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.

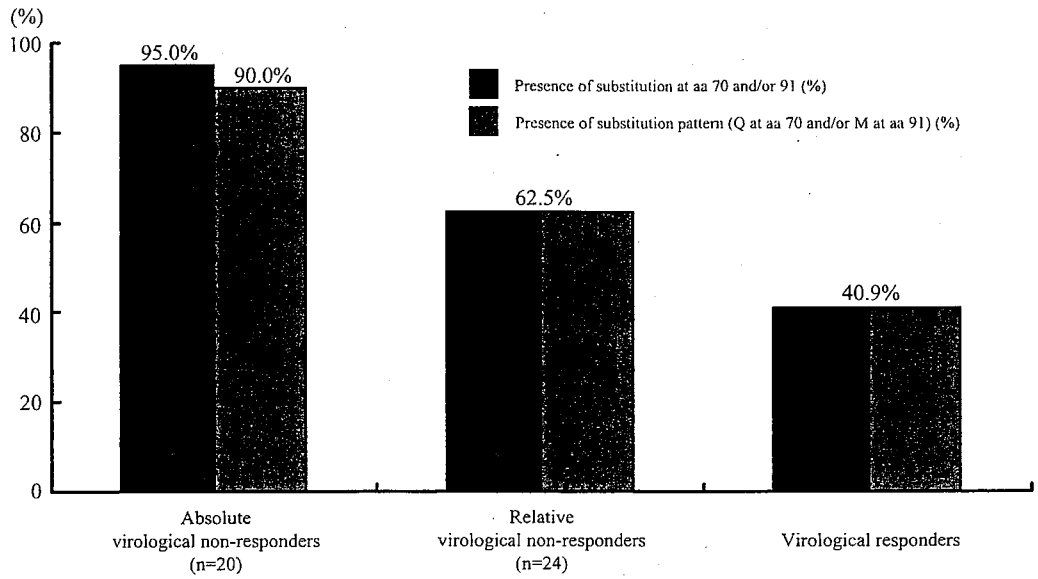

Fig. 1. Frequencies of substitutions at amino acid sites 70 and/or 91 and substitution patterns (glutamine [Q] at aa 70 and/or methionine [M] at aa 91) in HCV core region are evaluated in three groups of absolute virological non-responders, relative virological non-responders, and virological responders. 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, $P = 0.011$).

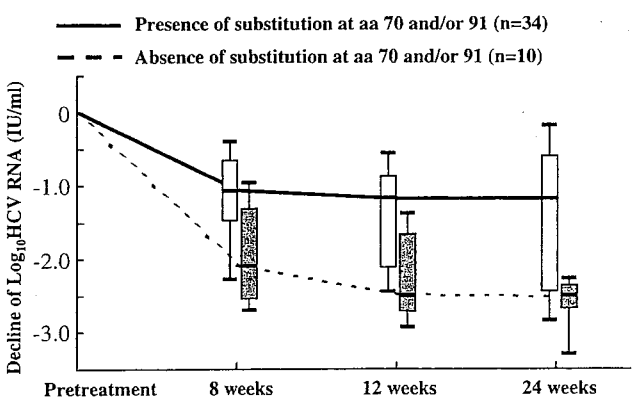

Fig. 2. Log changes in viral load from baseline at 8, 12, and 24 weeks during treatment, according to amino acid substitutions of the HCV core region. Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25th to 75th centiles, the lower and upper bars the 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.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

REFERENCES

Akuta N, Suzuki F, Tsubota A, Suzuki Y, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2002. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: Therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 37:831–836.

Akuta N, Suzuki F, Tsubota A, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. Association of amino acid substitution pattern in nonstructural protein 5A of hepatitis C virus genotype2a low viral load and response to interferon monotherapy. J Med Virol 69:376–383.

Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H. 2005a. Long-term follow-up of interferon monotherapy in 454 consecutive naive patients infected with hepatitis C virus. Multicourse interferon therapy may reduce the risk of hepatocellular carcinoma and increase survival. Scand J Gastroenterol 40:688–696.

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005b. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon–ribavirin combination therapy. Intervirology 48:372–380.

Alexander WS. 2002. Suppressors of cytokine signaling (SOCS) in the immune system. Nat Rev Immunol 2:410–416.

Antonelli G, Simeoni E, Turriziani O, Tesoro R, Redaelli A, Roffi L, Antonelli L, Pistello M, Dianzai F. 1999. Correlation of interferon-induced expression of MxA mRNA in peripheral blood mononuclear

cells with the response of patients with chronic hepatitis C to IFN-α therapy. J Interferon Cytokine Res 19:243–251.

Auernhammer CJ, Melmed S. 2001. The central role of SOCS-3 in integrating the neuro-immunoendocrine interface. J Clin Invest 108:1735–1740.

Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH. 2003. Expression of hepatitis C virus proteins inhibits interferon α signaling in the liver of transgenic mice. Gastroenterology 124:1465–1475.

Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Häussinger D. 2003. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 17:488–490.

Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, Matsumoto T, Kobayashi M, Iwasaki S, Koyama S, Morinaga T, Kumada H. 1993. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 8:150–156.

D'Alessandro AM, Kalayouglu M, Sollinger HW, Hoffmann RM, Reed A, Knechtle SJ, Pirsch JD, Hafez GR, Lorentzen D, Belzer FO. 1991. The predictive value of donor liver biopsies for the development of primary nonfunction after orthotopic liver transplantation. Transplantation 51:157–163.

de Lucas S, Bartolome J, Carreno V. 2005. Hepatitis C virus core protein down-regulates transcription of interferon-induced antiviral genes. J Infect Dis 191:93–99.

Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. 1995. Comparison of full-length sequences of interferon sensitive and resistant hepatitis C virus 1b. J Clin Invest 96:224–230.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Fried MW, Shiffman ML, Reddy R, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Fujimoto M, Naka T. 2003. Regulation of cytokine signaling by SOCS family molecules. Trends Immunol 24:659–666.

Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230:217–227.

Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M. 1999. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 29:1124–1130.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 87:9524–9528.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237–238.

Lalvakolanu DV. 2003. Alternate interferon signaling pathways. Pharmacol Ther 100:1–29.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling MH, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomized trial. Lancet 358:958–965.

Melén K, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. 2004. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 73:536–547.

Ronni T, Matikainen S, Lehtonen A, Palvimo J, Dellis J, Van Eylen F, Goetschy JF, Horisberger M, Content J, Julkunen I. 1998. The proximal interferon-stimulated response elements are essential for interferon responsiveness: A promoter analysis of the antiviral MxA gene. J Interferon Cytokine Res 18:773–781.

Song MM, Shuai K. 1998. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. J Biol Chem 273:35056–35062.

Stoiber D, Kovarik P, Cohney S, Johnston JA, Steinlein P, Decker T. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. J Immunol 163:2640–2647.

Vlotides G, Sörensen AS, Kopp F, Zitzmann K, Cengic N, Brand S, Zachoval R, Auernhammer CJ. 2004. SOCS-1 and SCOS-3 inhibit IFN-α-induced expression of the antiviral 2,5-OAS and MxA. Biochem Biophys Res Commun 320:1007–1014.

Wang Q, Floyd-Smith G. 1997. The p69/71 2-5A synthetase promoter contains multiple regulatory elements required for interferon-alpha-induced expression. DNA Cell Biol 16:1385–1394.

Journal of Medical Virology 78:60–67 (2006)

Virological Outcomes in Patients Infected Chronically With Hepatitis B Virus Genotype A in Comparison With Genotypes B and C

Mariko Kobayashi,[1] Norio Akuta,[2] Fumitaka Suzuki,[2] Yoshiyuki Suzuki,[2] Yasuji Arase,[2] Kenji Ikeda,[2] Tetsuya Hosaka,[2] Satoshi Saitoh,[1] Masahiro Kobayashi,[2]* Takashi Someya,[2] Junko Sato,[1] Sachiyo Watabiki,[1] Yuzo Miyakawa,[3] and Hiromitsu Kumada[2]

[1]*Research Institute for Hepatology, Toranomon Hospital, Tokyo, Japan*
[2]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan*
[3]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

In a single hospital in Tokyo, the 87 patients infected persistently with hepatitis B virus (HBV) genotype A, the 413 with B, and the 3,389 with C were compared for virological outcome. Hepatitis B surface antigen (HBsAg) was cleared from the serum in 12% (3/26), 2% (2/112), and 3% (23/826) of patients with genotypes A, B, and C, respectively, at 5 years of follow-up ($P=0.0395$). Hepatitis B e antigen (HBeAg) was cleared from serum more frequently in patients with genotype B than those with A or C (78% [32/41] vs. 58% [11/19] or 45% [251/562], $P=0.00001$) at 5 years. Of the 45 individuals infected with genotype A and followed for 3 years or longer, HBeAg was more frequent (16% [3/19] vs. 73% [19/26], $P=0.0002$) and levels of HBV DNA higher (median <2.6 [range: <2.6–5.6] vs. >7.6 [<2.6–>7.6] log copies/ml, $P=0.001$) in the 26 patients with biopsy-proven chronic hepatitis than the 19 asymptomatic carriers. Among the 26 hepatitis patients infected with HBV genotype A, decreases in HBV DNA were less frequent (20% [1/5] vs. 93% [13/14] or 86% [6/7], $P=0.0095$) and increases in serum levels of hyaluronic acid ≥10 ng/ml commoner (80% [4/5] vs. 14% [2/14] or 14% [1/7], $P=0.017$) in the patients who kept HBeAg than in those who seroconverted or who remained HBeAg-negative. In conclusion, patients persistently infected with HBV genotype A fare better than those with genotype B or C. However, high levels of HBV DNA continue in those in whom HBeAg persists along with fibrosis in the liver. ***J. Med. Virol. 78:60–67, 2006.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis; cirrhosis; hepatitis B e antigen; hepatitis B surface antigen; hepatocellular carcinoma; sexual transmission

INTRODUCTION

There are an estimated 350 million people in the world who are persistently infected with hepatitis B virus (HBV), some of whom develop a spectrum of chronic liver disease ranging from chronic hepatitis through cirrhosis to hepatocellular carcinoma [Lee, 1997]. New HBV infections have been prevented by mass vaccination of neonates [Tsen et al., 1991; Chen et al., 1996] and immunopropylaxis of babies born to mothers carrying HBV [Noto et al., 2003]. However, there are individuals who have been infected, and they need to be identified for receiving treatment as required. Clinical outcomes and the response to antiviral treatment are influenced by many host factors, such as ethnicity, gender, and the age at infection, as well as viral factors represented by HBV genotypes.

HBV has a partially double-stranded DNA genome of approximately 3,200 nucleotides (nt) [Tiollais et al., 1981]. Eight HBV genotypes have been classified by a sequence divergence in the entire genome exceeding 8% [Okamoto et al., 1988], and they are named by capital Alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1992; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Recently, HBV genotypes have attracted an increasing attention because they influence the clinical outcome and treatment response in patients with chronic liver disease [Tsubota et al., 2001; Kao, 2002; Miyakawa and Mizokami, 2003; Schaefer, 2005; Yu et al., 2005]. Due to their uneven geographical

Grant sponsor: Ministry of Health, Labour and Welfare of Japan.

*Correspondence to: Masahiro Kobayashi, BS, Research Institute for Hepatology, Toranomon Hospital, 1-3-1, Kajigaya, Takatsu-ku, Kawasaki City 213-8587, Japan.
E-mail: vj7m-kbys@asahi-net.or.jp

Accepted 29 September 2005
DOI 10.1002/jmv.20504
Published online in Wiley InterScience (www.interscience.wiley.com)

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.

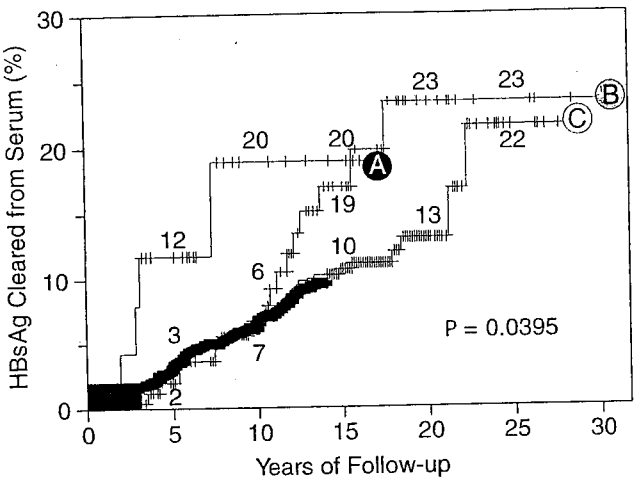

Fig. 1. Seroclearance of HBsAg during follow-up. Clearance rates of HBsAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant between genotype A and genotypes B and C at 5 and 10 years, as well as 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

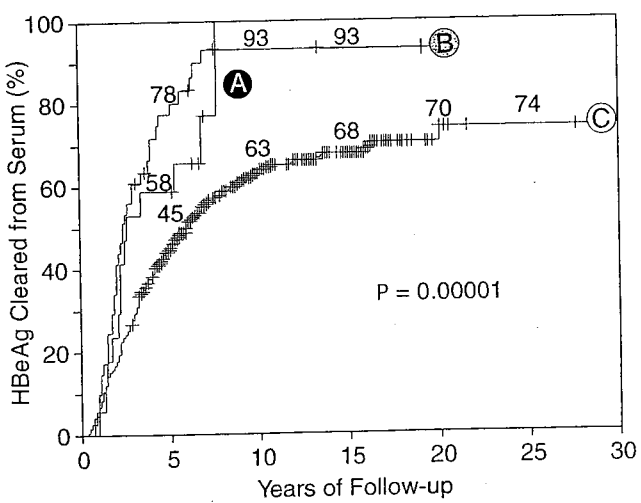

Fig. 2. Seroclearance of HBeAg during follow-up. Clearance rates of HBeAg are compared among patients with chronic hepatitis B who were infected with genotypes A, B, or C by the Kaplan–Meier life table. Differences are significant among genotypes A–C at 5 years as well as between genotypes B and C since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

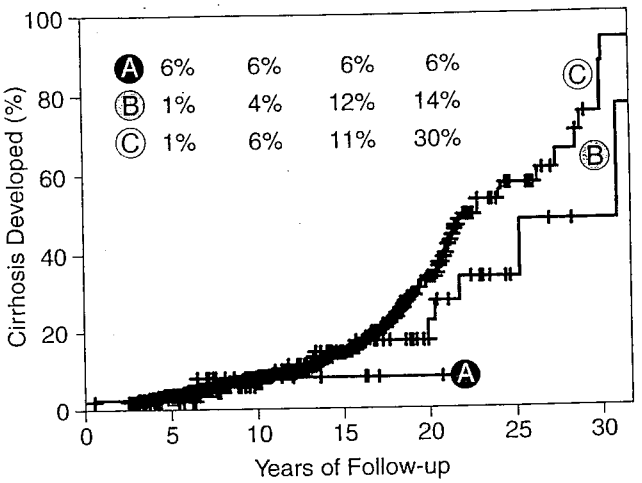

Fig. 3. Development of cirrhosis in patients infected with HBV genotype A, B, or C.

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

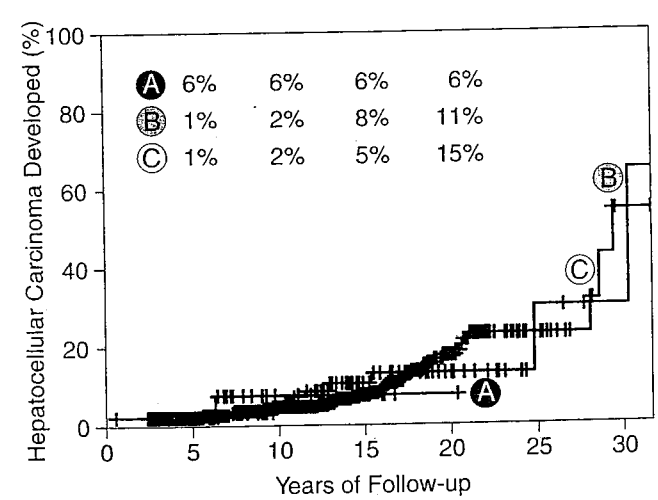

Fig. 4. Development of hepatocellular carcinoma in patients infected with HBV genotype A, B, or C.

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

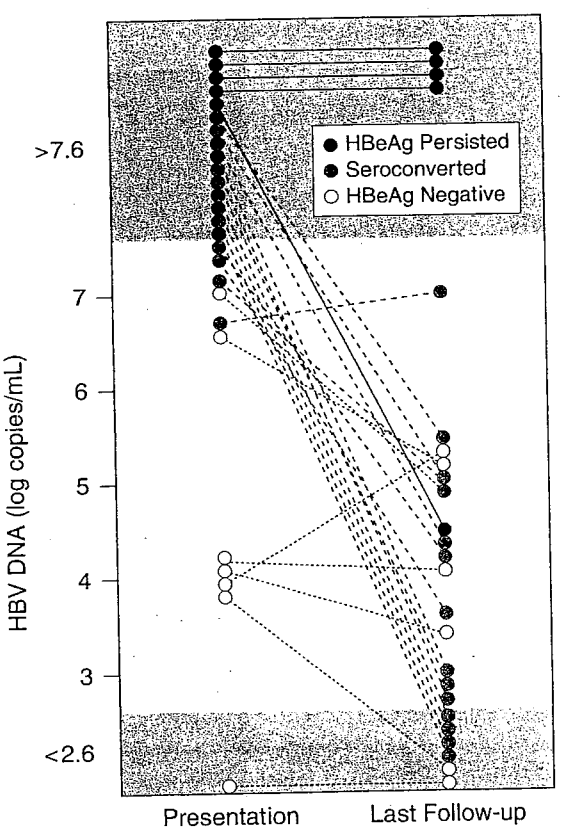

Fig. 5. Changes in serum levels of HBV DNA from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

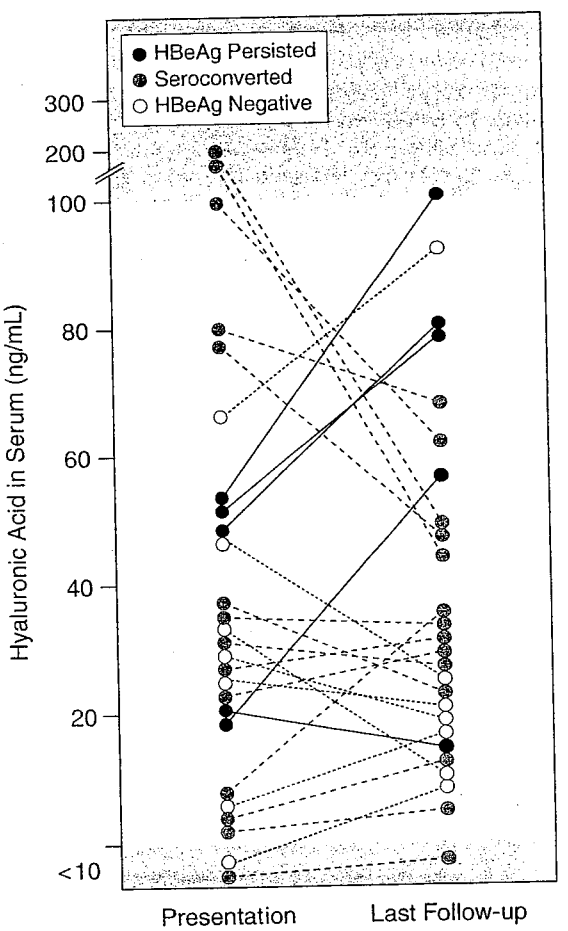

Fig. 6. Changes in serum levels of hyaluronic acid from the baseline to the last follow-up. Patients in whom HBeAg persisted, who seroconverted and who were without HBeAg at the baseline are compared.

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 ≥ 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].

REFERENCES

Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, Someya T, Saitoh S, Arase Y, Ikeda K, Kumada H. 2003. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 38:315–321.

Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 83:2059–2073.

Bowyer SM, van Staden L, Kew MC, Sim JG. 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 78:1719–1729.

Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ. 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 53:1494–1498.

Chen HL, Chang MH, Ni YH, Hsu HY, Lee PI, Lee CY, Chen DS. 1996. Seroepidemiology of hepatitis B virus infection in children: Ten years of mass vaccination in Taiwan. JAMA 276:906–908.

Chu CJ, Hussain M, Lok AS. 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 122:1756–1762.

Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS, Jr., Luketic VA, Terrault N, Lok AS. 2003. Hepatitis B virus genotypes in the United States: Results of a nationwide study. Gastroenterology 125:444–451.

Goilav C, Piot P. 1989. Vaccination against hepatitis B in homosexual men. A review. Am J Med 87:S21–S25.

Gojobori T, Ishii K, Nei M. 1982. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. J Mol Evol 18:414–423.

Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. N Engl J Med 348:800–807.

Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, Simon C, So TM, Gerken G, de Man RA, Niesters HG, Zondervan P, Hansen B, Schalm SW. 2005. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: A randomised trial. Lancet 365:123–129.

Kao JH. 2002. Hepatitis B viral genotypes: Clinical relevance and molecular characteristics. J Gastroenterol Hepatol 17:643–650.

Kao JH, Chen PJ, Lai MY, Chen DS. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 118:554–559.

Kato H, Orito E, Sugauchi F, Ueda R, Gish RG, Usuda S, Miyakawa Y, Mizokami M. 2001. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. J Virol Methods 98:153–159.

Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M. 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 76:6131–6137.

Kato H, Orito E, Sugauchi F, Ueda R, Koshizaka T, Yanaka S, Gish RG, Kurbanov F, Ruzibakiev R, Kramvis A, Kew MC, Ahmad N, Khan M, Usuda S, Miyakawa Y, Mizokami M. 2003. Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. J Virol Methods 110:29–35.

Kato H, Gish RG, Bzowej N, Newsom M, Sugauchi F, Tanaka Y, Kato T, Orito E, Usuda S, Ueda R, Miyakawa Y, Mizokami M. 2004. Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. J Med Virol 73:516–521.

Kew MC, Kramvis A, Yu MC, Arakawa K, Hodkinson J. 2005. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. J Med Virol 75: 513–521.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Takagi K, Miyakawa Y, Kumada H. 2002. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 68:522–528.

Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Hosaka T, Saitoh S, Suzuki F, Akuta N, Someya T, Matsuda M, Sato J, Kumada H. 2003. Clinical features of hepatitis B virus genotype A in Japanese patients. J Gastroenterol 38:656–662.

Kobayashi M, Suzuki F, Arase Y, Akuta N, Suzuki Y, Hosaka T, Saitoh S, Kobayashi M, Tsubota A, Someya T, Ikeda K, Matsuda M, Sato J, Kumada H. 2004. Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001. J Gastroenterol 39:844–850.

Kumada H. 2003. Continued lamivudine therapy in patients with chronic hepatitis B. Intervirology 46:377–387.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Magnius LO, Norder H. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 38:24–34.

Mayerat C, Mantegani A, Frei PC. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? J Viral Hepat 6:299–304.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. 1992. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 73:1201–1208.

Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO. 2004. Genetic diversity of hepatitis B virus strains derived worldwide: Genotypes, subgenotypes, and HBsAg subtypes. Intervirology 47:289–309.

Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. J Gastroenterol Hepatol 18:943–949.

Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. 2002. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res 23:167–177.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. e antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746–749.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, Okanoue T, Yotsuyanagi H, Iino S. 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology 33:218–223.

Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A. 2000. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. Hepatology 32:129–134.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Schaefer S. 2005. Hepatitis B virus: Significance of genotypes. J Viral Hepat 12:111–124.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 81:67–74.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Ueda R, Miyakawa Y, Mizokami M. 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 76:5985–5992.

Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, Chutaputti A, Lai CL, Gish RG, Ueda R, Miyakawa Y, Mizokami M. 2003. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124:925–932.

Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, Gish RG, Tanaka Y, Kato T, Orito E, Ueda R, Miyakawa Y, Mizokami M. 2004. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 85:811–820.

Sumi H, Yokosuka O, Seki N, Arai M, Imazeki F, Kurihara T, Kanda T, Fukai K, Kato M, Saisho H. 2003. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 37:19–26.

Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arase Y, Akuta N, Hosaka T, Saitoh S, Kobayashi M, Someya T, Matsuda M, Sato J, Watabiki S, Miyakawa Y, Kumada H. 2005. Persistence of acue infection with hepatitis B virus genotype A and the treatment in Japan. J Med Virol 76:33–39.

Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, Gish RG, Kramvis A, Kew MC, Yoshihara N, Shrestha SM, Khan M, Miyakawa Y, Mizokami M. 2004. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. Hepatology 40:747–755.

Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. 2002. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 17: 165–170.

Tiollais P, Charnay P, Vyas GN. 1981. Biology of hepatitis B virus. Science 213:406–411.

Tsen YJ, Chang MH, Hsu HY, Lee CY, Sung JL, Chen DS. 1991. Seroprevalence of hepatitis B virus infection in children in Taipei, 1989: Five years after a mass hepatitis B vaccination program. J Med Virol 34:96–99.

Tsubota A, Arase Y, Ren F, Tanaka H, Ikeda K, Kumada H. 2001. Genotype may correlate with liver carcinogenesis and tumor characteristics in cirrhotic patients infected with hepatitis B virus subtype adw. J Med Virol 65:257–265.

Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M. 1999. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 80:97–112.

Usuda S, Okamoto H, Tanaka T, Kidd-Ljunggren K, Holland PV, Miyakawa Y, Mayumi M. 2000. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 87:81–89.

Westland C, Delaney Wt, Yang H, Chen SS, Marcellin P, Hadziyannis S, Gish R, Fry J, Brosgart C, Gibbs C, Miller M, Xiong S. 2003. Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil1. Gastroenterology 125:107–116.

Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: A prospective study in men. J Natl Cancer Inst 97:265–272.

Yuen MF, Fung SK, Tanaka Y, Kato T, Mizokami M, Yuen JC, Wong DK, Yuan HJ, Sum SM, Chan AO, Wong BC, Lai CL. 2004. Longitudinal study of hepatitis activity and viral replication before and after HBeAg seroconversion in chronic hepatitis B patients infected with genotypes B and C. J Clin Microbiol 42:5036–5040.

Zhang X, Zoulim F, Habersetzer F, Xiong S, Trepo C. 1996. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. J Med Virol 48:8–16.

Journal of Medical Virology 78:1025–1034 (2006)

Changes in Viral Loads of Lamivudine-Resistant Mutants and Evolution of HBV Sequences During Adefovir Dipivoxil Therapy

Fumitaka Suzuki,[1,2]* Hiromitsu Kumada,[1] and Hirotoshi Nakamura[3]

[1]*Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan*
[2]*Okinaka Memorial Institute for Medical Research, Tokyo, Japan*
[3]*Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan*

The addition of adefovir dipivoxil (ADV) to ongoing lamivudine therapy is effective against lamivudine-resistant virus in patients with hepatitis B virus (HBV) infection. We studied 39 patients who received ADV added to lamivudine for breakthrough hepatitis. We determined early viral changes (12 weeks) in YMDD mutants (rtM204I [YIDD sequence], rtM204V [YVDD]) and rtL180M in all 39 patients as well as amino acid changes in the polymerase reverse transcriptase (rt) region and precore/core promoter mutations in 15 patients who received long-term treatment (more than 1 year). Changes in rtM204I and rtL180M viral loads were greater than that of the rtM204V, albeit statistically insignificant. Moreover, the greatest change in viral load was seen for rtM204I without hepatitis B e antigen (HBeAg). The precore mutant was replaced with wild-type virus in three of eight patients after 1 year of added ADV therapy. Compared to baseline with lamivudine therapy only, new amino acid mutations were seen in the rt region at baseline with ADV in seven patients. At 1 year after ADV coadministration, the YMDD motif was replaced with wild-type (rt204M) in two patients, in whom mutations were fewer and of a different type. We conclude that the rtM204I may be more sensitive to ADV in vivo. ADV tended to select wild-type virus from precore mutants. Moreover, viruses that were wild-type in the rt region reappeared after 1 year of ADV coadministration in some patients. ***J. Med. Virol. 78:1025–1034, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis B virus; breakthrough hepatitis; YMDD mutant; precore; core promoter; reverse transcriptase

INTRODUCTION

The goal of therapy in patients with hepatitis B virus (HBV) is to limit or reverse progression of the disease through the sustained suppression of HBV replication [Conjeevaram and Lok, 2003]. Several studies have reported that various nucleoside analogues such as lamivudine are effective in suppressing HBV replication, improving transaminase levels and liver histology, and enhancing the rate of loss of hepatitis B e antigen (HBeAg) [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. A major problem with the long-term use of lamivudine, however, is its potential to induce viral resistance, with associated increases in HBV DNA and serum transaminases [Honkoop et al., 1997; Chayama et al., 1998; Suzuki et al., 2003].

Adefovir dipivoxil (ADV) is a potent suppressor of both wild-type and lamivudine-resistant HBV in vitro and a suppressor of wild-type HBV in vivo [Hadziyannis et al., 2003; Marcellin et al., 2003]. Clinical trials to date show that the addition of ADV to ongoing lamivudine therapy in lamivudine-resistant patients, or its administration as monotherapy, produces virologic and biochemical improvements [Perrillo et al., 2000, 2004; Hosaka et al., 2004; Peters et al., 2004].

Recently, a rapid, highly sensitive and reproducible method for quantifying mutant HBV virus in lamivudine-treated patients was reported [Punia et al., 2004]. Using a real-time polymerase chain reaction (PCR; LightCycler) with a ResonSense probe, this method detects as little as 0.01% of YMDD mutant DNA among

Grant sponsor: Ministry of Health, Labor and Welfare, Japan.

*Correspondence to: Fumitaka Suzuki, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan. E-mail: fumitakas@toranomon.gr.jp

Accepted 22 March 2006

DOI 10.1002/jmv.20658

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

10^5–10^9 copies of wild-type DNA. However, there are few reports of changes in YMDD mutant (rtM204I [YIDD sequence], rtM204V [YVDD]) viral loads during ADV treatment added to ongoing lamivudine therapy [Punia et al., 2004].

During the course of chronic HBV infection, natural seroconversion to antibody to HBeAg (anti-HBe) usually correlates with the resolution of viremia and clinical recovery. Mutation in the precore region (nucleotide [nt] 1896) is related to the absence of HBeAg secretion [Carman et al., 1989] and may enhance the stability of the secondary structure of pregenome encapsidation signals, ensuring perpetuation of viral replication and thus contributing to viral persistence [Lok et al., 1994]. Buckword et al. [1996] showed that HBV genome carrying core promoter mutations (nt G1762A and A1764T) influenced viral replication. Cho et al. [2000] and our group [Suzuki et al., 2002] reported that lamivudine therapy resulted in reversion from precore and core promoter mutants to wild-type, but that these mutants reappeared during long-term therapy. However, it is not clear at this stage how ADV influences precore and core promoter mutants of lamivudine-resistant virus.

Analysis of mutations of the reverse transcriptase (rt) domain of HBV polymerase in patients who had received long-term (48 or 60 weeks) ADV monotherapy revealed the presence of several amino acid substitutions [Yang et al., 2002; Westland et al., 2003.]. Other studies showed that selection of the rtN236T polymerase mutant is associated with resistance to ADV [Angus et al., 2003; Villeneuve et al., 2003]. Further elucidation of this process requires the analysis of amino acid substitutions during coadministration of ADV with ongoing lamivudine therapy for lamivudine-resistant virus.

The aims of this prospective study were (1) to determine changes in YMDD mutant (rtM204I, rtM204V) and rtL180M viral loads during coadministration of ADV with ongoing lamivudine therapy in patients with HBV, and (2) to determine viral polymerase (rt region), precore and core promoter mutants during treatment with ADV by analyzing serial serum samples from patients with lamivudine resistance.

PATIENTS AND METHODS

Patients

The subjects were 39 consecutive adult Japanese patients who commenced ADV treatment between November 2002 and June 2004 at the Department of Gastroenterology, Toranomon Hospital. At entry, all 39 patients were being treated with lamivudine for chronic HBV infection when the emergence of YMDD motif mutations indicated the development of breakthrough hepatitis. They had not received other nucleoside analogue drugs before lamivudine and were therefore treated by the addition of ADV to the ongoing lamivudine therapy (Group 1). Moreover, viral load data for another group of nine patients who were previously cotreated with interferon (IFN) in addition to ongoing lamivudine against breakthrough hepatitis (before ADV therapy was instituted in Japan) were compared with the viral load of patients treated with ADV (Group 2). These nine patients received IFN therapy daily for 4 weeks, and then three times a week for 20 weeks (Table I). All patients were negative for hepatitis C serologic markers. Lamivudine and ADV were administered orally at 100 and 10 mg/day, respectively. Chronic hepatitis or cirrhosis was confirmed before lamivudine treatment by peritoneoscopy and/or needle biopsy (n = 23), or clinical features (n = 16) [Suzuki et al., 2003].

Blood Tests and Serum Viral Markers

Routine biochemical tests were performed using standard procedures before and during therapy at least once each month. HBsAg, HBeAg, and anti-HBe were determined by radioimmunoassay kits (Abbot Diagnostics, Chicago, IL) according the instructions provided by

TABLE I. Patient Characteristics at the Start of Therapy for Lamivudine Breakthrough Hepatitis

	ADV (Group 1)	IFN (Group 2)
Total number	39	9
Sex (female/male)	4/35	2/7
Age (years)[a]	48 (26–58)	46 (23–56)
Aspartate aminotransferase (IU/L)[a]	118 (37–478)	158 (42–495)
Alanine aminotransferase (IU/L)[a]	188 (24–858)	234 (72–727)
Bilirubin (mg/dl)[a]	0.8 (0.3–13.7)	0.8 (0.2–2.3)
Albumin (g/dl)[a]	3.7 (2.6–4.5)	3.9 (3.4–4.3)
Liver histology (CH/LC)[b]	23/16	7/2
Serum HBV DNA[c] (Amplicor: log copy/ml)[a]	7.3 (4.4 –> 7.6)	>7.6 (5.9 –> 7.6)
HBeAg (positive/negative)	24/15	7/2
HBV genotype (A/B/C)	2/3/34	0/0/9

[a]Data are median (range).
[b]Liver histology: CH, chronic hepatitis; LC, liver cirrhosis.
[c]HBV DNA levels were measured by Amplicor HBV Monitor assay. HBV DNA values below the lower limit of detection are listed as 2.6 Log copy/ml and those over the upper limit of detection as 7.6 Log copy/ml. For statistical analysis, all HBV DNA values over the upper limit of detection (>7.6 Log copy/ml) were set to 8.0.

the manufacturer. Serum HBV DNA was quantified using the Roche Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN), a PCR-based assay with a lower limit of detection of 400 copies of HBV DNA/ml (2.6 Log copy/ml).

Quantitation of Lamivudine-Resistant Mutants by Real-Time Amplification Refractory Mutation System (ARMS) PCR

DNA was extracted from 100 μl of serum. The assay was performed using a sensitive, real-time PCR-based assay for the detection of lamivudine resistance-associated mutations in the presence of high levels of wild-type virus, as reported recently [Punia et al., 2004]. Briefly, this method is based on ARMS PCR for the detection of single base mutations [Newton et al., 1989] and uses the same ARMS primers, reactions, and cycling conditions on the LightCycler. To prepare the standards (rt204M, rtM204I, and rtM204V), the first PCR product amplified using primers P1 and P2, as reported previously [Günther et al., 1995], was cloned into the plasmid vector pBluescript (Stratagene, La Jolla, CA). The concentration of purified plasmids was based on absorbance at 260 nm (GeneQuant II; Amersham Pharmacia Biotech, Tokyo, Japan). The standards for real-time PCR were prepared by serial dilution of a plasmid of known concentration. DNA values of those mutants below the lower limit of detection were expressed as 2.0 Log copy and those over the upper limit of detection as 9.0 Log copy. Selectivity of this assay was tested as described previously [Punia et al., 2004] using reactions containing 10^9 copies of wild-type DNA (rt204M) template and from 10^9 to 0 copies of mutant virus (rtM204I or rtM204V) template. Under these conditions, the mutant primers (for rtM204I and rtM204V) detected the number of copies of mutant template present within the range of 10^9–10^4 copies. Moreover, one primer (rtM204I or rtM204V) detected the number of copies of mutant template present within the range of 10^9–10^4 copies (mixed with 10^9 copies of the other mutant virus [rtM204V DNA or rtM204I DNA], respectively). The detection limit for mutation of rt180 (rtL180M) was the same. Total HBV DNA levels were measured by real-time PCR as described previously [Punia et al., 2004]. Serum samples were assayed at five time points, namely before (baseline) and at 2, 4, 8, and 12 weeks after the start of coadministration of ADV with ongoing lamivudine therapy. Moreover, in some patients, serum samples were also assayed at two other time points; at 24 and 52 weeks. Data for the time-dependent decline in viral load relative to baseline were log transformed, and thus all results for quantitative HBV level are expressed as Log_{10} copy.

Determination of Nucleotide Sequences of HBV DNA

We determined the nucleotide sequences of HBV DNA of the initial 15 patients who received ADV treatment. Among these 15 patients, 4 received combination therapy of lamivudine, ADV, and IFN because of severe hepatitis. The remaining 11 patients were cotreated with ADV in addition to the ongoing lamivudine therapy and belonged to Group 1. DNA was extracted from 100 μl of serum. PCR reactions for detection of the rt region (nt 130–1161) of HBV DNA were performed in two parts. The first and second PCR reactions for detection of the first part of the rt region were performed using primers BGF1 (sense: 5′-CTGTGGAAGGCTGGCATTCT-3′) and BGR2 (antisense: 5′-GGCAGGATAGCCGCATTGTG-3′), and PreSBamH1 (sense: 5′-CTTGGGATCCAGAGC-TACAGCATGG-3′) and BR112 (antisense: 5′-TTCC GTCGACATATCCCATGAAGTTAAGGGA-3′), respectively, under conditions of initial denaturation for 4 min, 35 cycles of amplification with 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and final extension at 72°C for 7 min. The first and second PCR reactions for detection of the second part of the same region were performed using primer pairs B11F (sense: 5′-GGCCAAGTCTG-TACAACATC-3′) and B12R (antisense: 5′-TGCA-GAGGTGAAGCGAAGTG-3′), and B11F and B14R (antisense: 5′-GATCCAGTTGGCAGCACACC-3′), respectively, under the same conditions. The amplified PCR products were used for direct sequencing. Measurement of sequences in the rt region was performed at three time points; at the start of lamivudine, start of ADV, and 1 year after the start of ADV therapy. Nucleotide sequences of the core promoter and precore regions were determined as described previously [Suzuki et al., 2002], with measurements taken at the same three time points. All HBV genomes analyzed in detail by sequencing were found to be of genotype C. All sequence alignments were performed in comparison with genotype C wild-type sequences (accession no. AB014378, AB014394, AB033550, AB033551, AB0335 56, AB042283).

Mutation of the HBV DNA polymerase gene (rtM204I/V) was determined using PCR and restriction fragment length polymorphism (PCR-RFLP) as described previously [Chayama et al., 1998].

Statistical Analysis

Differences between groups were examined for statistical significance using the χ^2 and Mann–Whitney test (*U*-test) where appropriate. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Changes in Viral Loads of Lamivudine-Resistant Mutants During ADV Therapy

Changes in rtM204I, rtM204V, and rtL180M viral loads were measured in all 39 patients. At the start of ADV coadministration, the number of patients with detectable rtM204I alone, rtM204V alone and mixed-type (rtM204I and rtM204V) among the 39 patients was 17, 4, and 18, respectively. Viral load of rtL180M was detected in 36 patients. Figure 1 shows the median log changes from baseline in rtM204I, rtM204V, and

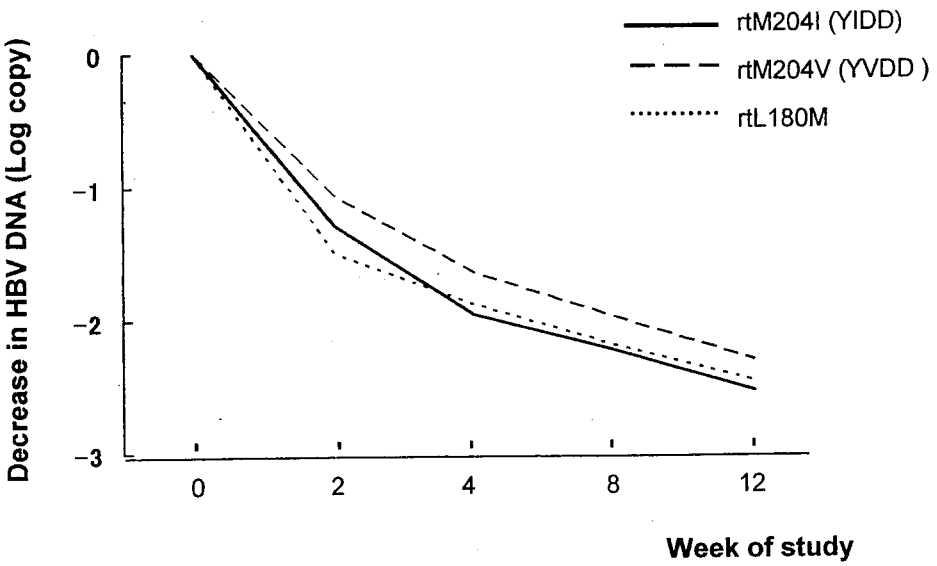

Fig. 1. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline during the initial 12 weeks of coadministration of ADV added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR. rt, HBV polymerase reverse transcriptase.

rtL180M viral loads during the initial 12 weeks of ADV and lamivudine coadministration. The changes in viral load of rtM204I and rtL180M were greater than that of rtM204V, although the difference was not statistically significant. The rate of decrease of all mutants at 12 weeks was about one-hundredth (1/100) that at baseline. Moreover, the change of viral load of HBV DNA in HBeAg-negative patients was greater than that in HBeAg-positive patients at 12 weeks (median log changes in viral load; HBeAg-positive vs. negative = −2.14: −2.71; $P = 0.077$). The numbers of rtM204I and rtM204V with HBeAg, and rtM204I and rtM204V without HBeAg were 24, 13, 11, and 9, respectively. The change of viral load of rtM204I without HBeAg was the greatest among the groups.

Among the nine patients coadministered IFN with ongoing lamivudine therapy, rtM204I only was detected in three and mixed-type was detected in six patients. Viral load of rtL180M was detected in eight patients. Log changes in rtM204I, rtM204V, and rtL180M viral loads under IFN coadministration are shown in Figure 2. The log viral load change for the rtM204V was greater than that for the rtM204I, although the difference was not statistically significant.

Normalization of alanine aminotransferase (ALT) level at 1 year was noted in 35 of 39 patients of Group

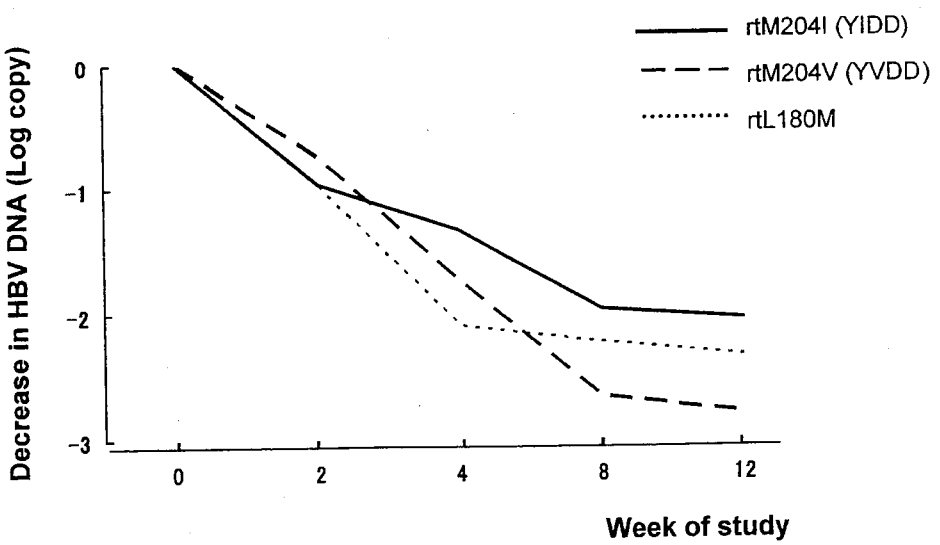

Fig. 2. Median log changes in rtM204I, rtM204V, and rtL180M viral loads from baseline and during the initial 12 weeks of coadministration of IFN added to ongoing lamivudine therapy. HBV DNA levels of rtM204I, rtM204V, and rtL180M were measured by real-time PCR.

1. Moreover, HBV DNA levels in 11 of 39 patients of Group 1 were more than 2.6 Log copy/ml by Amplicor HBV Monitor assay at 52 weeks. Those 11 patients were persistently HBeAg-positive and had mutant viral loads that were over 10^6 copies at the commencement of ADV and lamivudine coadministration. The number of patients with detectable rtM204I alone and mixed-type (rtM204I and rtM204V) was five and six, respectively. The rtM204I and rtM204V viral loads in these 11 patients were also measured at 24 and 52 weeks (Fig. 3). Viral loads of five patients with rtM204I alone gradually decreased but were still detectable at 52 weeks

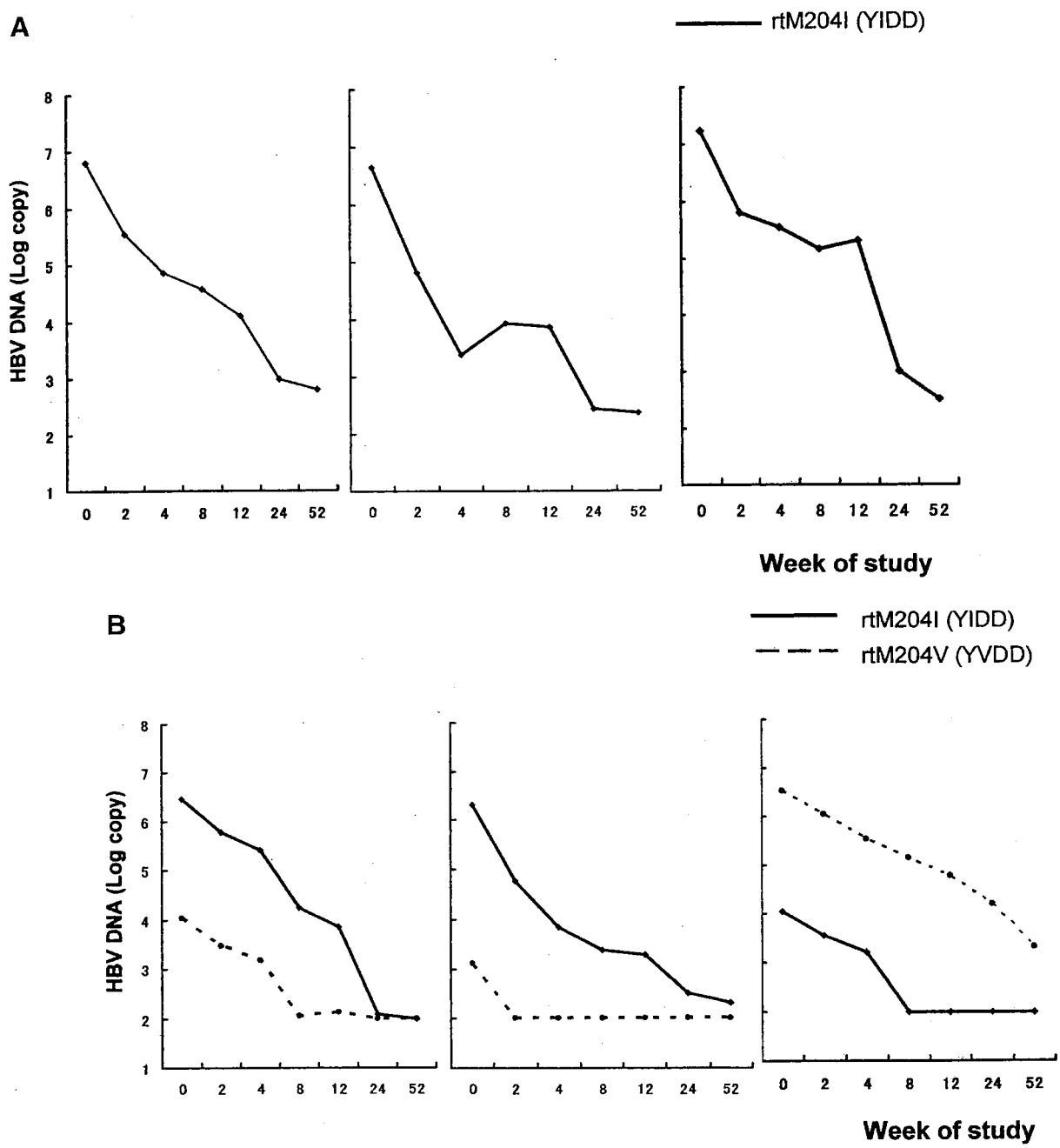

Fig. 3. Changes in rtM204I and/or rtM204V viral loads in nine patients with HBV DNA levels of >2.6 Log copy/ml as determined by Amplicor HBV Monitor assay at 52 weeks during ADV and lamivudine coadministration. HBV DNA levels of rtM204I and rtM204V were measured by real-time PCR. **A**: Viral loads in three of the five patients with rtM204I alone at commencement of ADV plus lamivudine combination therapy. Similar changes in viral loads were noted in the other two patients. Viral loads of these five patients with rtM204I alone gradually decreased but were still detectable at 52 weeks. **B**: Viral loads in three patients in whom either rtM204I or rtM204V was the major mutant (viral load of major mutant was over 10-fold that of minor mutant). Only the major mutant was detected at 52 weeks. **C**: Viral loads in three patients with two similar mutants (viral load of major mutant was within 10-fold that of minor mutant). Mutant rtM204V predominated over rtM204I at 52 weeks.