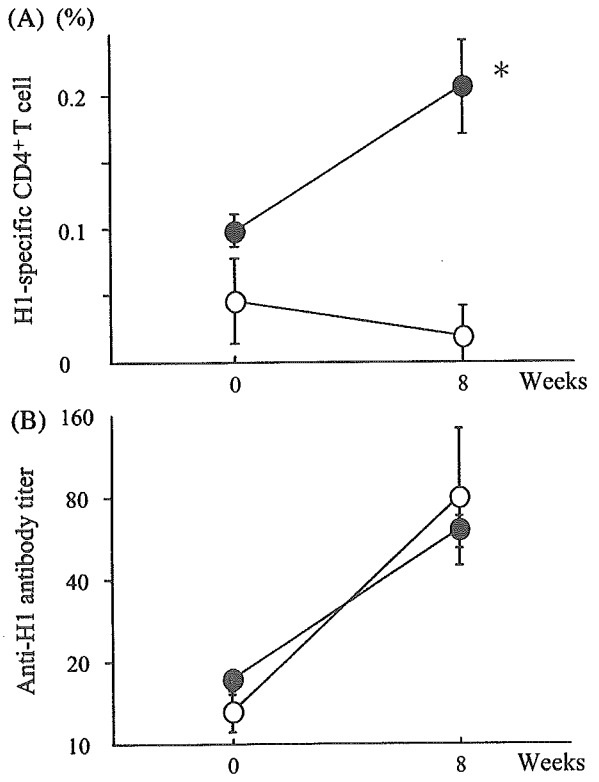

FIGURE 3. H1-specific CD4+ T cells and anti-H1 antibody responses at week 8 after vaccination in influenza A/H1N1–infected patients. Five vaccinated individuals were infected with influenza A/H1N1. A, Percentage of H1-specific CD4+ T-cell responses in infected (○; n = 4) and noninfected (●; n = 119) individuals. *H1-specific CD4+ T cells responded better to influenza A/H1N1 in noninfected patients than in infected patients ($P < 0.05$). One sample of 5 influenza A/H1N1–infected individuals was not examined because the sample was not among the first 10 samples per day as stated in the text. B, Anti-H1 antibody titers in infected (○; n = 5) and noninfected (●; n = 249) individuals. The anti-H1 antibody response at week 8 was similar in both groups. Data are mean ± SEM.

baseline antibody-negative HIV-1–infected patients, the antibody responses to the single-shot vaccination were less effective than those in healthy patients. In contrast, however, in baseline antibody-positive HIV-1–infected patients, the antibody responses were similar or more effective than those in the healthy controls and the titers exceeded >40 U in most cases, irrespective of CD4 count. Previous studies demonstrated that an antibody titer >40 U could be used as an index of vaccine protection.[12,25] In our study, the antibody titer was <40 U in most patients who became infected with influenza. Considered together, these results suggest that the antibody response may support the clinical efficacy of influenza vaccination. Kroon et al[8] reported that postvaccination antibody titers were higher in previously vaccinated HIV-1–infected patients than in nonvaccinated patients, although the difference was not significant. In the present study, the antibody titers showed a better response in individuals positive at baseline for anti-HA antibody than in those negative for the antibody. Furthermore, the response was well sustained, irrespective of CD4 count. Thus, it is conceivable that annual vaccination is specifically important for all HIV-1–infected patients. Sustainability of the antibody titer raised by the vaccination is to be followed in a future study.

In the immunologic part of our study, we examined antibody responses and specific CD4 T cells. The antibody response was almost the same as that reported previously[8,9]; the response correlated with the CD4 count. In contrast, specific CD4 T cells were much more influenced by HIV VL than by CD4 count.[1,8–15] Therefore, the specific CD4 T cells were higher in patients treated with HAART than in those untreated. This result indicates that HAART improves HA-specific CD4 T cells like in other infections,[21] or, in other words, the heightened cellular response to the influenza vaccine suggests functional reconstitution of the immune system after HAART.

Our data indicate that the specific CD4 T-cell responses may be related to HIV VL. The specific CD4 T-cell response needs antigen presentation by dendritic cells.[26] HIV-1 infection impairs the function of antigen presentation of dendritic cells.[27] Therefore, specific CD4 T-cell responses may be profoundly decreased in patients with a high HIV VL.

It is interesting to note that the percentage of H1-specific CD4 T cells at week 8 was significantly lower in influenza A/H1N1–infected patients. It is conceivable that the response of HA-specific CD4 T cells at week 8 can predict the efficacy of influenza vaccine. Influenza-specific CD4 T cells provide help (as Th cells) to B cells for the production of antibody to influenza HA and neuraminidase[28,29] and also promote the generation of virus-specific CD8+ cytotoxic T lymphocytes (CTLs).[26,30–33] Therefore, the specific CD4 T cell must have a protective role. This concept would be more reliable if we had analyzed H3-specific CD4 T cells rather than H1-specific CD4 T cells, because influenza A/H3N2 was the predominant subtype in this season. Further studies are necessary to elucidate this point.

Our study was designed as a prospective but nonrandomized study, because influenza vaccine has been already recommended for HIV-1–infected patients.[7] Practically, the number of nonvaccinated patients who did not participate in our study was higher than that of vaccinated patients (13% of nonvaccinated patients vs. 4.5% of vaccinated patients), and the violation rate of the study protocol was higher in nonvaccinated patients than in vaccinated patients (24.1% vs. 17.4%). Thus, 262 (78.9%) of 332 vaccinated patients and 66 (66%) of 100 nonvaccinated patients were analyzed in this study. Although a relatively high proportion of patients failed to complete the protocol, the main reason for the drop out may have been the lack of incentives and the need to visit our clinic on a fixed date for blood sampling. The vaccinated and nonvaccinated groups were well balanced in terms of baseline characteristics, however. Finally, we believe that the selection bias of participants, if any, is negligible.

In conclusion, our prospective study in a large population demonstrated that influenza vaccine provides protection of HIV-1–infected patients. In baseline antibody-negative patients, the antibody responses to the vaccination were significant in those patients with a CD4 count >200 cells/μL compared with those with a CD4 count <200 cells/μL. In contrast, in baseline antibody-positive patients, good antibody responses were observed, irrespective of CD4 counts. Annual vaccination of

HIV-1–infected patients is thus recommended. Therapy with HAART improves the cellular immune response to influenza HA.

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APPENDIX

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Homozygous *CYP2B6* *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens

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Abstract

Efavirenz (EFV) is metabolized by cytochrome P450 2B6 (*CYP2B6*) in the liver. We analyzed the genotypes of *CYP2B6* and their contribution to plasma EFV concentrations in 35 EFV-treated patients in International Medical Center of Japan. The mean plasma EFV concentration of patients with *CYP2B6* *6/*6 (Q172H and K262R) (25.4 ± 7.5 μM, ± SD, $n = 2$) was significantly higher than that of patients with genotypes *6 heterozygote (9.9 ± 3.3 μM, $n = 10$) or without alleles *6 (8.0 ± 2.6 μM, $n = 23$) ($p < 0.0001$). To confirm our result, we further analyzed nine patients (three with high EFV concentrations and arbitrarily selected six with normal EFV concentrations) treated in Osaka National Hospital, and it resulted that the only three patients with the high concentrations were the *6/*6 holder. EFV dose could be decreased in those patients harboring the genotype to reduce toxicity with compromising potency, representing the first step of the Tailor-Made therapy of HIV-1 infection.

Keywords: Cytochrome P450; Genetic polymorphism; HIV-1; Efavirenz; Plasma concentration

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor that shows potent inhibitory activity against HIV-1 and is stated as a key drug of the first line regimens in the HIV-1 treatment Guideline [1]. However, a number of patients treated with EFV develop central nervous system symptoms including headache, dizziness, insomnia, and fatigue. These side effects are more frequent in patients with high plasma concentration of EFV [2,3] as well as worsen with long-term therapy, and are the main reason for poor adherence or interruption of therapy. EFV is reported to be metabolized by cytochrome P450 (CYP) 3A4 (*CYP3A4*) and 2B6 (*CYP2B6*) to hydroxylated metabolites in the liver [4]. The recent HIV-1 treatment Guideline stated that EFV is metabolized by *CYP3A* [1], whereas an in vitro study indicated that EFV is mainly metabolized by *CYP2B6* [5]. Furthermore, a pharmacogenetic study demonstrated the association of the homozygous variant of multidrug-resistance transporter (*MDR1*; gene product P-glycoprotein) C3435T and good immune recovery in patients treated with EFV-containing regimens [6]. In order to clarify the contribution of polymorphisms to plasma EFV concentration in vivo, we analyzed genotypes of *CYP2B6*, *CYP3A4*, and *MDR1*, and their correlation with plasma EFV concentrations.

Materials and methods

Patients. A total of 60 HIV-1 patients who were treated with EFV-containing regimens at the AIDS Clinical Center, International

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Medical Center of Japan (IMCJ), were examined for their allelic variants of *CYP2B6*, *CYP3A4*, and *MDR1*. Among them, 35 patients were on standard therapy of EFV-containing regimens (600 mg EFV once daily dosing with two nucleotide reverse transcriptase inhibitors) and fully adhered to the regimens based on self-reports. Their plasma EFV concentrations were measured and the correlation between variants and EFV concentrations was analyzed. We excluded those patients who were taking other agents that could potentially interact with plasma EFV concentration such as protease inhibitors and those taking EFV twice daily from the analysis of the correlation. The mean age and body weight of these 35 patients (34 males and 1 female) were 41.6 ± 11.5 years and 63.4 ± 10.9 kg, respectively. The median latency between commencement of treatment and analysis of EFV concentration was 76.9 weeks (range, 4–200). The means ± SD alanine aminotransferase level was 33.1 ± 18.4 U/L. Blood samples were taken between 10 and 14 h (mean, 12.0 h) after dosing. To confirm the results of patients treated at the IMCJ, we further analyzed the allelic variants of nine patients who were treated at the Osaka National Hospital (ONH) [three patients with high plasma EFV concentrations (one patient was taking only 200 mg EFV once daily due to severe side effects) and six patients with normal EFV concentrations]. The Ethics Committee for the Study of Human Genome in each hospital approved this study (IMCJ-H14-36, ONH-23) and all patients gave a written informed consent.

Genotyping. Genomic DNA was isolated from peripheral blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of allelic variants of *CYP2B6* [7] [**1* (wild type), **2* (C64T), **3* (C777A), **4* (A785G), **5* (C1459T), **6* (G516T and A785G), **7* (G516T, A785G, and C1459T), and **8* (A415G)], CYP3A4 [**11* (C1088T; unstable form [8]), **12* (C1117T; has an altered testosterone hydroxylase activity [8]), **13* (C1247T; lack of expression [8]), **17* (T566C; exhibits lower turnover numbers for testosterone and chlorpyrifos [9]), and **18* (T878C; exhibits higher turnover numbers for testosterone and chlorpyrifos) [9] and *MDR1* C3435T was carried out using the allelic-specific fluorogenic 5′ nuclease chain reaction assay by the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Each 25 μl PCR mixture contained 20 ng genomic DNA, 900 nM primers, 200 nM TaqMan minor groove binder (MGB) probes, and 12.5 μl TaqMan universal PCR master mix (Applied Biosystems). The primers and TaqMan MGB probes used in this study are summarized in Table 1. The thermal cycler program was set up at 50 °C for 2 min and 95 °C for 10 min, and then repeated 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

Plasma efavirenz concentration. Plasma was isolated by centrifugation (10 min at 1800*g*) on the same day as blood sampling and stored at −80 °C until analysis. EFV concentration was measured by reverse-phase high performance liquid chromatography (HPLC) method [10] at BioMedical Laboratory (Saitama, Japan). HPLC was performed on an Inertsil ODS-3 column (5 μm, 250 × 4.6 mm; GL Sciences, Tokyo, Japan) at a flow rate of 1.2 ml/min with ultraviolet-detection at 247 nm. The mobile phase consisted of acetonitrile and water (65:35, v/v).

Statistical analysis. StatView version 5.0 software (SAS Institute, Cary, NC) was used for the comparison of different genotype groups. If one-way analysis of variance (ANOVA) was significant ($p < 0.05$), post hoc Scheffe's F test was applied.

Table 1
Primers and TaqMan MGB probes used in this study

	Forward primer	Reverse primer	VIC probe (wild-type)	6-FAM probe (mutant)
CYP2B6				
C64T	CCTCACAGGACTCTTGCTACTC	AGGCGGTCATGGGTGTTAG	TGGTTCAGCGCCACC	CTGGTTCAGTGCCACC
A415G	CTGTGACCACTATGAGGGACTTC	CTGAGCCTCCTCCTGAATCC	ACACTCCGCTTTCCCAT	CACTCCGCTCTCCCAT
G516T	TCATGGACCCCACCTTCCT	GACGATGGAGCAGATGATGTTG	TTCCAGTCCATTACC	CTTCCATTCCATTACC
C777A	TGGAGAAGCACCGTGAAACC	GAGCAGGTAGGTGTCGATGAG	CCCAGCGCCCCCA	CCCCAGAGCCCCCA
A785G	TGGAGAAGCACCGTGAAACC	TGGAGCAGGTAGGTGTCGAT	CCCCCAAGGACCTC	CCCCAGGGACCTC
C1459T	CCCAGAAGACATCGATCTGACA	GAATGACCCTGGAATCCTTTGAC	AGATCCGCTTCCTG	AGATCTGCTTCCTGC
CYP3A4				
T566C	GGCCTACAGCATGGATGTGAT	TGGATTGTTGAGAGAGTCGATGTT	AGCACATCATTTGGA	AGCACATCATCTGGA
T878C	TCTTTCCTCTCCTTTCAGCTCTGT	GGTTTCATAGCCAGCAAAAATAAAG	CGATCTGGAGCTCG	CGATCCGGAGCTC
C1088T	TGTGCTACAGATGGAGTATCTTGACA	CATCCCATTGATCTCAACATCTTTT	TCTGAGCGTTTCATT	CTGAGCATTTCATTCA
C1117T	TCTTGACATGGTGGTGAATGAAA	CATCCCATTGATCTCAACATCTTTT	CCCTCTCAAGTCTC	CCTCTCAAATCTC
C1247T	AAAGTACTGGACAGAGCCTGAGAAG	GGAGGGCTCCCTTCCCA	TTCCTCCCTGAAAGG	CCTCCTTGAAAGGTA
MDR1				
C3435T	AACAGCCGGGTGGTGTCA	ATGTATGTTGGCCTCCTTTGCT	CTCACGATCTCTTC	CCTCACAATCTCTT

MGB, minor groove binder; VIC, vasoactive intestinal contractor; 6-FAM: 6-carboxyfluorescein.
Bold indicates the site of substitution.

Results and discussion

Frequency of genotypic variants of CYP2B6, CYP3A4, and MDR1

We first analyzed the frequency of genotypic variants of the 60 patients seen at IMCJ. The *CYP2B6* genotypes were **1/*1* in 28 patients, **1/*2* in 4, **1/*4* in 5, **1/*6* in

Table 2
Frequency of CYP2B6 alleles and genotypes in 60 HIV-1 patients at IMCJ[a]

	Frequency (%)	95% CI
CYP2B6 allele		
**1*	78 (65)	56.5–73.5
**2*	9 (7.5)	3.8–14.2
**4*	10 (8.3)	4.4–15.3
**5*	2 (1.7)	0.3–6.0
**6*	21 (17.5)	10.7–24.3
CYP2B6 genotype		
**1/*1*	28 (46.7)	33.7–60.0
**1/*2*	4 (6.7)	1.8–16.2
**1/*4*	5 (8.3)	2.7–18.4
**1/*6*	13 (21.7)	12.1–34.2
**2/*4*	2 (3.3)	0.4–11.5
**2/*6*	3 (5.0)	1.0–13.9
**4/*4*	1 (1.7)	0.02–8.9
**4/*6*	1 (1.7)	0.02–8.9
**5/*5*	1 (1.7)	0.02–8.9
**6/*6*	2 (3.3)	0.4–11.5

95% CI, 95% confidence intervals.
[a] IMCJ, International Medical Center of Japan.

13, **2/*4* in 2, **2/*6* in 3, **4/*4* in 1, **4/*6* in 1, **5/*5* in 1, and **6/*6* in 2 (Table 2). The *CYP3A4* polymorphisms were only shown in T878C T/C heterozygote in three patients and other alleles were not found. *MDR1* C3435T polymorphisms were C/C in 19 patients, C/T in 31, and T/T in 10.

Correlation between the genotypic variants and EFV concentrations

Among the 35 patients who were on standard therapy of EFV-containing regimens, two had significantly higher plasma EFV concentrations (30.7 and 20.0 μM) than the other patients. *CYP2B6* genotype of the two patients was **6/*6* homozygote. The mean plasma EFV concentrations of patients with *CYP2B6 *6/*6* genotype (25.4 ± 7.5 μM, $n = 2$) were significantly higher than those of patients with **6* heterozygous genotypes (9.9 ± 3.3 μM, $n = 10$) and non-**6* alleles (8.0 ± 2.6 μM, $n = 23$) [one-way ANOVA ($p < 0.0001$) and post hoc Scheffe's F test showed statistically significant difference in plasma EFV concentration between **6/*6* genotype and **6* heterozygous genotypes ($p < 0.0001$), and non-**6* alleles ($p < 0.0001$)]. As shown in Table 3, the differences of patients' characteristics in each *CYP2B6* genotype were not significant, indicating that these characteristics did not influence the difference of EFV concentrations among the three genotypes. Then, we analyzed the additional nine samples (three with high EFV concentrations) obtained from the ONH and found that *CYP2B6* genotypes of the three patients with high EFV concentration were also **6/*6* genotype. Consequently, only five patients whose EFV concentrations were >20 μM had *CYP2B6 *6/*6* genotype (Fig. 1A). There was a significant correlation between *CYP2B6 *6/*6* genotype and high plasma EFV concentrations. In contrast, there was no correlation between *CYP2B6 *5*, *CYP3A4*, *MDR1* genotypes, and plasma EFV concentrations (Figs. 1B–D) in our small number of patients examined in this study.

Homozygous variant of *MDR1* C3435T has been shown to associate with responsiveness to EFV therapy [6]. However, no correlation was found between the C3435T polymorphisms and plasma EFV concentration in our study. Then, the plasma EFV concentration could not explain the favorable clinical result. EFV is a non-nucleoside reverse transcriptase inhibitor and, therefore, plays an anti-HIV-1 activity within HIV-1 infected cells but not in plasma. It remains to be elucidated whether or not the C3435T polymorphisms correlate with high intracellular EFV concentration.

Genetic polymorphism is known to be associated with variable level of *CYP2B6* expression in the liver. Especially, the expression levels of *CYP2B6 *6/*6* genotype are significantly lower than those of wild and other genotypes [7,11]. The high plasma EFV concentration may be explained by the low expression level of this genotype. Based on our new finding, extremely high plasma EFV concentration can be predicted by determining the genotype before commencement of EFV-containing therapy. In such patients, the EFV dose could be decreased to reduce the cost and more importantly the associated toxicity, without compromising its potency. In fact, one patient was treated with 200 mg EFV once daily due to severe side effects but had higher EFV concentrations than other patients with other genotypes. The frequency of the *CYP2B6 *6/*6* genotype in IMCJ patients was 3.3% (2 in 60 patients), whereas

Table 3
Patients' characteristics in each CYP2B6 genotype in 35 patients who were treated with standard EFV-containing therapy at IMCJ[a]

	Non-**6* genotypes	**6* heterozygote genotypes	**6/*6* genotype	*p*
n	23	10	2	
Male:female	23:0	9:1	2:0	n.s.
Age (years) (mean ± SD)	38.8 ± 8.2	45.3 ± 14.8	55.5 ± 19.1	n.s.
Weight (kg) (mean ± SD)	64.3 ± 11.5	58.6 ± 7.5	77.0 ± 5.1	n.s.
Alanine aminotransferase level (U/L) (mean ± SD)	31.0 ± 20.4	35.3 ± 14.0	46.5 ± 3.5	n.s.

n.s., not significant.
[a] IMCJ, International Medical Center of Japan.

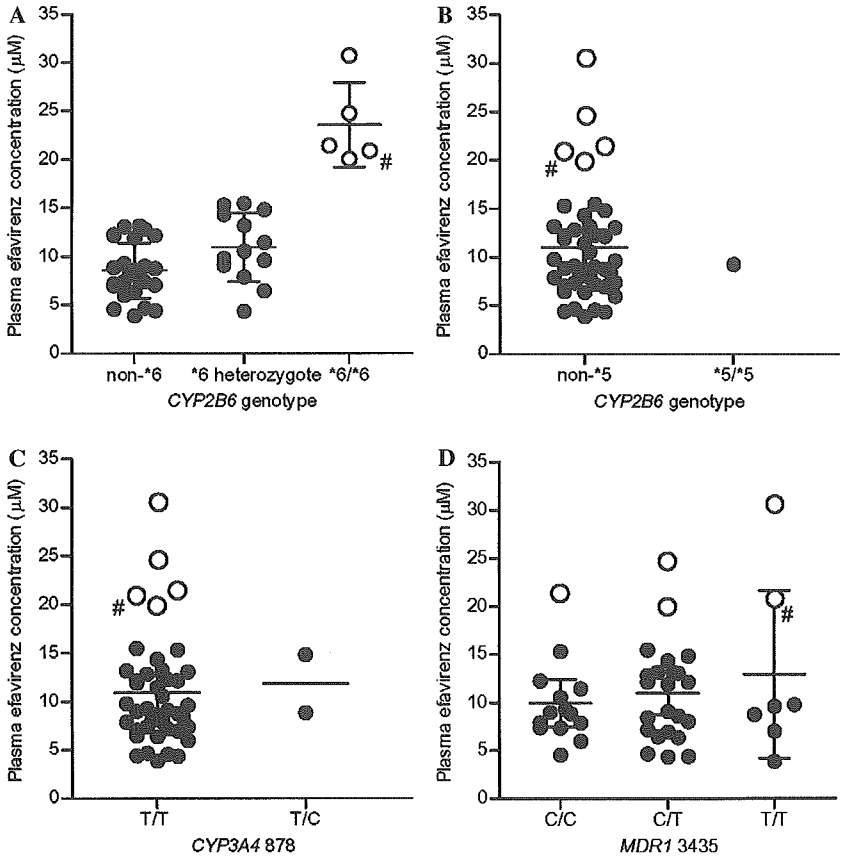

Fig. 1. Correlation between *CYP2B6 *6* genotypes (A), *CYP2B6 *5/*5* genotype (B), *CYP3A4* T878C genotype (C), *MDR1* 3435 genotypes (D), and plasma efavirenz concentrations. A total of 44 HIV-1 patients treated with standard EFV-containing regimens (35 from IMCJ and 9 from ONH) are depicted. Only homozygous genotypes of *CYP2B6* are represented in this figure [A (**6* genotypes) and B (**5/*5* genotype)]. Non-**6* genotypes ($n = 26$) include **1/*1* ($n = 18$), **1/*2* ($n = 2$), **1/*4* ($n = 3$), **2/*4* ($n = 2$), and **5/*5* ($n = 1$). **6* heterozygote genotypes ($n = 13$) include **1/*6* ($n = 9$), **2/*6* ($n = 3$), and **4/*6* ($n = 1$). Numbers of patients of *MDR1* 3435 C/C, C/T, and T/T genotypes are 14, 23, and 7 patients, respectively. Open circles: *CYP2B6 *6/*6* genotype holders, closed circles: other *CYP2B6* genotypes holders. Middle bar indicates mean, and upper and lower bars SD. (#) Patient on 200 mg EFV once daily.

the frequency was 6% in Caucasian population [7]. If these patients could be treated with low dose EFV based on genetic data of *CYP2B6 *6/*6* genotype, it could represent the first step of the Tailor-Made therapy of HIV-1 infection.

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Carvedilol, Metoprolol, and Insulin Resistance

To the Editor: The study by Dr Bakris and colleagues[1] comparing the metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension found that insulin sensitivity improved with carvedilol (–9.1%; $P=.004$) but not metoprolol (–2.0%; $P=.48$), with a between-group difference of –7.2% (95% confidence interval [CI], –13.8% to –0.2%). The reference the article cites supporting the use of the Homeostasis Model Assessment–Insulin Resistance (HOMA-IR) as the tool to measure insulin resistance was the study by Haffner et al.[2] This was a prospective study assessing predictors of development of diabetes mellitus in 3.5 years of follow-up. Jayagopal et al[3] studied the variation of HOMA indices and showed that to consider a change of HOMA as clinically significant, the new value must represent at least a 90% increase or a 47% decrease. The mean improvement in HOMA-IR of 9% in the present study may be statistically significant but not clinically significant.

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1. Bakris GL, Fonseca V, Katholi RE, et al. Metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension: a randomized controlled trial. *JAMA*. 2004;292:2227-2236.
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In Reply: Dr Gonzalez-Feldman raises concerns about interpretation of changes in HOMA-IR. The study by Haffner et al[1] was cited to document the validity of HOMA-IR as a measure of insulin resistance in large numbers of patients. HOMA-IR correlates well with more complex testing such as the euglycemic clamp method ($r^2=-0.88$; $P<.001$) and the minimal model ($r=0.7$; $P<.001$).[2,3] A review of this test in the context of large studies documents its validity.[4]

Although a change in 2 measurements in the same patient may be due to biological variation, as suggested by Jayagopal et al,[5] this is not applicable to large groups, as in the Glycemic Effects in Diabetes Mellitus: Carvedilol-Metoprolol Comparison in Hypertensives (GEMINI) study.[6] This is exemplified by data from the UK Prospective Diabetes Study,[7] in which metformin increased insulin sensitivity by 11% in the group that received it; although the group sensitivity remained at that higher level for 6 years, individuals within the group had varying results. Similarly, in the GEMINI study,[6] the difference in insulin sensitivity over time was maintained and closely paralleled differences in glycosylated hemoglobin (HbA_{1c}) between the groups. Because of the congruency of data between changes in HOMA-IR and HbA_{1c} in this and other studies, large magnitudes of change in HOMA-IR are not needed to support a clinically important benefit. Thus, we believe that the difference in insulin sensitivity between carvedilol and metoprolol was clinically as well as statistically significant.

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RESEARCH LETTER

Ribavirin and Use of Clotting Factors in Patients With Hemophilia and Chronic Hepatitis C

To the Editor: Ribavirin has been used in combination with interferon (IFN) alfa to treat chronic hepatitis C. This combination therapy has been reported to be more effective than IFN monotherapy for eradicating hepatitis C virus (HCV),[1-3] including that occurring in patients with concomitant hemophilia.[4,5] We are unaware of previous reports of reduction in the use of clotting factors in HCV-

Table. Patient Characteristics and Treatment Responses*

Patient No.	Age, y	Hemophilia†			HCV‡				
		Type	Severity	Duration, y	HCV-RNA Level, kIU/mL	HCV Genotype	Duration of HCV Infection, y	Ribavirin Load, mg/d	Eradication of HCV
1	28	A	Moderate	28	44	3a	27	800	Yes
2	61	A	Severe	61	640	3a	29	800	Yes
3	50	A	Severe	50	850	1b	34	600/400§	No
4	42	B	Mild	42	510	2a + 1b	30	800	Yes
5	44	A	Severe	44	600	3a	26	800	Yes
6	52	A	Mild	52	750	2b	22	600	Yes
7	37	A	Mild	37	59	1a	29	800/600§	No
8	44	B	Moderate	44	310	1a	33	800	Yes

*Human immunodeficiency virus (HIV) infection status, as determined by HIV antibody detection using a particle agglutination assay kit (Fuji Rebio, Tokyo, Japan), was negative for all patients except patient 7.
†Severity of hemophilia was classified as mild when clotting factor was above 5%, moderate when clotting factor was 1 to 5%, and severe when clotting factor was below 1%.
‡Serum hepatitis C virus (HCV) RNA levels were measured by Amplicor HCV assay version 2.0 (Roche Diagnostic Systems, Tokyo) at start of treatment. Hepatitis C virus genotype was determined by direct sequence of the 5'UTR region. Eradication of HCV was considered positive when the absence of serum HCV RNA was maintained for 24 weeks after treatment was completed.
§Patient 3 received 600 mg/d for 12 weeks and 400 mg/d for 12 weeks. Patient 7 received 800 mg/d for 8 weeks and 600 mg/d for 16 weeks.

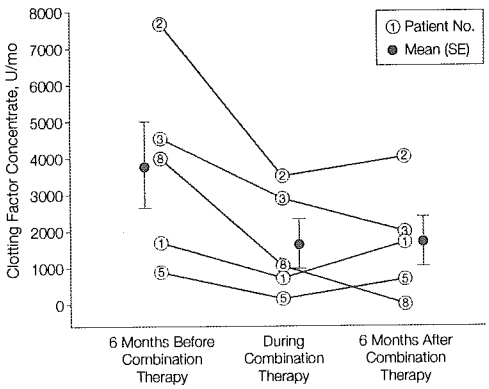
positive patients with hemophilia treated with ribavirin and IFN.

Methods. Eight consecutive patients with hemophilia were treated as outpatients for HCV infection with IFN and ribavirin between June 2002 and November 2003 at Nagoya University Hospital (TABLE). All patients were men with a mean (SD) age of 44.8 (10.0) years. Six patients had hemophilia A, and 2 had hemophilia B. Hemophilia was severe in 3 patients, moderate in 2, and mild in 3. Four patients (patients 1, 2, 3, and 5) had been previously treated with IFN alfa-2b alone (Intron A; Schering Plough K. K., Osaka, Japan) (6 MU/d for 2 weeks, followed by 3 times/wk for 22 weeks), but HCV had not been eradicated. During this study, all patients were treated with the same 24-week regimen of IFN. Oral ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ) was administered at a dose of 600 mg/d for patients who weighed 60 kg or less and 800 mg/d for those who weighed more than 60 kg during 24 weeks. The dose of ribavirin was reduced by 200 mg/d if the patient's hemoglobin concentration decreased to below 10 g/dL due to hemolytic anemia induced by ribavirin; the hemoglobin level did not fall this low from any other causes.

The use of clotting factors was assessed by patient logs. Because the patients with mild hemophilia rarely used clotting factors, we compared the average use of clotting factors from the 5 patients with moderate and severe hemophilia before, during, and after treatment. Pairwise comparisons were made by 2-sided t test, with statistical significance defined as $P<.05$. All statistical analyses were done using JMP version 5.0.1 (SAS Institute Inc, Cary, NC). Ability to perform activities of daily living was assessed by interview. The protocol was approved by the Nagoya University institutional review board and written informed consent was obtained from each patient before treatment.

Results. In the 5 patients requiring clotting factor, the mean (SD) use of clotting factors per month for the 6 months prior to combination therapy was 3783 [2646] U/mo; during therapy it was 1605 [1488] U/mo; and for the 6 months after therapy it was 1667 [1528] U/mo (FIGURE). The mean use during treatment was significantly lower than before treatment ($P=.03$). The mean use after treatment was lower than before treatment, approaching statistical significance ($P=.06$). Seven of 8 patients noted no limitation of life activities before or after the start of IFN and ribavirin. One patient (patient 1) noted slight limitation of daily activities because of fatigue associated with combination therapy. The use of clotting factors in this patient was reduced during treatment but returned to the pretreatment level afterward. The average

Figure. Use of Clotting Factor Concentrates 6 Months Before, During, and 6 Months After Combination Therapy With Ribavirin and Interferon for the 5 Patients Requiring Clotting Factor During the Study



Differences in each period were analyzed by paired t test. $P=.03$ for comparison of pretreatment and active treatment; $P=.06$ for comparison of pretreatment and posttreatment. Large data markers indicate mean clotting factor use; error bars, SEs.

numbers of bleeding episodes treated per month in the 6 months before combination therapy, during the therapy period, and in the 6 months after therapy, respectively, were 1.7, 0.7, and 1.6 (patient 1); 6.0, 3.0, and 4.1 (patient 2); 4.0, 2.6, and 2.2 (patient 3); 1.0, 0.2, and 0.7 (patient 5); and 3.6, 0.8, and 0.0 (patient 8). Most bleeding episodes were hemarthroses; a few involved mucosal or intramuscular bleeding.

Comment. In our hospital, 47 patients with hemophilia who had been treated for chronic hepatitis C with IFN alone had no observed reduction in the use of clotting factor, including 4 of the patients in this study. This strongly suggests that the reduced use of clotting factors was associated with the addition of ribavirin.

The reason for the reduction in the use of clotting factors by these patients with hemophilia is not clear. One suggestion comes from a case report that described an increase in warfarin dose requirement in a patient with a heart valve prosthesis after the start of this combination therapy,[6] and a change in coagulation status after starting ribavirin could be associated with our observations.

In 2 patients in this study, the decreased use of clotting factors continued after combination therapy ended. Being able to increase physical activities during treatment might strengthen muscles recovering from atrophy caused by joint bleeding. This increased strength could reduce stress on joints, further decreasing the risk of spontaneous hemarthrosis.

Based on this case series, ribavirin in combination with IFN, and possibly alone, may reduce the use of clotting factors in patients with hemophilia and chronic hepatitis C. Further studies, including replication of these findings and clinical trials, would help to clarify the potential role of ribavirin in these patients.

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LETTERS TO THE EDITOR

Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin

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The combination therapy with ribavirin and interferon-α (IFN-α) has been reported to be more effective than IFN-α monotherapy for eradicating hepatitis C virus (HCV) [1,2], including patients with concomitant hemophilia [3]. We observed significant decreases in doses of clotting factors used for hemostatic therapy in hemophiliacs during ribavirin administration (e.g. 3780 units per month before ribavirin treatment and 1600 units per month during ribavirin on the average) [4]. In our hospital, 47 hemophilic patients who had been treated for chronic hepatitis C with IFN-α alone demonstrated no significant reduction in the use of clotting factor. This observation strongly suggests that the addition of ribavirin leads to the reduction of clotting factors used for bleeding in hemophiliacs. One suggestion comes from a case report that described an increase in warfarin dose requirement in a patient with heart valve prosthesis after starting this anti-HCV combination therapy [5].

These observations led us to investigate the ribavirin-induced change in vitamin K-dependent coagulation factors. To this purpose, we have measured the clotting activity of factor (F)VII, X, and prothrombin in hemophilic patients who were receiving the anti-HCV combination therapy. The protocol of therapy and analysis was approved by the Nagoya University institutional review board and written informed consent was obtained from each patient before treatment. Nine hemophilic patients, including seven hemophilia A and two hemophilia B (mean age ± SD: 42.5 ± 10.4 years old), whose characteristics were previously described [4], were entered in this study.

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The liver biopsy performed before starting the combination therapy did not show cirrhosis but chronic hepatitis in all patients analyzed. During this study, all patients were treated with the same 24-week regimen of IFN-α 2b (Intron A®, Schering Plough, K.K., Osaka, Japan) and oral ribavirin (600–800 mg day^{-1} of Rebetol; Schering-Plough, Kenilworth, NJ, USA). All statistical analyses were performed with STATA ver.7 software (STATA Corp., College Station, TX, USA) and the P-value < 0.05 was considered statistically significant.

The procoagulant activity of FVII in plasma has been elevated in all of nine ribavirin-treated hemophilic patients in comparison with that before ribavirin administration (Fig. 1A). The average and standard deviation for the elevation of FVII activity was 15.7% ± 8.8% ($P < 0.04$ in before vs. during ribavirin treatment; max. 28%; min. 5%). This elevation of FVII activity was independent of improvement of liver function (i.e. albumin, total billirubin, cholinesterase) in the patients (not shown). Only two patients, one has HIV infection and the other has hepatitis B virus concomitant with HCV, did not show a substantial elevation of FVII activity (i.e. 5% and 8%, respectively). We then measured activated FVII (FVIIa) levels in patients' plasma before and during ribavirin treatment using STACLOT® VIIa-rTF (Diagnostica Stago, Asnieres, France) [6] and observed substantial increases in FVIIa (e.g. 25.3 ± 14.8 mU mL^{-1}), which were almost compatible with elevation of FVII clotting activity. The plasma levels of FX and prothrombin were unchanged by ribavirin treatment in all of nine hemophilic patients (not shown). The elevation of FVII clotting activity by ribavirin is consistent with the previous observation of warfarin resistance in a ribavirin-treated patient [5].

To investigate the mechanism of ribavirin-induced elevation of FVII activity, we analyzed the gene expression of FVII in cultured normal human hepatocytes (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) or human hepatoma cell line, HepG2 cells (ATCC, Manassas, VA, USA),

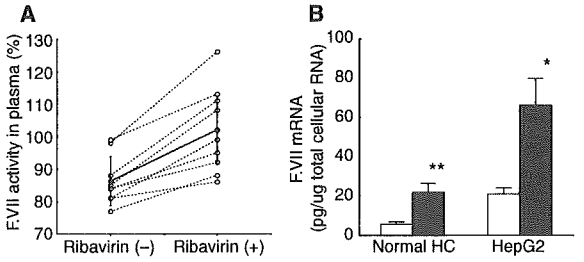

Fig. 1. Clotting activity of FVII in plasma of hemophilic patients and the mRNA expression of FVII in cultured human hepatocytes with or without ribavirin. (A) Each clotting activity of FVII in plasma of nine hemophilic patients before and at 4 weeks after starting ribavirin therapy was shown as open circle and dashed line, respectively. The average and SD of all patients was expressed as closed circle and error bar (without ribavirin: 86.3% ± 7.6%; with ribavirin: 102.0% ± 10.3%; $P < 0.04$). (B) Normal human hepatocytes or HepG2 cells had been cultured with (■) or without (□) ribavirin for 48 h. The mRNA expression of FVII was quantitated by real-time RT-PCR assay. Each value is expressed as the mean and SD from three sets of experiments. All of real-time RT-PCR assays were performed in duplicate. $*P < 0.02$; $**P < 0.01$.

which were cultured in medium with ribavirin at clinically therapeutic concentration (150 μg mL^{-1}) in the presence of IFN-α 2b (0.75 μg mL^{-1}; kindly provided by Schering Plough, K.K.). The expression level of mRNA for FVII, FX, and prothrombin, was determined by real-time quantitative RT-PCR with the ABI Prisms 7700 Sequence Detection (Perkin-Elmer Biosystems, Foster City, CA, USA) and SYBR Green PCR Kit (Perkin-Elmer Biosystems), according to the manufacturer's recommendations. The sequences of primer pairs used to quantify mRNA of the above genes were described in the NCBI Sequence Viewer. Variations in sample loading were assessed by measuring β-actin mRNA. Comparison of quantitative RT-PCR results between two groups was performed with the two-sample *t*-test. Welch's method was applied when variance between two groups was unequal (statistical significance: $P < 0.05$).

Significant induction of FVII mRNA was demonstrated in cultured normal hepatocytes (fourfold; $P < 0.01$) or HepG2 cells (threefold; $P < 0.02$) at 48 h after ribavirin treatment (Fig. 1B). No significant induction of mRNAs for FX and prothrombin was detected in ribavirin-treated cultured hepatocytes or HepG2 cells (not shown). In hepatocytes, ribavirin may stimulate to synthesize FVII by binding specifically to the promoter region of FVII gene (under current investigation).

It is possible that not only the induction of FVII but also changes in other coagulation factors during ribavirin therapy may be responsible for the decreased events of bleeding in hemophiliacs. However, the elevation of FVII activity in plasma could contribute most to the increased hemostatic potential in hemophilic patients because the cell-based tissue factor-activated FVII would play a central role in initiating coagulation and in activating platelets followed by large scale thrombin generation [7]. Clinically, recombinant activated FVII has been widely used as an antidote to control and prevent excessive hemorrhage in hemophilic patients with inhibitors [8]. Meanwhile, it was reported that even 10–20% of increase in plasma FVII/FVIIa would be an independent risk factor for coronary heart disease in healthy individuals [9,10], suggesting that a substantial elevation of endogenous FVII levels could result in an increased thrombotic potential. In general, the occurrence of spontaneous bleeding events in hemophiliacs is dependent on the critical hemostatic balance. In these conditions, 15–20% elevation of intrinsic FVII activity in plasma (Fig. 1A), because of the continuous induction of endogenous FVII by ribavirin (Fig. 1B), would contribute to the prevention from spontaneous bleeding in hemophiliacs. As a half-life of FVII in plasma is the shortest in all of coagulation factors, the continuous induction of FVII can maintain or increase the hemostatic value *in vivo*. If the prophylaxis to bleeding in hemophilic patients by ribavirin treatment were executable, it would result in much improvement of quality of their life and in large reduction of medical expenses in the country.

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BRIEF REPORT

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Clearance of GB virus C during highly active antiretroviral therapy and course of HIV disease progression in HIV-infected patients with hemophilia

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Highly active antiretroviral therapy (HAART) has been shown to improve the prognosis for HIV-infected patients through dramatic improvement of immune function. In the long-term follow-up of individuals receiving HAART, the status of other concomitant viruses, such as hepatitis C virus (HCV) or GB virus C (GBV-C), may also be altered [1]. It was recently reported that GBV-C viremia has a favorable effect on the prognosis of HIV infection [2–5], but a more recent study reported that loss of GBV-C viremia during follow-up of HIV-infected patients suggests a poor prognosis [6]. The prevalence of GBV-C clearance during HAART and the effect of GBV-C clearance on the clinical course of HIV infection have not yet been clarified. In this report, GBV-C persistence and clearance in HIV-infected patients receiving HAART and the effect of GBV-C clearance on HIV disease are discussed.

In a previous study, we investigated the presence of GBV-C RNA in sera obtained prior to 1988 from 41 HIV-infected patients with hemophilia [2]. GBV-C RNA was detected in 11 of these 41 patients. Two of the 11 patients died from AIDS before HAART was introduced, and the remaining nine patients began receiving HAART between 1996 and 1997. We monitored these nine patients until September 2004, and the findings of this long-term follow-up are presented here.

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All nine patients tested positive for persistence of GBV-C RNA and negative for E2 antibody against GBV-C at the start of HAART. Annual serum samples from all patients were tested for the presence of GBV-C RNA using nested reverse transcription polymerase chain reaction [7]. HIV RNA levels, determined using an Amplicor HIV Monitor Assay (Roche Molecular Systems, Branchburg, NJ, USA) and CD4+ T-cell counts were also monitored annually. In patients in whom GBV-C RNA disappeared during follow-up, GBV-C E2 antibody was measured annually thereafter using an enzyme-linked immunosorbent assay [8]. Written informed consent was obtained from all nine patients before HAART was started. The study was approved by the University Ethics Committee and carried out in compliance with the Helsinki Declaration.

HIV RNA concentrations, CD4+ T-cell counts and GBV-C viremia status for all nine patients are summarized in Table 1. All of the patients were followed up for more than 7.5 years. In all patients, HIV RNA levels decreased after the start of HAART and fell below the detection limit within 2 years. CD4+ T-cell counts increased accordingly and were within the normal range, also within 2 years. All patients tolerated the therapy well and were compliant.

As of the end of September 2004, no patient had discontinued therapy. In three of the nine patients (patients 2, 8, and 9), GBV-C RNA was no longer detected in sera obtained after the start of HAART, whereas it was detected throughout the therapy in the remaining six patients. Clearance of GBV-C was accompanied by the appearance of GBV-C E2 antibody in two patients (patients 2 and 9). In contrast, in patient 8, E2 antibody was not detected in any of the annual serum samples obtained after the clearance of GBV-C, and serum was still negative at the end of September 2004. During observation, no patient showed HAART failure or advancement to AIDS, and no patient died. HIV RNA levels remained below the detection limit and CD4+ T-cell counts were normal in all patients regardless of GBV-C RNA and E2 antibody status.

It has been reported that 40–70% of immunocompetent persons show spontaneous clearance of GBV-C with the development of E2 antibody [9, 10]. Therefore, it should be

Table 1 Clinical features of HIV-infected patients with GB virus C who received highly active antiretroviral therapy (HAART)

Patient	Age (year)	Status at start of HAART		Clearance of GBV-C[a]	Appearance of E2 antibody	Status at end of September 2004	
		HIV RNA concentration (copies/ml)	CD4+ T-cell counts (cells/mm^3)			HIV RNA concentration (copies/ml)	CD4+ T-cell counts (cells/mm^3)
1	24	160,000	15	No	NT	UDL	553
2	15	5,800	110	Yes (2.5 years)	Yes	UDL	748
3	36	14,000	275	No	NT	UDL	474
4	13	810	228	No	NT	UDL	810
5	42	26,000	362	No	NT	UDL	445
6	19	58,000	36	No	NT	UDL	594
7	28	28,000	379	No	NT	UDL	540
8	21	1,100	476	Yes (0.5 year)	No[b]	UDL	852
9	39	77,000	260	Yes (2.0 years)	Yes	UDL	676

GBV-C GB virus C, *NT* not tested, *UDL* under the detection limit
[a]Number of years of GBV-C clearance after start of HAART is given in parentheses
[b]E2 antibody was not detected in annual serum samples between 1998 and 2004, and the enzyme immunosorbent assay was still negative at the end of September 2004

clarified whether GBV-C clearance during HAART also has an adverse effect on the clinical course of HIV infection. We observed clearance of GBV-C in three of nine HIV-infected patients. Of the three patients with GBV-C clearance, two had developed an E2 antibody response to GBV-C, whereas the remaining patient had not. A previous study reported a poorer prognosis for HIV-infected patients in whom GBV-C had been cleared and who failed to develop E2 antibodies [6]. We found no differences in immune status or HIV RNA concentration between patients with and without clearance of GBV-C RNA, regardless of the development of E2 antibody.

In conclusion, GBV-C clearance was observed during HAART in one-third of our cohort of HIV-infected patients, but this had no adverse effect on HIV disease progression. Larger studies with longer observation periods may clarify the effect of GBV-C clearance and presence of E2 antibody on HIV disease.

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

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

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INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Patients

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

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

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

Determination of HBV DNA

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

Genotyping HBV

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

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

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

Amplifying and Sequencing HBV DNA of Genotype A Isolates

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

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

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

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

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

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

Statistical Analysis

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

RESULTS

Distribution of HBV Genotypes

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

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

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

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

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

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

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

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

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

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

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

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

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

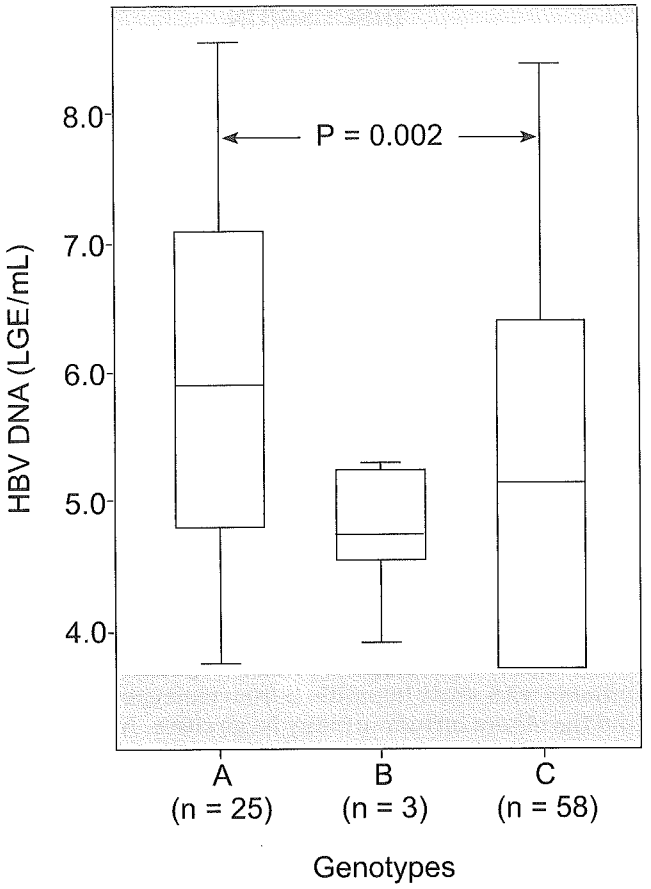

Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded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

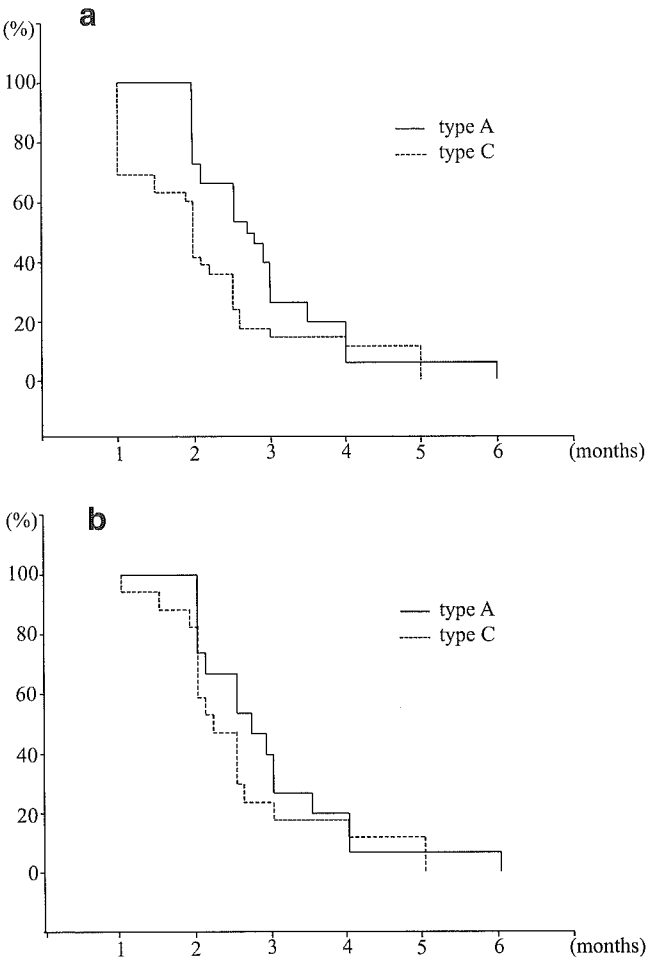

Fig. 2. The duration of HBsAg in patients with acute hepatitis B with genotypes A or C. The results are shown for (**a**) all patients, and (**b**) patients with the wild-type sequences both in precore and BCP regions of HBV.

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

Subtypes of Genotypes A and B

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

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

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

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

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

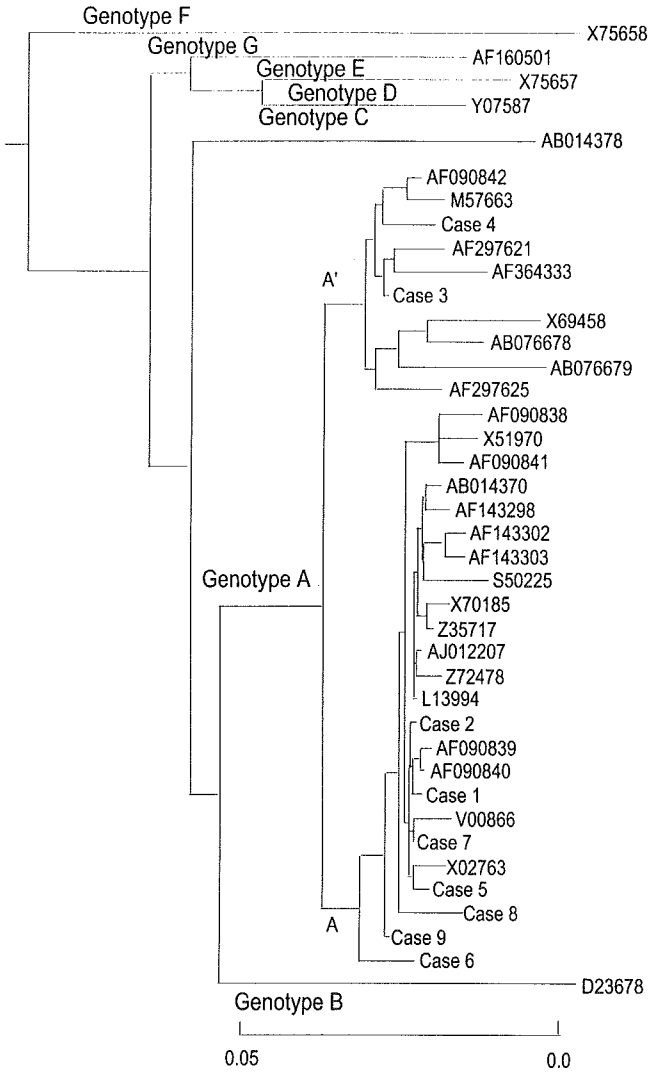

Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major *S*-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1015 IU/L, respectively, $P = 0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09 vs. 1.58 ± 1.24 months, $P = 0.35$) (Fig. 2b).

DISCUSSION

The salient finding in this study is that infection with HBV genotype A is frequent in patients with acute hepatitis in Japan, lending support to previous studies [Kobayashi et al., 2002; Ogawa et al., 2002]. Substantial portion of patients with acute hepatitis were infected with genotype A, which is detected rarely among patients with chronic hepatitis in Japan [Orito et al., 2001a; Kobayashi et al., 2002]. Genotype A prevails in North-Western Europe, United States, Central Africa, and India [Kao, 2002; Miyakawa and Mizokami, 2003]. This genotype may be prevalent in countries elsewhere, since the distribution of HBV genotypes has not been examined in many districts of the world. Phylogenetic analysis has shown that seven (78%) HBV/A strains of the nine patients examined with acute hepatitis B were of the European-American type. Although the HBV/A sequences from four, (cases 1, 2, 5, and 7) clustered with those reported previously, those from three (cases 6, 8, and 9) were separated genetically (Fig. 3), which suggests their distinct geographic origin.

Notably, the genotype distribution differed between patients with acute hepatitis B from metropolitan areas and the others including many large cities. As genotype A is seen rarely in patients with chronic hepatitis [Orito et al., 2001a; Kobayashi et al., 2002], it is suspected that genotype A in metropolitan areas has a distinct geographic origin. Many patients with genotype A infection in these areas had a history of extramarital sexual contacts with plural unspecified partners. Such sexual behavior may increase the risk of infection with genotype A. In support of this view, most homosexual people in Tokyo who have human immunodeficiency virus type I are coinfected with HBV genotype A [Koibuchi et al., 2001]. Taken together, homosexual activity would increase the risk of genotype A infection in metropolitan areas. Further molecular analysis on HBV isolates from transmitters and recipients will verify this hypothesis. With respect to genotype B, both Ba, and Bj subtypes [Sugauchi et al., 2002b] were detected. Although the number of studied patients was small, patients with subtype Ba were found in the Tokyo metropolitan area exclusively. Whether subtype Ba intrinsic to the metropolitan area has a peculiar geographic origin is currently unknown and awaits further analyses.

Another point made in this study is that HBV genotypes influence clinical features and the outcome of acute hepatitis B. It has been shown that the proportion of patients who develop chronic HBV infection is close to 10% in European and American countries [Sherlock S, 1997] but rare in Japan [Kobayashi et al., 2002]. Recent studies suggest that chances for evolution into chronicity may differ among patients acutely infected with HBV of distinct genotypes [Mayerat et al., 1999; Ogawa et al., 2002]. Our study has shown that patients with genotype A had higher HBV DNA and lower ALT levels, as well as a longer duration of HBsAg in serum. Development of chronic hepatitis was seen in one of the 27 (4%) patients with genotype A as against one of the 109 (1%)