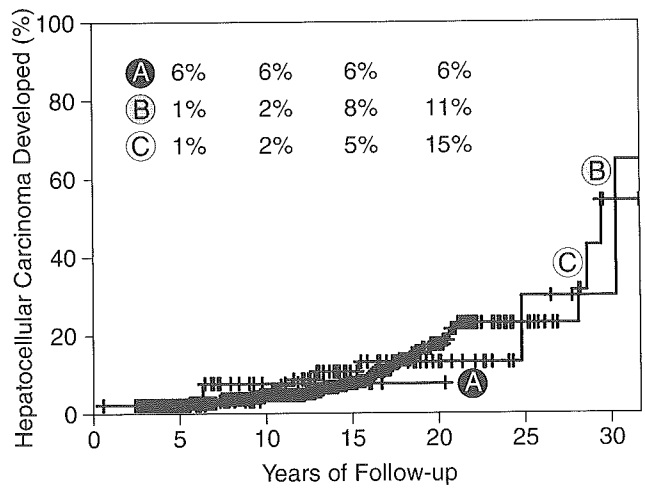

Fig. 4. Development of hepatocellular carcinoma in patients infected with HBV genotype A, B, or C.

TABLE II. Baseline Characteristics of the 26 Patients Infected With HBV Genotype A in Whom HBeAg Persisted, Who Seroconverted and Who Were Without HBeAg at the Presentation

Feature	HBeAg persisted (n = 5)	Seroconverted (n = 14)	Without HBeAg (n = 7)	Differences
Age (years)[a]	49 (24–59)	30 (13–60)	33 (14–41)	NS[c]
Male	5 (100%)	14 (100%)	5 (71%)	NS
Follow-up (years)[a]	6.2 (3.7–7.4)	9.2 (3.0–21)	8.1 (3.9–17)	NS
History of transfusion	0	1 (7%)	0	NS
Anti-HCV	0	0	1 (14%)	NS
HBV DNA (log copies/ml)	>7.6 (all patients)	>7.6 (6.7–>7.6)	4.1 (<2.6–7.1)	$P = 0.03$
Subgroups (Aa/Ae/ND[b])	(0%/80%/20%)	(7%/79%/14%)	(29%/57%/14%)	NS

[a]Median values are shown with the range in parentheses.
[b]Not determined.
[c]Not significant.

patients with persistent HBeAg than in those with seroconversion and those without HBeAg (80% [4/5] vs. 14% [2/14] and 14% [1/7], $P = 0.017$ by the Fisher's exact test).

Of the 19 hepatitis patients presenting with serum HBeAg, 16 received antiviral and/or steroid withdrawal therapies, and 11 (69%) responded by the loss of HBeAg, while the remaining 4 failed to do so (Table III). There were three patients in whom HBeAg disappeared without receiving treatments. In total, therefore, seroconversion was accomplished in 14 of the 19 (74%) patients with genotype A.

DISCUSSION

Of the eight genotypes of HBV, E, and F are local, and confined to Central Africa and Central/South America, respectively [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003]. Genotype H is genetically close to F and distributes in Central America [Arauz-Ruiz et al., 2002]. Genotype G occurs very rarely [Stuyver et al., 2000; Chu et al., 2003; Kato et al., 2004], and is always

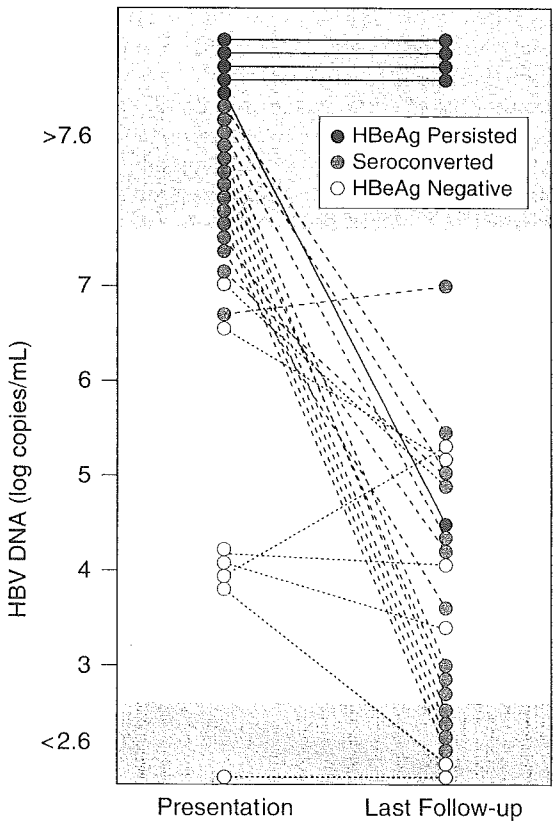

Fig. 5. Changes in serum levels of HBV DNA from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

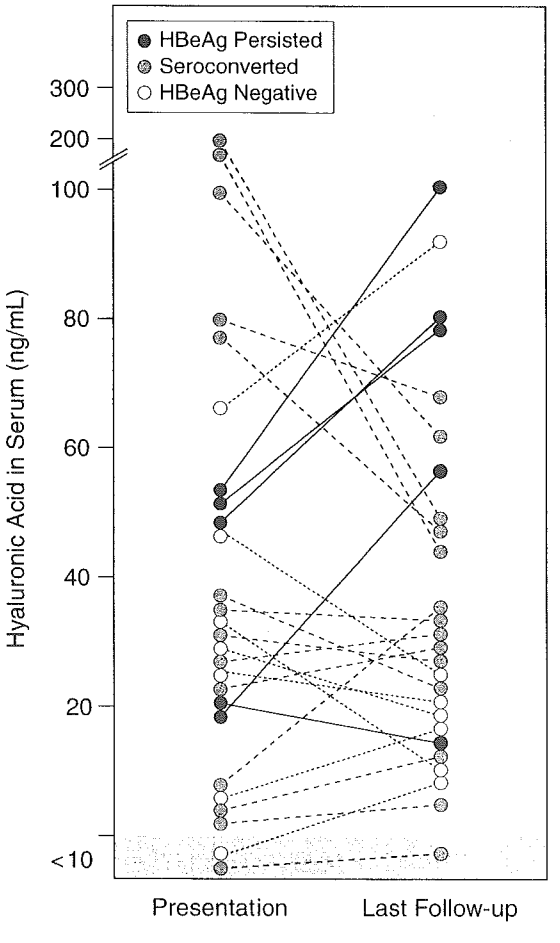

Fig. 6. Changes in serum levels of hyaluronic acid from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

TABLE III. Loss of HBeAg in the 19 Hepatitis Patients Infected With HBV Genotype A Who Had Been Followed for Longer Than 3 Years

Case No.	Sex/age	Pathology	Sub-group	Treatment	HBeAg Lost
1	M23	F1/A1	Ae	Interferon	Yes
2	M33	F2/A1	Ae	Interferon	Yes
3	M44	F3/A1	Ae	Interferon	Yes
4	M57	F2/A1	Ae	Interferon	Yes
5	M13	F1/A1	Ae	Steroid withdrawal	Yes
6	M16	F1/A1	Ae	Steroid withdrawal	Yes
7	M28	F1/A1	ND	Steroid withdrawal	Yes
8	M47	F2/A1	Aa	Steroid withdrawal	Yes
9	M17	F1/A1	Ae	Steroid/Interferon	Yes
10	M29	F1/A1	Ae	Lamivudine	Yes
11	M38	F1/A1	Ae	Lamivudine	Yes
12	M30	F1/A0	Ae	None	Yes
13	M39	F1A1	Ae	None	Yes
14	M47	F3/A2	Ae	None	Yes
15	M24	F2/A2	Ae	Interferon and others[b]	No
16	M43	F2/A1	Ae	Steroid/Interferon	No
17	M48	F1/A2	Ae	Interferon/Lamivudine	No
18	M49	F1/A1	ND[a]	Steroid withdrawal	No
19	M59	F1A1	Ae	Interferon	No

[a]Not determined.
[b]The patient received interferon, lamivudine interferon/lamivudine, and then lamivudine plus entecavir.

co-infected with HBV of the other genotypes [Kato et al., 2002, 2003]. Thus, only four genotypes (A–D) are left for comparison in epidemiological and clinical studies in most countries of the world. Since even these four genotypes have distinct geographical distributions, comparison with respect to severity of liver disease or response to antiviral treatment is hardly feasible among them, except in multi-national studies on patients of diverse ethnicities [Westland et al., 2003; Janssen et al., 2005].

In the Toranomon Hospital in Tokyo, by far the most patients presenting with HBsAg were infected with HBV of genotype B (10.5%) or C (83.3%), and genotype A infected only a minority (3.10%) of them. During 31 years, 128 patients with genotype A visited there. Unlike most infections with genotype B and C transmitted perinatally from carrier mothers with HBeAg [Okada et al., 1976], genotype A infection in Japan is often acquired in the adulthood by men having extramarital sexual contacts either with men or women; there has been no evidence for maternal transmission of HBV genotype A in Japan [Kobayashi et al., 2002, 2003; Ogawa et al., 2002; Suzuki et al., 2005]. HBV infection prevails among homosexuals in Western countries where genotype A is frequent, who poorly respond to vaccines [Goilav and Piot, 1989]. Genotype A infection in Japan has a propensity to become chronic and tends to respond to antiviral therapies better than genotype B or C infection [Kobayashi et al., 2002, 2003 Suzuki et al., 2005].

In the present study, we have compared the virological outcome among infections with HBV genotypes A, B, and C, and found substantial differences. Patients with genotype A fared better than those with genotype B or C in that they cleared HBsAg and HBeAg faster during follow-up (Figs. 1 and 2). It is not certain, however, whether or not the observed differences are influenced by the duration of HBV infection. HBV genotype A is contracted predominantly by men in the adulthood and genotypes B or C had been transmitted perinatally until 1986 when the national immunoprophylaxis started. It needs to be pointed out that this study is retrospective in nature, and most patients with HBeAg had received interferon, lamivudine or steroid withdrawal, or combination thereof. Of the 16 patients with genotype A who received treatment, 11 (69%) responded and cleared HBeAg from serum. In addition, three patients lost HBeAg spontaneously. Hence seroconversion was achieved in 14 of the 19 (74%) patients with genotype A. In view of lamivudine, adefovir dipivoxil, and pegylated interferon that are reported efficacious in treatment of chronic hepatitis B [Perrillo et al., 2000; Hadziyannis et al., 2003; Kumada, 2003; Janssen et al., 2005], it would be unethical to evaluate genotype-dependent differences in the natural course of persistent HBV infection.

Of the 45 individuals chronically infected with HBV genotype A and had been followed for 3 years or longer, HBeAg was more frequent and HBV DNA levels higher in the 26 patients with biopsy-proven chronic hepatitis than in the 19 asymptomatic carriers. Among the 26 patients with genotype A, HBeAg persisted throughout the observation in 5 (19%) and disappeared in 14 (54%); HBeAg remained negative in the other 7 (27%) patients. HBV DNA stayed in high levels more frequently ($P = 0.0095$) in the patients with persistent HBeAg (80% [4/5]) than in those who seroconverted (7% [1/14]) or remained HBeAg-negative (29% [2/7]). Furthermore, increases in serum hyaluronic acid $\geq$10 ng/ml were more frequent ($P = 0.017$) in the patients with persistent HBeAg (80% [4/5]) than in those with seroconversion (14% [2/17]) or HBeAg-negative (14% [1/7]). Although the patients with genotype A fare better than those with genotype B or C, persistent HBeAg refractory to

treatment would predict ongoing liver disease with fibrosis in progress.

Recently, subgenotypes have been recognized and they may influence the biology of HBV and liver disease. For instance, a subgenotype of B having the recombination with genotype C (Ba) induces more severe liver disease with poorer response to lamivudine than that without the recombination (Bj) [Sugauchi et al., 2002, 2003; Akuta et al., 2003]. As for genotype A, there are two subgenotypes with different geographical distributions. Subgenotype Ae is common in Europe and the United States, while Aa is prevalent in Asia and Africa [Bowyer et al., 1997; Sugauchi et al., 2004]. In a case-control study, HBeAg was more frequent and HBV DNA levels higher in carriers of Ae than Aa [Tanaka et al., 2004]. The majority of genotype A strains from our patients (86%) were found to be Ae; they were probably introduced to Japan by immigrants and visitors from foreign countries [Kobayashi et al., 2004]. Cirrhosis and hepatocellular carcinoma developed in only one of the 19 (5%) patients infected with subgenotype Ae, in remarkable contrast to frequent hepatocellular carcinoma in Africa where infection with subgenotype Aa is common during the infancy [Kew et al., 2005].

Although there have been accumulating lines of evidence for virological and clinical influence of HBV genotypes, there are conflicting views on them. Differences between genotypes B and C in Asia [Kao et al., 2000; Orito et al., 2001; Tsubota et al., 2001; Chan et al., 2004; Yu et al., 2005] have not been reproduced, probably due to selection bias for the patients with severe disease [Sumi et al., 2003] or subgenotypes of B different between Japan (Bj) and Hong Kong (Ba) [Yuen et al., 2004]. Liver disease, once advanced beyond a certain severity, will progress spontaneously irrespective of HBV genotypes. Subgenotype Ba having the recombination with genotype C may be endowed with a higher disease-inducing capacity than subgenotype Bj without the recombination [Sugauchi et al., 2002].

Of patients infected with three different genotypes in Japan, the virological outcome of persistent HBV infection was more favorable for those with genotype A than B and C in that order. It is not known where genotype D stands, although it fares worse than genotype A in chronic HBV infection [Thakur et al., 2002; Janssen et al., 2005]. In ranking the four major genotypes (A–D) in disease-inducing capacity and response to antiviral therapies, perinatals, or adulthood transmission, as well as subgenotypes inherent to countries, would have to be taken into considerations [Sugauchi et al., 2002, 2004; Norder et al., 2004].

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Predictive Factors of Virological Non-Response to Interferon–Ribavirin Combination Therapy for Patients Infected With Hepatitis C Virus of Genotype1b and High Viral Load

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Patients with high viral load ($\geq 1.0 \times 10^5$ IU/ml) of hepatitis C virus (HCV) genotype 1b do not achieve high sustained virological response rates to interferon (IFN)/ribavirin combination therapy. Previous studies suggested that pretreatment amino acid (aa) substitution patterns in the HCV core region could affect virological non-response especially in patients who could not achieve HCV-RNA negativity during treatment. The present study evaluated 167 consecutive Japanese adults with high HCV genotype 1b viral load who received combination therapy for ≥24 weeks. A case-control study matched for age, sex, genotype, and viral load was conducted to investigate the predictive factors for virological non-response, especially absolute virological non-response (patients who could not achieve >2 log decline of HCV RNA from baseline during the initial 24 weeks of therapy). Virological non-response was identified in 26.3% of patients, and 45.5% of these were absolute virological non-responders. Multivariate analysis identified ribavirin dose <11.0 mg/kg, moderate-to-severe hepatocyte steatosis, and substitutions of aa 70 and/or 91 in the core region as significant independent factors associated with virological non-response. The majority of absolute virological non-responders had such substitutions in the core region (95.0%), as well as substitution of glutamine at aa 70 and/or methionine at aa 91 (90.0%). In the present work, such substitutions significantly affected the viral kinetics in virological non-responders. The results suggest that viral, host, and treatment-related factors determine the response to IFN/ribavirin combination therapy in patients with high HCV genotype1b viral load, and that amino acid substitution patterns in the core region is potentially useful pretreatment predictor of virological non-response. ***J. Med. Virol. 78:83–90, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; core region; hepatocyte steatosis; interferon; ribavirin; virological non-response; case-control study

INTRODUCTION

The aims of IFN therapy for chronic hepatitis C virus (HCV) infection include reduction of the risk of development of HCC and liver-related death by viral clearance, and then by normalization of alanine aminotransferase (ALT) even if viral clearance cannot be achieved [Ikeda et al., 1999; Akuta et al., 2005a]. The most effective initial therapy for viral clearance is the combination of interferon (IFN) and ribavirin administered for 48 weeks [Manns et al., 2001; Fried et al., 2002]. However, patients with high load of genotype 1b virus ($\geq 1.0 \times 10^5$ IU/ml), dominant in Japan, do not achieve high sustained virological response rates (less than 50%), even when the most effective combination treatment (pegylated IFN plus ribavirin) is administered for 48 weeks [Manns et al., 2001; Fried et al., 2002]. Furthermore, in genotype 1b, virological non-responders are seen frequently who do not achieve HCV-RNA

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negativity, as determined by polymerase chain reaction (PCR), during treatment. The underlying mechanism(s) of the different virological response to treatment in patients with 1b strain infection is still not clear.

Using multivariate analysis, Akuta et al. [2005b] identified hypoalbuminemia, pretreatment substitutions of amino acid (aa) 70 in the core region and pretreatment substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response, based on 48-week combination therapy of IFN plus ribavirin [Akuta et al., 2005b]. Especially, substitutions of arginine (R) by glutamine (Q) at aa 70, and/or leucine (L) by methionine (M) at aa 91 were significantly more common in virological non-responders. Decline of HCV-RNA levels during treatment in patients with specific substitutions in the core region was significantly less than in those without such substitutions [Akuta et al., 2005b].

The aims of the present study were the following: (1) to investigate the proportion of virological non-responders among a large number of Japanese adult patients who received combination therapy. Especially, to determine the proportion of absolute virological non-responders (i.e., ultimate resistant cases) who did not achieve a log decline of more than 2 from baseline HCV RNA during the initial 24 weeks of therapy, (2) to conduct a case-control study between groups matched for age, sex, genotype, and viral loads, to identify the predictive factors associated with virological non-response, including pretreatment amino acid substitution patterns in the core region, (3) to examine the initial viral kinetics in virological non-responders according to the virological features of the core region.

PATIENTS AND METHODS

Study Population

A total of 323 HCV-infected Japanese adult patients were recruited consecutively into the study of combination therapy with IFN (pegylated [PEG]-IFNα-2b or IFNα-2b) plus ribavirin for 24 weeks or more between 1999 and 2004 at Toranomon Hospital, Tokyo, Japan. Among these, 167 patients were selected in the present study based on the following criteria. (1) They were negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emerville, CA), and positive for HCV RNA qualitative analysis with PCR (Amplicor, Roche Diagnostic Systems, California). (2) They were naive to ribavirin therapy. (3) They were infected with HCV genotype 1b alone. (4) Each had a high viral load ($\geq 1.0 \times 10^5$ IU/ml) by quantitative analysis of HCV RNA with PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Tokyo, Japan) at the start of treatment. (5) Each had chronic hepatitis, without cirrhosis or hepatocellular carcinoma (HCC), as confirmed by biopsy examination within the preceding 12 months of enrolment. (6) They had abnormal serum ALT levels (the upper limit of normal for ALT; 50 IU/L) within the preceding 2 months of enrolment. (7) Their body weight was >40 kg. (8) All were free of coinfection with human immunodeficiency virus. (9) None had been treated with antiviral or immunosuppressive agents within the preceding 3 months of enrolment. (10) None was an alcoholic; lifetime cumulative alcohol intake was <500 kg (mild to moderate alcohol intake). (11) None had diabetes, other forms of hepatitis, such as hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease. (12) None of the females was pregnant or lactating mother. (13) All accepted treatment for 24 weeks or more as outlined in the study protocol, as well as repeated evaluation of HCV-RNA levels during treatment (at least once every month). (14) Each signed a consent form of the study protocol that had been approved by the Human Ethics Review Committee of Toranomon Hospital.

With regard to the treatment protocol, 21 (31.8%) patients received PEG-IFNα-2b at a dose of 1.5 μg/kg subcutaneously each week plus oral ribavirin at 600–800 mg/day for 24 weeks or more. The remaining 45 (68.2%) patients received 6 million units of IFNα-2b intramuscularly each day for 24 weeks or more (daily for the initial 2 weeks, followed by three times per week for 22 weeks or more), and oral ribavirin at a dose of 600–800 mg/day for 24 weeks or more.

Table I summarizes the profiles and data of the 167 patients at the commencement of combination therapy of IFN plus ribavirin. They included 119 men and 48 women, aged 22–68 years (median, 54 years). The median total duration of treatment was 24 weeks (range, 24–48 weeks). In 46 (27.5%) patients, the dose of ribavirin was reduced during treatment due to a fall in hemoglobin concentration.

Patients who remained positive for HCV RNA based on quantitative and/or qualitative PCR analyses during and at the end of initial 24 weeks of combination therapy, were defined as virological non-responders. On the other hand, patients who became HCV RNA negative by qualitative PCR analysis during and/or at the end of initial 24 weeks were defined as virological responders. Virological non-responders who could not or could achieve a log decline of more than 2 from baseline of HCV RNA based on quantitative PCR analyses during the initial 24 weeks of combination therapy, were defined as absolute virological non-responders or relative virological non-responders, respectively.

Applying multivariate analysis, previous studies identified substitutions of aa 70 in the core region and substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response to combination therapy in patients with high viral load of genotype 1b [Akuta et al., 2005b]. Therefore, based on the larger numbers of patients, a case-control study was conducted to compare the substitution patterns in aa 70 and/or aa 91 of the core region, between virological non-responders and virological responders who were matched for age, sex, genotype, and viral load, in the present study.

TABLE I. Patient Profile and Laboratory Data at Commencement of Combination Therapy of Interferon Plus Ribavirin

n	167
Age (years)*	54 (22–68)
Sex (M/F)	119/48
Positive history of blood transfusion	50 (29.9%)
Positive family history of liver disease	52 (31.1%)
Genotype 1b	167 (100%)
High viral load ($\geq 1.0 \times 10^5$ IU/ml)	167 (100%)
Serum alanine aminotransferase (IU/l)*	90 (24–398)
Serum albumin (g/dl)*	3.8 (2.7–4.7)
Hemoglobin (g/dl)*	14.8 (11.1–18.2)
Platelet count ($\times 10^4/mm^3$)*	17.3 (7.1–26.4)
Stage (F1/F2/F3)[a]	94/44/29

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Stage of chronic hepatitis by Desmet et al. [1994]. ALT levels were abnormal in all patients at recruitment. Normal reference ranges: 6–50 IU/L for alanine aminotransferase and 3.9–5.2 g/dl for albumin.

Laboratory Tests

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for ALT and HCV-RNA levels. The serum samples were frozen at −80°C within 4 hr of collection and were thawed at the time of measurement. HCV genotype was determined by PCR using a mixed primer set derived from the nucleotide sequences of NS5 region [Chayama et al., 1993]. HCV-RNA levels were measured quantitatively by PCR (Cobas Amplicor HCV monitor v 2.0 using the 10-fold dilution method, Roche Diagnostics, Tokyo, Japan) at least once every month before, during, and after therapy. The dynamic range of the assay was 5.0×10^3 to 5.0×10^6 IU/ml. Samples collected during and after therapy that showed undetectable levels of HCV-RNA ($<5.0 \times 10^3$ IU/ml) were checked also by qualitative PCR (Amplicor, Roche Diagnostic Systems, California), which has a higher sensitivity than quantitative analysis, and the results were expressed as positive or negative. The lower limit of the assay was 50 IU/ml.

Histopathological Examination of Liver Biopsies

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examinations contained six or more portal areas. Histopathological diagnosis was confirmed by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was graded as either none (absent), mild (less than 1/3 of hepatocytes involved), moderate (greater than 1/3 but less than 2/3 of hepatocytes involved), or severe (greater than 2/3 of hepatocytes involved) [D'Alessandro et al., 1991].

Nucleotide Sequencing of the Core and NS5A Gene

The core amino acids (aa) 1–191 and NS5A aa 2209–2248 (IFN-sensitivity determining region [ISDR]) [Enomoto et al., 1995, 1996] sequences were determined by the direct sequencing method using pretreatment sera of 66 patients. These sequences were compared with the consensus sequence of genotype 1b, which was determined by comparing the sequences obtained in this study and prototype sequence (HCV J) [Kato et al., 1990]. HCV RNA was extracted from serum samples at the start of treatment and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′) primers, and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (antisense) primers. (b) Nucleotide sequences of ISDR in NS5A: The first-round PCR was performed with ISDR1 (sense, 5′-ATG CCC ATG CCA GGT TCC AG-3′) and ISDR2 (antisense, 5′-AGC TCC GCC AAG GCA GAA GA-3′) primers, and the second-round PCR with ISDR3 (sense, 5′-ACC GGA TGT GGC AGT GCT CA-3′) and ISDR4 (antisense, 5′-GTA ATC CGG GCG TGC CCA TA-3′) primers. ([a], hemi-nested PCR; [b], nested PCR). All samples were denatured initially at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first PCR product was transferred to the second PCR reaction. The conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy

Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan).

To avoid false-positive results, the procedures recommended by Kwok and Higuchi [1989] to prevent contamination were strictly applied to these PCR assays. No false positive results were observed in this study.

Viral Kinetic Study of Virological Non-Response

Viral kinetics in the initial 24 weeks was evaluated in the two groups of absolute virological non-responders and relative virological non-responders at three time points (8, 12, and 24 weeks during treatment). Decline of HCV-RNA levels from baseline was expressed using $\log_{10}$ of viral load at each time point, in comparison with the pretreatment viral load. For data analysis, $\log_{10}$ of the cut-off value (5.0×10^3 IU/ml) was used for HCV-RNA values below the limit of detection.

Statistical Analysis

Non-parametric tests were used to compare the characteristics of the groups, including the Mann–Whitney U test, Chi-squared test, and Fisher's exact probability test. Multiple comparisons were examined by the Bonferroni test. Univariate and multivariate logistic regression analyses were used to determine the factors that significantly contributed to virological non-response. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ($P<0.05$) or marginal significance ($P<0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential predictive factors associated with virological non-response included the following variables: sex, age, history of blood transfusion, familial history of liver disease, body mass index, ALT, albumin, hemoglobin, platelet count, indocyanine green retention rate at 15 min (ICG R15), serum iron, serum ferritin, creatinine clearance, viremia level, pathological staging, hepatocyte steatosis, type of IFN, ribavirin dose relative to body weight, dose reduction, and pretreatment amino acid substitution in the core and ISDR of NS5A. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

The response to IFN/ribavirin combination treatment protocol among the 167 patients included virological non-response in 44 (26.3%) and virological response in 123 (73.7%). Furthermore, the first group of 44 virological non-responders consisted of 20 absolute virological non-responders (45.5%) and 24 relative virological non-responders (54.5%). To compare the pretreatment features between virological non-responders and virological responders, all 44 virological non-responders entered a case-control study along with 22 virological responders. The latter group was selected from among the 123 because they matched patients of the virological non-response group with respect to sex, age, genotype, and viral load. Table II lists the clinical and virological features of patients who entered the matched case-control study.

Predictive Factors Associated With Virological Non-Response in Multivariate Analysis

The clinical and virological data listed in Table II for the whole population sample were analyzed to determine the factors that could predict virological non-response. Univariate analysis identified six parameters that tended to or significantly influenced the virological non-response. These included ribavirin dose according to body weight ($P=0.019$), staging ($P=0.024$), serum albumin ($P=0.062$), hepatocyte steatosis ($P=0.049$), and presence of aa substitution in HCV core in the pretreatment sample (substitution of aa 70, $P=0.030$; and aa 70 and/or 91, $P=0.006$). ISDR amino acid substitutions, which had been reported as one predictor of sustained virological response by IFN monotherapy [Enomoto et al., 1995, 1996], were not identified as a predictor of virological non-response to the combination therapy of IFN/ribavirin.

Multivariate analysis identified three parameters that independently influenced virological non-response; ribavirin dose ($P=0.019$), hepatocyte steatosis ($P=0.040$), and substitutions of aa 70 and/or 91 ($P=0.005$) (Table III).

Treatment Efficacy According to Amino Acid Substitution Patterns in HCV Core Region

Frequencies of the substitution site at aa 70 were 60.0% (12/20), 37.5% (9/24), and 18.2% (4/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution site in absolute virological non-responders was significantly higher than that in virological responders ($P=0.015$; Bonferroni test). Frequencies of substitution pattern of glutamine (Q) at aa 70 were 55.0% (11/20), 37.5% (9/24), and 13.6% (3/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution pattern in absolute virological non-responders was significantly higher than that in virological responders ($P=0.014$; Bonferroni test).

The frequencies of substitution sites at aa 70 and/ or 91, which were a significant predictor of virological non-response based on multivariate analysis, were 95.0% (19/20), 62.5% (15/24), and 40.9% (9/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution sites in absolute virological non-responders was significantly higher than that in relative virological non-responders ($P=0.049$; Bonferroni test) and virological responders

TABLE II. Clinical and Virological Features of Patients Infected With HCV Genotype1b With or Without Virological Response to Combination Therapy of Interferon Plus Ribavirin (Matched Case-Control study)

	Virological non-responders (case; n = 44)	Virological responders (control; n = 22)
Matching data		
Age (years)*	53 (24–67)	53 (20–64)
Sex (M/F)	33/11	17/5
Genotype 1b	44 (100%)	22 (100%)
High viral load ($\geq 1.0 \times 10^5$ IU/ml)[b]	44 (100%)	22 (100%)
Demographic data		
Positive history of blood transfusion	8 (18.2%)	6 (27.3%)
Positive family history of liver disease	11 (25.0%)	7 (31.8%)
Body mass index (kg/m^2)*	23.5 (17.3–32.3)	22.9 (19.3–28.8)
Laboratory data*		
Serum alanine aminotransferase (IU/L)	78.5 (24–247)	100.5 (43–276)
Serum albumin (g/dl)	3.7 (3.3–4.7)	3.9 (3.4–4.2)
Hemoglobin (g/dl)	14.7 (12.0–17.0)	15.0 (12.2–17.4)
Platelet count ($\times 10^4/mm^3$)	16.2 (7.1–26.6)	15.7 (10.1–30.9)
ICG R15 (%)[a]	18 (7–49)	12 (7–26)
Serum iron (μg/dl)	149 (51–253)	142 (52–308)
Serum ferritin (μg/L)	158 (19–696)	136 (<10–644)
Creatinine clearance (ml/min)	95.7 (42.6–174.6)	106.3 (45.7–131.0)
Viral load (KIU/ml)	1,650 (160–5100)	1,700 (650–4900)
Histological findings		
Stage (F1/F2/F3)[b]	19/15/10	15/7/0
Hepatocyte steatosis (none-mild/ moderate-severe)	33/11	21/1
Treatment		
PEG-IFNα-2b/IFNα-2b	11/33	10/12
Ribavirin dose (mg/kg)*	10.8 (7.3–14.2)	11.4 (9.7–13.0)
Virological features		
Number of amino acid substitutions in ISDR (0/1–3/≥4/ND)	26/11/3/4	10/10/2/0
Presence of amino acid substitutions sites in the core region		
aa 70	21 (47.7%)	4 (18.2%)
aa 91	22 (50.0%)	7 (31.8%)
aa 70 and/ or 91	34 (77.3%)	9 (40.9%)

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]ICG R15: indocyanine green retention rate at 15 min.
[b]Stage of chronic hepatitis by Desmet et al. [1994]. ALT levels were abnormal in all patients at recruitment. Normal reference ranges: 6–50 IU/L for alanine aminotransferase and 3.9–5.2 g/dl for albumin.

($P < 0.001$; Bonferroni test). Frequencies of substitution patterns of glutamine (Q) at aa 70 and/or methionine (M) at aa 91 were 90.0% (18/20), 62.5% (15/24), and 40.9% (9/22) in the three groups of absolute virological non-responders, relative virological non-responders, and virological responders, respectively. The proportion of such substitution patterns in absolute virological non-responders was significantly higher than in virological responders ($P = 0.002$; Bonferroni test). Figure 1 shows the association of aa substitution patterns at aa 70 and/or 91 and response to combination therapy. There were no significant differences in other substitution sites, patterns and treatment efficacy among the three groups.

Viral Kinetics in Virological Non-Responderstpb

The decline of HCV-RNA levels at 8, 12, and 24 weeks relative to baseline was evaluated in absolute virological non-responders and relative virological non-responders. The decline at each time point was significantly lower in

TABLE III. Factors Associated With Virological Non-Response to Combination Therapy of Interferon Plus Ribavirin in 66 Patients Infected With HCV Genotype 1b, Identified by Multivariate Analysis

Factor	Category	Odds ratio (95% confidence interval)	P
Ribavirin dose (mg/kg)	1: <11.0	1	
	2: ≥11.0	0.195 (0.050–0.765)	0.019
Hepatocyte steatosis	1: None, mild	1	
	2: Moderate, severe	14.299 (1.127–181.344)	0.040
Substitution of aa 70 and/or 91	1: Absent	1	
	2: Present	7.343 (1.841–29.285)	0.005

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

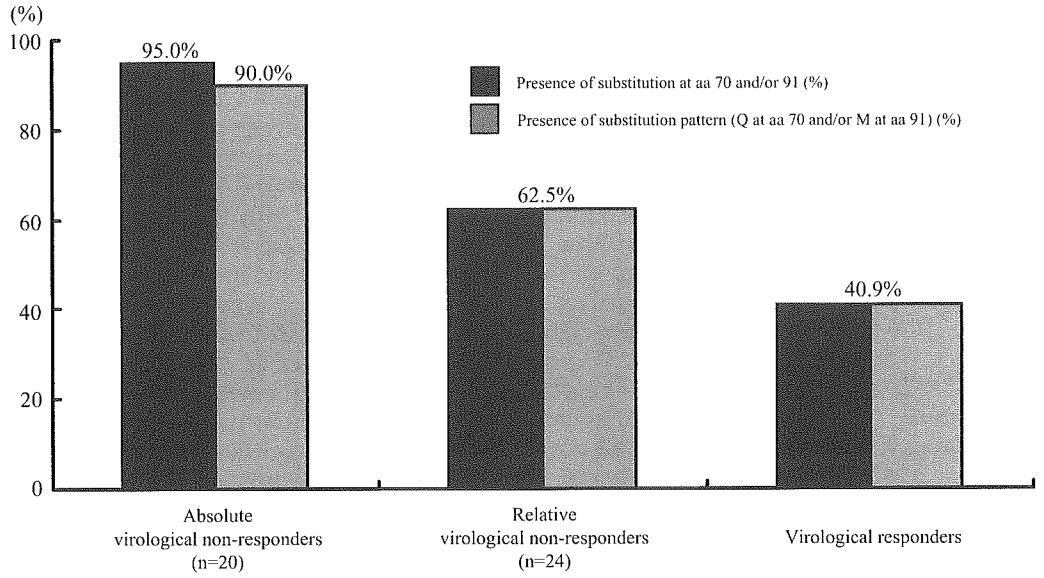

Fig. 1. Frequencies of substitutions at amino acid sites 70 and/or 91 and substitution patterns (glutamine [Q] at aa 70 and/or methionine [M] at aa 91) in HCV core region are evaluated in three groups of absolute virological non-responders, relative virological non-responders, and virological responders. The proportion of such substitution sites in absolute virological non-responders was significantly higher than that in relative virological non-responders ($P = 0.049$; Bonferroni test) and virological responders ($P < 0.001$; Bonferroni test). The proportion of such substitution patterns in absolute virological non-responders was significantly higher than that in virological responders ($P = 0.002$; Bonferroni test).

absolute virological non-responders than in relative virological non-responders (8 weeks, $P = 0.001$; 12 weeks, $P < 0.001$; 24 weeks, $P < 0.001$). Figure 2 shows the decline of HCV-RNA levels in virological non-responders, according to aa substitutions of the core region. The decline at each time point was significantly lower in patients with substitution sites of aa 70 and/or 91 than in those without them (8 weeks, $P = 0.004$; 12 weeks, $P = 0.005$; 24 weeks, $P = 0.013$), and with substitution patterns of Q at aa 70 and/or M at aa 91 than in those without them (8 weeks, $P = 0.008$; 12 weeks, $P = 0.015$; 24 weeks, $P = 0.011$).

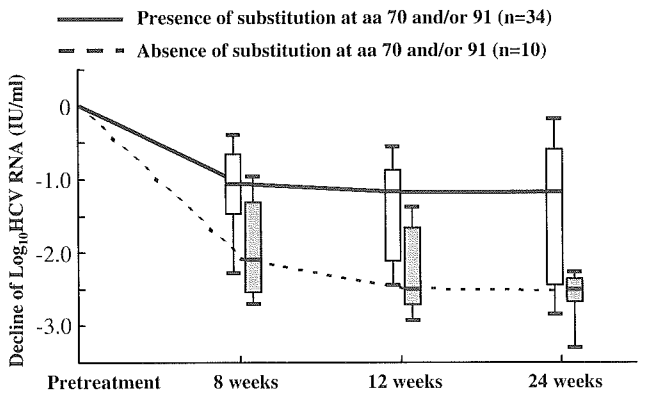

Fig. 2. Log changes in viral load from baseline at 8, 12, and 24 weeks during treatment, according to amino acid substitutions of the HCV core region. Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25th to 75th centiles, the lower and upper bars the 10th and 90th centiles, respectively. The decline of HCV-RNA levels at each time point was significantly lower in patients with substitution sites of aa 70 and/or 91 than in those without them (Mann–Whitney U test).

DISCUSSION

Using multivariate analysis, Akuta et al. [2005b] identified pretreatment substitutions of aa 70 in the core region and substitutions of aa 91 as independent and significant pretreatment factors associated with virological non-response to 48-week combination therapy of IFN plus ribavirin. Substitutions of R by Q at aa 70 and/or L by M at aa 91, were significantly more common in virological non-responders. Furthermore, decline of HCV-RNA levels during treatment in patients with specific substitutions in the core region was significantly less than in those without such substitutions [Akuta et al., 2005b]. Using the same analysis, the present study based on a larger number of patients has also identified substitution patterns in aa 70 and/or aa 91 as independent and significant pretreatment factors associated with virological non-response to combination therapy, by a case-control study matched for age, sex, genotype, viral loads. Especially, most absolute virological non-responders, as ultimate resistant cases, were found to have such specific substitution sites (95.0%), and also had substitution patterns of glutamine (Q) at aa 70 and/or methionine (M) at aa 91 (90.0%).

Furthermore, such specific substitutions also significantly affected the viral kinetics in absolute virological non-responders and relative virological non-responders. Hence, we propose that the aa substitution pattern in the core region is useful as a pretreatment predictor of virological non-response to IFN/ribavirin combination therapy.

IFN-α and IFN-β bind to type I IFN receptor, and one major pathway in type I IFN signaling involve the Jak-STAT signaling cascade [Song and Shuai, 1998; Stoiber

et al., 1999; Auernhammer and Melmed, 2001; Alexander, 2002; Fujimoto and Naka, 2003; Lalvakolanu, 2003; Vlotides et al., 2004]. Previous studies reported that the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade [Blindenbacher et al., 2003; Bode et al., 2003; Melén et al., 2004; de Lucas et al., 2005]. The present study identified amino acid substitutions in the HCV core as a predictor of virological non-response to IFN/ribavirin combination therapy. This result suggests that substitutions of amino acids in the HCV core region might be associated with resistance to the antiviral actions of IFN therapy involving the Jak-STAT signaling cascade. Further studies that examine the structural and functional impact of core amino acid 70 and/or 91 substitutions during IFN/ribavirin combination therapy should be conducted in the future to confirm the above finding.

In the present study, virological non-response was noted in 26.3% of patients with high viral load of genotype 1b who received IFN/ribavirin combination therapy. This rate is worse than that of only 2.0% in patients with high viral load of genotype 2a treated with IFN alone [Akuta et al., 2002]. Akuta et al. [2002] examined patients infected with genotype 2a and reported that virological non-responders had higher viral load and one or more of other negative predictive factors associated with sustained virological response (i.e., lower total dose of IFN, moderate-to-severe grade of hepatocyte steatosis, lower levels of albumin, and ALT). Based on the above findings, it was concluded that a complex of negative predictive factors, including viral, host, and treatment-related factors, was the underlying cause of resistance to IFN treatment [Akuta et al., 2002]. Using multivariate analysis, the present study of patients with high viral load of genotype 1b who were treated with IFN/ribavirin, also identified lower ribavirin dose (as treatment-related factor), moderate-to-severe grade of hepatocyte steatosis (as host factor), and substitutions of aa 70 and/or 91 in the core region (as viral factor) as independent and significant factors associated with virological non-response. In this regard, another recent study did not identify ribavirin dose as an independent and significant predictor of virological non-response [Akuta et al., 2005b]. This discrepant finding may be due to the non-uniform dose of ribavirin used in the treatment of patients, which was not strictly adjusted according to body weight (e.g., 600 mg for weight $\leq$60 kg, and 800 mg for weight >60 kg). Thus, the response to combination therapy of IFN/ribavirin is based on a dynamic tripartite interaction of the virus, host, and treatment-related factors. Further understanding of the complex interactions between these factors should facilitate the development of more effective therapeutic regimens.

Akuta et al. [2005b] reported that virological response to 48-week combination therapy of IFN/ribavirin was significantly influenced as negative predictive factor by the presence of pretreatment hypoalbuminemia, which might reflect liver function, based on multivariate analysis. However, the same analysis in the present study did not identify serum albumin concentration as a significant predictor of virological non-response, although univariate analysis identified it as one of the parameters that tended to influence virological non-response. This discrepant finding could be due to one or more factors. The first is probably related to the design of the present study based on a case-control study matched for age and sex. The second is probably related to the relatively small number of patients in the previous study. A large-scale prospective study should be conducted in the future to establish the role of pretreatment hypoalbuminemia in virological non-response to 48-week IFN/ribavirin combination therapy.

In conclusion, the present study demonstrated that amino acid substitution patterns in the core region is a potentially useful predictor of virological non-response. One limitation of this study was that it did not examine other viral factors, such as amino acid substitutions in areas other than the core region and ISDR of HCV genome, as well as other host factors such as IFN-inducible protein kinase, MxA, and 2′,5′-OAS protein [Gale et al., 1997; Wang and Floyd-Smith, 1997; Ronni et al., 1998; Antonelli et al., 1999; Akuta et al., 2003; Vlotides et al., 2004]. These factors should be investigated together with other factors in future studies. Moreover, further large-scale prospective studies are necessary to investigate whether the present results also explain resistance to combination therapy of IFN/ribavirin.

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Distinct Geographic Distributions of Hepatitis B Virus Genotypes in Patients With Acute Infection in Japan

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Genotypes of hepatitis B virus (HBV) were determined in 145 patients with acute hepatitis B from various districts in Japan to establish their geographic distribution and evaluating the influence on the clinical illness and outcome. Genotypes were A in 27 (19%) patients, B in 8 (5%), C in 109 (75%) and mixed with B and C in the remaining one (1%). Genotype A was more frequent in metropolitan than the other areas (21/69 (30%) vs. 6/76 (8%), $P<0.001$). On phylogenetic analysis, seven of the nine (78%) HBV/A isolates selected at random clustered with those from Europe and the United States, while the remaining two with those of subgroup A′ prevalent in Asia and Africa. Maximum ALT levels were lower (2069 ± 1075 vs. 2889 ± 1867 IU/L, $P=0.03$) and baseline HBV DNA titers were higher (5.90 ± 1.45 vs. 5.13 ± 1.36 log genome equivalents (LGE)/ml, $P=0.002$) in patients infected with genotype A than C. Hepatitis B surface antigen persisted longer in patients infected with genotype A than C (1.95 ± 1.09 vs. 1.28 ± 1.42 months, $P=0.02$). HBV infection became chronic in one (4%) patient with genotype A and one (1%) with genotype C infection. Fulminant hepatic failure developed in none of the patients with genotype A, one (13%) with genotype B and five (5%) with genotype C. The point mutation in the precore region (A1896) or the double mutations in the basic core promoter (BCP) region (T1762/A1764) were detected in none of the patients with genotype A, two (25%) with genotype B and 27 (26%) with genotype C. In conclusion, genotype A is frequent in patients with acute hepatitis B in metropolitan areas of Japan, probably reflecting particular transmission routes, and associated with longer and milder clinical course than genotype C. *J. Med. Virol. 77:39–46, 2005.* © 2005 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; genotypes; epidemiology; hepatitis B virus; hepatitis B e antigen; sexuality; Japan

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INTRODUCTION

The clinical outcome in patients with acute hepatitis B varies widely. Although hepatitis is self-limited in most patients, the clinical features range from asymptomatic to fulminant hepatic failure, while some patients become carriers of hepatitis B virus (HBV) [Chan HL and Lok, 1999; Chan HLY, 1999]. Factors that determine the clinical outcome remain unknown.

Viral nucleotide (nt) mutations have been shown to influence the clinical outcome of acute hepatitis B. Mutations in the precore region (A1896) and the basic core promoter (BCP) region (T1762/A1764) are common in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996]. Viral factors other than these mutations may influence the clinical outcome of acute hepatitis B.

Eight genotypes of HBV have been identified by sequence divergence greater than 8% in the entire genome, and they are designated by capital alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Araúz-Ruiz et al., 2002]. Furthermore, recombinant HBV strains consisting of two different genotypes have been reported [Bollyky et al., 1996; Morozov et al., 2000]. Genotype distribution is different in different countries and even in distinct areas of the same country [Orito et al., 2001a; Kao, 2002; Kato et al., 2002; Miyakawa and Mizokami, 2003]. Therefore, surveys on genotype distribution may be helpful in identifying transmission routes and evaluating clinical relevance.

It has been shown that the clinical outcome of chronic hepatitis B is influenced by HBV genotypes. In Asian patients with chronic hepatitis B, genotype C is associated with later seroconversion of hepatitis B e antigen (HBeAg) and more severe liver damage than genotype B [Kao et al., 2000; Orito et al., 2001b; Chu et al., 2002; Ding et al., 2002; Sugauchi et al., 2002a]. Likewise, a study from India has shown that genotype D is associated with more severe liver disease than genotype A [Thakur et al., 2002]. Genotype A is peculiar in that A1896 in the precore region occurs infrequently, because it causes instability of the stem-loop structures of the pregenome encapsidation signal [Li et al., 1993; Lok et al., 1994]. These reports suggest that HBV genotypes also influence the clinical characteristics of acute hepatitis. Recent studies on small numbers of patients with acute hepatitis B suggest that the clinical course may differ among infections with distinct HBV genotypes [Mayerat et al., 1999; Kobayashi et al., 2002; Ogawa et al., 2002]. However, the association between viral genotype and severity of liver disease remains uncertain in acute HBV infection.

To evaluate the effect of HBV genotypes on the clinical characteristics of acute hepatitis B, a multi-center study on 145 patients was conducted in Japan.

MATERIALS AND METHODS

Patients

During 1992 through 2001, serum samples were collected from 147 patients diagnosed with acute hepatitis B in our institutions. Only patients from whom sera at the onset of hepatitis were stored were included in this study. Sixty-nine (47%) patients lived in metropolitan areas (Kawasaki, Tokyo and Tokorozawa), while the others in Kurume, Ube, Osaka, Gifu, Nagoya and Sapporo. Criteria for the diagnosis of acute hepatitis B were: (1) Acute onset of liver injury without a history of liver dysfunction and detection of hepatitis B surface antigen (HBsAg) in serum, and (2) IgM antibody to HBV core (anti-HBc) in high titer. Co-infection with hepatitis A virus or hepatitis C virus was excluded by serological tests.

Among the 147 patients, acute hepatitis B in six (4%) was complicated by hepatic encephalopathy and prolonged prothrombin time for the diagnosis of fulminant hepatic failure. Other two (1%) patients remained positive for HBsAg for longer than 6 months, and they were considered to have acquired chronic infection.

Sera from the 147 patients with acute hepatitis B were examined virologically, and the results were correlated with clinical and demographic characteristics. Informed consent was obtained from each patient for the purpose of this study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committees of our institutions.

Determination of HBV DNA

Levels of HBV DNA were determined using transcription-mediated amplification (TMA) and hybridization-protection assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan) after the protocol as reported [Kamisango et al., 1999]. The range of detection by TMA was from 3.7 log genome equivalents (LGE)/ml ($10^{3.7}$ copies/ml corresponding to 5,000 copies/ml) to 8.7 LGE/ml ($10^{8.7}$ copies/ml). In 16 of 86 studied sera, levels of HBV DNA were under 3.7 LGE/ml and categorized in 3.7 LGE/ml.

Genotyping HBV

HBV genotypes in most samples were determined with commercial enzyme immunoassay kits (HBV Genotype EIA, Institute of Immunology Co. Ltd., Tokyo, Japan) involving monoclonal antibodies to genotype-specific epitopes in the preS2-region, as reported previously [Usuda et al., 1999, 2000; Kato et al., 2001]. Genotypes in 18 (12%) samples were determined by genotype-specific probe assay (Smitest HBV Genotyping Kit, Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by the polymerase chain reaction (PCR) with three sense primers (s1: 5′-ACC AAC CCT CTG GGA TTC TTT CC-3′, s2: 5′-ACC AAT CCT CTG GGA TTC TTC CC-3′ and s3: 5′-AGC AAT CCT CTA GGA TTC CTT CC-3′ [nt 2902–2924]) and an antisense primer (as1: 5′-GAG CCT GAG GGC TCC ACC C-3′ [nt 3091–3073]) biotinated at the 5′-end;

they were deduced from conserved sequences in the preS1 region of HBV. The biotin-labeled and amplified HBV DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one or other of the seven genotypes (A–G) immobilized on wells of a 96-well microplate. Thereafter, hybiridization was detected by staining with the streptavidine-horseradish peroxidase (HRP) conjugate [Kato et al., 2003].

Subtypes of genotype B, in terms of Ba with the recombination with genotype C and Bj without it were determined by direct sequencing of precore and core regions by the method reported previously [Sugauchi et al., 2002b].

Amplifying and Sequencing HBV DNA of Genotype A Isolates

A subgroup of genotype A is reported with the designation of A' from South Africa, Philippines, Malawi, and Belgium [Bowyer et al., 1997; Kramvis et al., 2002; Sugauchi et al., 2004]. Randomly selected HBV/A samples were classified into genotype A and subtype A' by sequencing the S region. For amplification and sequencing, the entire S region was divided into two fragments, spanning nt 3130–478 and nt 378–878, respectively, and they were amplified by two-stage PCR. The outer primers for amplification of the 1st fragment were: 5'-ACC AAT CGG CAG TCA GGA AG-3' (sense: nt 3121–3140) and 5'-CTG GAA TTA GAG GAC AAA CG-3' (antisense: nt 488–469) and the inner primers were: 5'-CAG TCA GGA AGG CAG CCT ACT-3' (sense: nt 3130–3150) and 5-AGG ACA AAC GGG CAA CAT AC-3' (antisense: nt 478–459). The outer primers for amplification of the 2nd fragment were: 5'-TGT CCT GGT TAT CGC TGG AT-3' (sense: nt 359–378) and 5'-CAA CGT ACC CCA ACT TCC AA-3' (antisense: nt 909–890) and the inner primers were: 5'-TGT GTC TGC GGC GTT TTA TC-3' (sense: nt 378–397) and 5'-ATG AAG TTT AGG GAA TAA CC-3' (antisense: nt 878–859).

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μl of the reaction mixture containing 200 μM dNTPs, 1.0 μM each of primers and 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Connecticut). PCR products (2 μl) were subjected to the second stage of amplification under the same conditions as the first stage. Standard precautions to avoid contamination were exercised during PCR, with a negative control serum included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Wisconsin), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California) using the PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The nucleotide sequences of HBV/A isolates from patients were compared with those of 25 reference HBV/A strains including subtype A' retrieved from the DDBJ/EMBL/GenBank database, as well as representatives of the other six major genotypes (B–G). Phylogenetic trees were constructed with the mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method [Sugita et al., 1991]. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling, and reconstruction were carried out 500 times.

Detection of Point Mutations in the Precore and BCP Regions of HBV

Mutation in the precore region for A1896 was detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) and mutations in the BCP region for T1762/A1764 were detected by enzyme-linked specific probe assay (Smitest HBV Core Promoter Mutation Detection Kit; Genome Science Laboratory, Tokyo, Japan) according to the manufacturer's instructions, after the principles described previously [Orito et al., 2001b]. The results were recorded as "the wild-type" and "the mutant-type" expressed dominantly by HBV isolates.

Statistical Analysis

Data were analyzed by chi-square test or Fisher's exact test for categorical data and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant. Logistic regression (backward logistic regression) was used in the multivariate analysis to evaluate the factors associated with differences between genotypes A and C.

RESULTS

Distribution of HBV Genotypes

HBV genotypes were determined in 145 of the 147 (99%) patients with acute hepatitis B; they were untypeable in the remaining two patients (Table I). Genotype A was detected in 27 (19%) patients, B in 8 (5%), C in 109 (75%), and mixed genotypes with B and C in the remaining one (1%). In the 69 patients with acute hepatitis B from metropolitan areas (Tokyo, Kawasaki, and Tokorozawa), genotype A was found in 21 (30%), B in 5 (7%), and C in 43 (63%). In the 76 patients from the other areas in the mainland, by contrast, genotype A occurred in 6 (8%), B in 3 (4%), C in 66 (87%), and mixed genotypes with B and C in one (1%). Thus, genotype A was significantly more frequent in patients with acute hepatitis B from the metropolitan than the other areas (30% vs. 8%; $P < 0.001$).

Demographic and Clinical Differences Among Patients Infected With HBV of Distinct Genotypes

Clinical and demographic backgrounds in patients with acute hepatitis B who were infected with HBV of

TABLE I. Demographic and Clinical Differences Among Patients With Acute Hepatitis Who Were Infected With HBV of Distinct Genotypes

Features	Genotypes of HBV A (n = 27)	B (n = 8)	C (n = 109)	B/C (n = 1)	Differences (A vs. C) Univariate (*P*-value)	Multivariate logistic regression (*P*-value)
Areas					<0.001	0.03
Metropolitan (n = 69)	21 (30%)	5 (7%)	43 (63%)	0		
Others (n = 76)	6 (8%)	3 (4%)	66 (87%)	1 (1%)		
Age (years)	29.3 ± 8.0	35.7 ± 10.1	36.6 ± 13.6	51	0.016	0.152
Male	25 (93%)	7 (88%)	69 (57%)	1 (100%)	0.003	0.018
Transmission routes						
Heterosexual	15 (56%)	3 (37%)	52 (48%)	0	0.197	
Homosexual	5 (19%)	1 (13%)	2 (2%)	0	<0.001	0.133
IV drugs	0	0	8 (7%)	0	0.280	
Unknown	7 (25%)	4 (50%)	47 (43%)	1 (100%)	0.102	
Fulminant hepatic failure	0	1 (13%)	5 (5%)	0	0.582	
ALT (IU/L)[a]	2069 ± 1075	2952 ± 1106	2889 ± 1867	646	0.030	0.084
Bilirubin (mg/dl)[a]	10.7 ± 14.1	10.3 ± 4.9	7.8 ± 6.7	4.8	0.533	
ALP (IU/L)[a]	476 ± 161	501 ± 94	432 ± 116	No data	0.542	
HBeAg	24/26 (92%)	4/8 (50%)	57/93 (61%)	1/1 (100%)	0.357	
Precore and BCP mutations						
Precore (1896A)	0/27	1/8 (13%)	20/102 (20%)	No data	0.250	
BCP (1762T/1764A)	0/27	1/6 (17%)	14/75 (19%)	No data	0.357	
Precore or BCP	0/27	2/8 (25%)	27/102 (26%)	No data	0.096	

[a]Maximum data are shown for alanine aminotransferase (ALT), bilirubin and alkaline phosphatase (ALP).

different genotypes are compared in Table I. Patients with genotype A were younger than those with genotype C (29.3 ± 8.0 vs. 36.6 ± 13.6 years, $P = 0.016$). The proportion of male patients was higher in genotype A than C infection (93% vs. 57%, $P = 0.003$). The main route of transmission identified in the patients with acute hepatitis B was extramarital heterosexual contacts. Homosexual activity was more frequent in patients with genotype A than C (5/27 (19%) vs. 2/109 (1.8%), $P < 0.001$).

The maximum ALT levels were lower in patients with genotype A than B or C infection (2069 ± 1075, 2952 ± 1106 and 2889 ± 1867 IU/L, respectively; A vs. B, $P = 0.02$; A vs. C, $P = 0.03$). The maximum bilirubin and alkaline phosphatase levels were no different among patients infected with HBV of different genotypes. Fulminant hepatic failure developed in one (13%) patient with genotype B and five (5%) with genotype C; no patients with genotype A came down with it. Evolution into chronic infection occurred in two patients (one with genotype A and one with genotype C). The remaining 137 (96%) patients ran a non-fulminant and self-limited disease.

HBeAg was found in 24 of the 26 (92%) patients with genotype A, 4 of the 8 (50%) with genotype B and 57 of the 93 (61%) with genotype C; it was no different between genotype A than genotype C infection ($P = 0.357$). Of the six patients with fulminant hepatic failure, only one (17%) had HBeAg.

With logistic multivariate regression analysis, the variables for differences between genotypes A and C were sex (odds ratio (OR), 6.45; 95% confidence interval (CI), 1.378–30.213; $P = 0.0018$) and area (OR, 0.25; 95% CI, 0.076–0.830; $P = 0.0024$).

Routes of transmission were compared between genotypes A and C in patients with acute hepatitis B from metropolitan areas. Although the mean age was no different, frequently the proportion of male patients was higher in genotype A than C infection (20/21 (95%) vs. 28/43 (65%), $P = 0.012$). Homosexual patients had more frequently genotype A than C infection (5/21 (24%) vs. 1/44 (2%), $P = 0.012$). Additionally heterosexuals with multiple unspecific partners had in genotype A more frequently than C infection (7/12 (58%) vs. 6/26 (23%), $P = 0.035$, respectively). However, with logistic multivariate regression analysis, none of these variables differed between genotype A and C infections.

Figure 1 compares serum HBV DNA levels on admission among patients infected with different genotypes. HBV DNA levels were higher in patients with genotype A than C (5.90 ± 1.45 vs. 5.13 ± 1.36 LGE/ml, $P = 0.002$).

Among the 145 patients whose HBV genotypes could be determined, 54 (A: 15, B: 4, and C: 35) were followed for HBsAg in serum every 2–4 weeks until it disappeared. The time between the first and last detection of HBsAg was defined as the duration of HBsAg, and compared between patients infected with HBV of genotypes A and C (Fig. 2a). The duration of HBsAg was longer in patients with genotype A than C infection (1.95 ± 1.09 (n = 15) vs. 1.28 ± 1.42 months (n = 35), $P = 0.02$). When patients with fulminant hepatic failure were excluded, the mean duration of HBsAg in patients with genotype C became longer, but it was still shorter

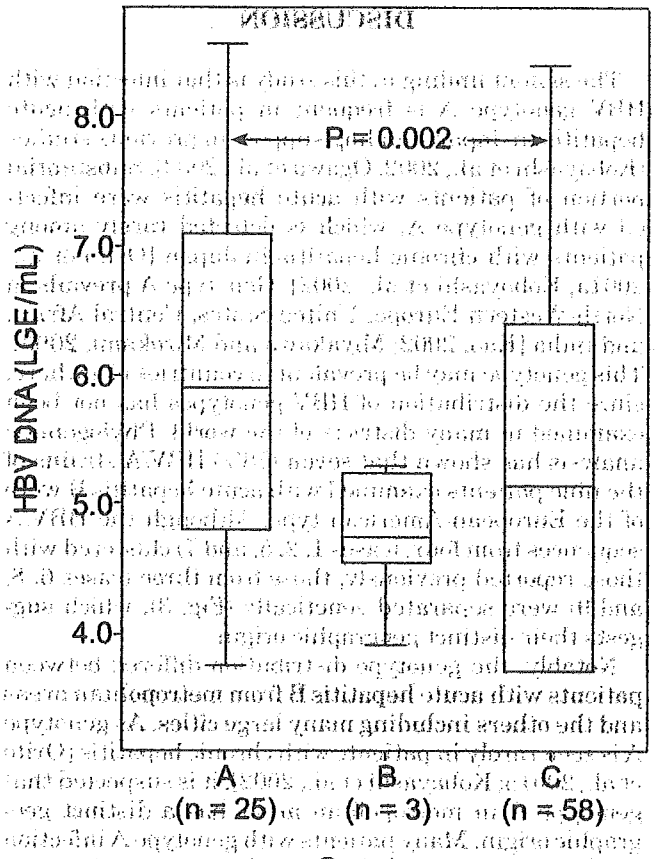

Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded areas are outside the range of detection by the TMA method.

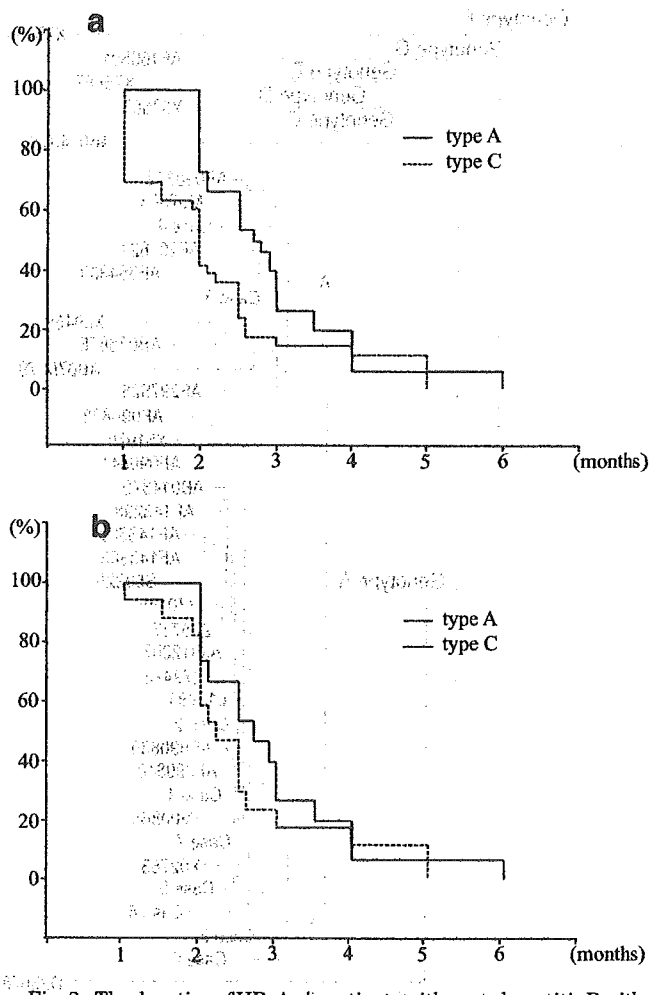

Fig. 2. The duration of HBsAg in patients with acute hepatitis B with genotypes A or C. The results are shown for (a) all patients, and (b) patients with the wild-type sequences both in precore and BCP regions of HBV.

than that in those with genotype A (1.95 ± 1.09 (n = 15) vs. 1.41 ± 1.42 (n = 31) months, $P = 0.03$).

Subtypes of Genotypes A and B

Among the 27 HBV/A isolates, 9 were selected at random and the entire S region was amplified and sequenced for them. Seven of them were classified into genotype A and the remaining 2 into subgroup A′. The sequence divergence within the seven genotype A isolates ranged from 0.12% to 2.01% in pair-wise comparison, while that between two subgroup A′ and seven genotype A isolates spanned from 5.70% to 6.53%.

A phylogenetic tree was constructed on the entire *S*-gene sequences from these nine sequences along with those from 31 HBV isolates retrieved from the database (Fig. 3). The seven (78%) HBV isolates classified into genotype A clustered with reported HBV/A isolates, while the remaining two isolates classified into subgroup A′ (cases 3 and 4) joined the branch of subgroup A′.

Six of the eight HBV/B isolates were available for analysis of subtypes. Two (both from the metropolitan area) were classified as Ba and the remaining four, including two from Tokyo and two from the other areas, as Bj. One of the four patients infected with subtype Bj developed fulminant hepatic failure, while the remaining three with subtype Bj as well as the two with subtype Ba ran a non-fulminant course.

Point Mutations in the Precore and Basic Core Promoter Regions of HBV

All the 27 HBV isolates of genotype A in which mutations were sought had the wild-type sequences both in the precore and BCP regions. In contrast, of the 102 genotype C isolates whose precore and BCP sequences were examined, 27 (26%) had mutations in the precore or BCP regions ($P = 0.096$). Furthermore, of the four genotype C isolates from patients with fulminant hepatic failure whose genetic mutations could be determined, three had mutations in the BCP region (T1762/A1764) and two had a mutation in the precore region (A1896). Only one isolate had the wild-type sequences both in the precore and BCP regions. Of