MATERIALS AND METHODS

Patients

A total of 441 therapy-naive HIV-1-infected patients underwent their initial consultation at Nagoya Medical Center in Nagoya, Japan, between January 1999 and December 2006. Genotypic drug-resistance testing for HIV-1 was performed on 402 of the 441 patients (91%) after obtaining patient consent. The characteristics of the 402 patients are shown in Table 1.

Genotypic drug-resistance testing for HIV-1

Genotypic drug-resistance testing for HIV-1 was performed as previously reported.[21,22] HIV-1 RNA was purified from a plasma sample using a QIAamp viral RNA mini kit (QIAGEN, Tokyo, Japan). A single DNA fragment containing both protease (PR) and reverse transcriptase (RT) genes was amplified by reverse transcription-nested polymerase chain reaction (RT-nested PCR) using the Superscript one-step RT-PCR for long templates kit (Invitrogen, Tokyo, Japan) and LA Taq polymerase (Takara, Shiga, Japan). A labeling reaction for DNA sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan), and DNA sequences were determined by the direct sequencing method using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequences were converted to amino acid sequences, and then amino acid mutations were extracted through comparison with amino acid sequences of the HIV-1 HXB2 strain. Judgment of drug-resistant amino acid mutations was performed according to the latest version of the International AIDS Society USA panel, Fall 2006.[23]

TABLE 1. CHARACTERISTICS OF 402 THERAPY-NAÏVE HIV-1-INFECTED PATIENTS

Age, years		
Median (IQR[a])	33 (28–41)	
Sex		
Male	362	90.0%
Female	40	10.0%
Nationality		
Japanese	335	83.3%
Foreign	67	16.7%
Risk factor for infection		
Homosexual	237	59.0%
Heterosexual	87	21.6%
Bisexual	32	8.0%
Unknown	46	11.4%
CD4 cell count, cells/μl		
Median (IQR[a])	270 (94–400)	
Viral load, $\log_{10}$ copies/ml		
Median (IQR[a])	4.77 (4.26–5.26)	
HIV-1 subtype		
B	346	86.1%
Non-B[b]	56	13.9%

[a]IQR, interquartile range.
[b]CRF01_AE, 30; A, 9; C, 8; D, 4; F, 2; G, 2; unclassified, 1.

Phylogenetic analysis

Phylogenetic analysis was performed using the nucleotide sequences of HIV-1 obtained from all 402 therapy-naive patients. Nucleotide sequences (1005 bases) containing both PR (codons 1–99) and RT (codons 1–236) genes were used. Multiple sequence alignment was performed using CLUSTAL W, and evolutionary distances were calculated using the Kimura two-parameter model. A phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates. These analyses were performed using MEGA software version 3.1.[24] Nucleotide sequences of 32 reference HIV-1 strains were obtained from the HIV sequence database in the Los Alamos National Laboratory. Subtyping of HIV-1 was also performed using the phylogenetic tree.

Measurement of viral load and CD4 cell count

Viral load was measured using an Amplicor HIV-1 monitor v1.5 system (Roche Diagnostics, Tokyo, Japan). CD4 cell counts were measured using a FACSCalibur flow cytometry system (Becton Dickinson, Tokyo, Japan).

Statistics

Multiple logistic regression analysis was performed to assess associations between patient characteristics and infection with drug-resistant or 215-revertant HIV-1 variants. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using SYSTAT version 10.2 software (SYSTAT Software, California, USA).

RESULTS

Emergence trend of drug-resistant HIV-1 in therapy-naive patients

The prevalence of drug-resistant HIV-1 fluctuated between 2.3% and 10.0% through the period from 1999 to 2006 (Fig. 1). The first wave was observed from 2001 to 2003, with prevalence increasing from a trough of 2.3% in 2001 and peaking at 10.0% in 2003. After that, the prevalence dropped to 4.2% in 2004, but increased again to reach 8.8% by 2006. The mean prevalence for the past 8 years was 6.7% (27/402).

Variants with NRTI-resistant mutations were sporadically found (Fig. 2A). Concerning nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant variants, none was found from 1999 to 2002 (Fig. 2B). However, two variants with the K103N mutation first emerged in 2003, and this type of variant was continuously detected thereafter. Variants with the V108I and P225H mutations first emerged in 2004 and 2006, respectively. Variants with protease inhibitor (PI)-resistant mutations appeared continuously from 2000 (Fig. 2C). The most abundant variant was that with the M46I mutation alone, found in a total of 12 cases (2000, $n = 1$; 2002, $n = 2$; 2003 and 2004, $n = 1$ each; 2005, $n = 2$; and 2006, $n = 5$). In contrast, variants with the L90M, L33F, or M46L mutation alone appeared once each in 2001, 2003, and 2006, respectively. A variant possessing

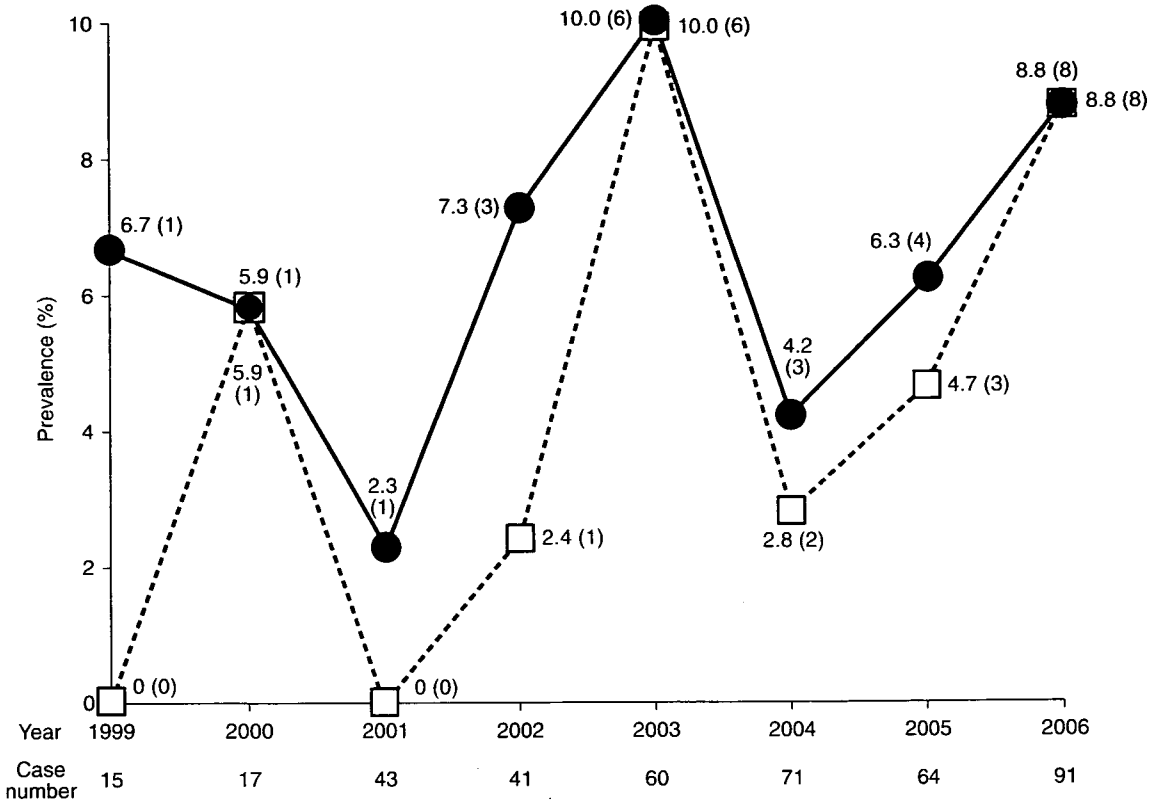

FIG. 1. Changes in prevalence of drug-resistant HIV-1 with major amino acid mutations (●—●) and 215-revertant variants (□—□) in therapy-naive patients. Genotypic drug-resistance testing was performed for 402 therapy-naive patients from 1999 to 2006. Detected numbers are shown in parentheses.

multiple mutations of V32I, M46I, I47V, and L90M was found very recently.

Characteristics of drug-resistant HIV-1

Characteristics of drug-resistant HIV-1 found in our surveillance are shown in Table 2. The most frequently found variant was a PI-resistant virus with the M46I mutation alone (n = 12), followed by an NNRTI-resistant virus with the K103N mutation alone (n = 4). Variants with two-class resistance were found in two cases, one possessing both PR- and NNRTI-resistant mutations, and the other with both PI- and NRTI-resistant mutations. Of note is the fact that no virus with resistance against all three classes was found in our surveillance.

Emergence trends for HIV-1 variants possessing the 215-revertant amino acid mutation in the reverse transcriptase

T215A/C/D/E/G/H/I/L/N/S/V amino acid substitutions in the RT represent revertant mutations of the T215Y/F NRTI-resistant mutation.[23] The 215-revertant mutations do not exhibit NRTI resistance by themselves, but most can reconvert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation. In other word, most 215-revertant variants can much more easily change to NRTI-resistant variants under the pressure of NRTIs than wild-type HIV-1.[17–20] We feel drug-resistant variants with the T215Y/F mutation are difficult to survive in the drug-free condition, as only one variant with the T215Y mutation has been found during an 8-year surveillance. The results of other researchers support our feelings.[17,18] Examination of the emergence of the 215-revertant variant in addition to the T215Y/F-possessing resistant variant is thus important. In our surveillance, variants possessing the T215A/C/D/E/G/L/S mutation were found in 21 cases; since T215G/D was found in 2000, such variants have been increasing (Fig. 2D). Among these, 17 cases (81%) can reconvert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation.

Phylogenetic analysis

This study identified 27 drug-resistant variants from 402 therapy-naive patients. We next performed phylogenetic analysis to clarify whether specific drug-resistant strains were spreading. Three different clusters were identified from 20 of 27 drug-resistant variants (#1–13, #14–18, and #19–20) on a phylogenetic tree (Fig. 3A). All the clusters were consisted of subtype B viruses. The remaining seven variants were dispersed over the tree (Fig. 3A, #21–27). Two out of the seven were non-B viruses, subtype D and CRF01_AE. Detailed divergence of

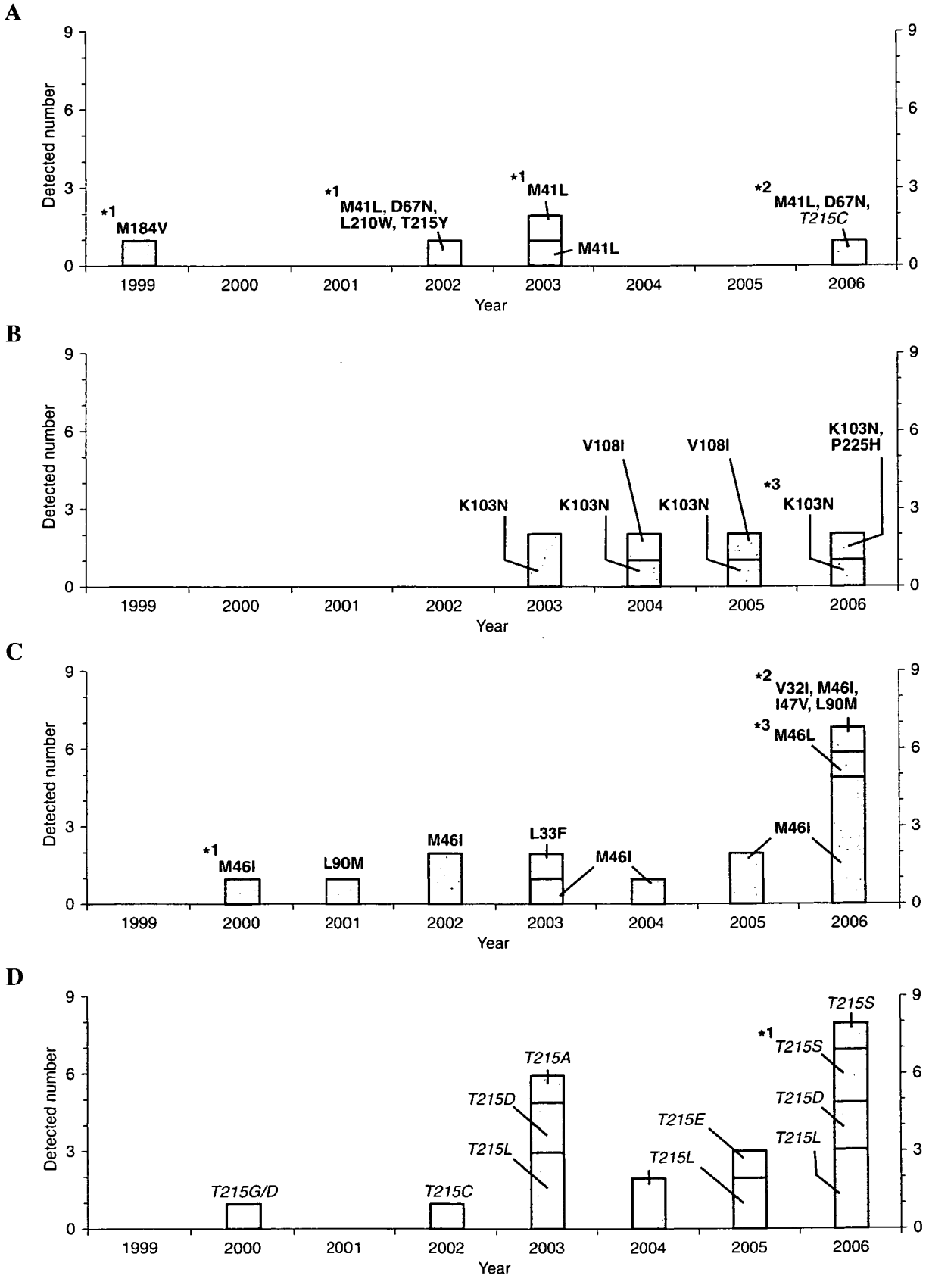
A
Detected number
Year
1999
2000
2001
2002
2003
2004
2005
2006
*1 M184V
*1 M41L, D67N, L210W, T215Y
*1 M41L
M41L
*2 M41L, D67N, T215C
B
K103N
K103N
V108I
K103N
V108I
*3 K103N
K103N, P225H
C
*1 M46I
L90M
M46I
L33F
M46I
*2 V32I, M46I, I47V, L90M
*3 M46L
M46I
D
T215G/D
T215C
T215A
T215D
T215L
T215L
T215E
*1 T215S
T215D
T215L
T215S

TABLE 2. CHARACTERISTICS OF DRUG-RESISTANT HIV-1

Type of drug resistance	n	*Major drug-resistant amino acid mutations*[a]
PI resistance	12	PR: **M46I**
alone	1	PR: **L33F**
14 (3.5%)	1	PR: **L90M**
NNRTI resistance	4	RT: **K103N**
alone	2	RT: **V108I**
7 (1.7%)	1	RT: **K103N, P225H**
NRTI resistance	2	RT: **M41L**
alone	1	RT: **M41L, D67N, L210W, T215Y**
4 (1.0%)	1	RT: **M184V**
Two class	1	PR: **M46L**
resistance		RT: **K103N**
2 (0.5%)	1	PR: **V32I, M46I, I47V, L90M**
		RT: **M41L, D67N,** ***T215C***

[a]Major drug-resistant mutations and 215-revertant mutations are shown in bold and italics, respectively. PI, protease inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PR, protease; RT, reverse transcriptase.

the 13 PI-resistant variants (#1–13) with the M46I or L90M mutation is shown in Fig. 3B, indicating derivatives from a common ancestral strain. Four NNRTI-resistant variants and a two class-resistant variant possessing the K103N mutation formed another cluster (Fig. 3C, #14–18). Concerning the 215-revertant variants, 19 of 21 variants formed an independent cluster (Fig. 3A, #28–46; Fig. 3D). The clusters B and D were made by continual detection of the corresponding viruses from 2000 to 2006, but the cluster C from 2003 to 2006 (Fig. 3).

Statistical analysis

No significant differences in age, sex, nationality, risk factors for infection, CD4 cell count, viral load, or HIV-1 subtype were seen between patients with drug-resistant or 215-revertant variants and patients with wild-type viruses (data not shown).

DISCUSSION

The prevalence of drug-resistant HIV-1 among therapy-naive patients in Nagoya, Japan, was studied from 1999 to 2006. The mean prevalence was 6.7% (27/402), which is lower than that reported recently from European and North American countries (8.1–25.2%),[25–37] but a tendency has recently been seen for increasing prevalence. Actually, prevalence has already exceeded the level at which the implementation of drug-resistance testing on therapy-naive patients is cost effective.[38,39]

Over the past 8 years, the most abundant drug-resistant HIV-1 strains have been PI-resistant variants (3.5%, $n = 14$), and most have possessed the M46I mutation alone. The second most abundant variants were NNRTI-resistant HIV-1 (1.7%, $n = 7$), most of which possessed the K103N mutation. This type of variant with K103N was first found in therapy-naive patients in 2003. As the corresponding NNRTIs of nevirapine, efavirenz, and delavirdine were approved in Japan from 1998 to 2000, 3–5 years will be needed for the appearance of drug-resistant amino acid mutations in therapy-naive individuals after the start of drug usage. The sporadic finding of NRTI-resistant variants (1.0%, $n = 4$) in our surveillance seems curious, as NRTIs have been in use since 1987 in Japan. However, this may be explained by the finding that many HIV-1 variants with revertant mutations of the T215Y/F NRTI-resistant mutation have frequently been identified since 2000. Moreover, most (81%, 17/21) possessed 215-reveratant mutations that could reconvert to the T215Y/F NRTI-resistant mutation through a single nucleotide change. Such highly resistant variants as three class-resistant variants have not yet been found, but two class-resistant variants were first identified in 2006.

Phylogenetic analysis yielded very important information, indicating that two independent major drug-resistant strains have been spreading in the Nagoya area, one possessing the M46I or L90M mutation and another possessing the K103N mutation. Furthermore, for 215-revertant variants, 19 of 21 variants were derivatives from the same strain, and have been independently spreading from 2000.

The present study succeeded in clarifying the epidemiological nature of drug-resistant variants and 215-reveratant variants in Nagoya, Japan. Our data will provide information valuable for attempts to block the spread of these variants.

SEQUENCE DATA

The base sequences of drug-resistant HIV-1, 215-revertant HIV-1, and wild-type HIV-1 have been registered in the DNA databank of Japan (DDBJ) as #AB356098–AB356124, #AB356125–AB356145, and #AB356146–AB356499, respectively.

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FIG. 2. Emergence trends for drug-resistant HIV-1 and 215-revertant variants. The *y*-axis shows detected numbers of drug-resistant HIV-1 or 215-revertant variants: NRTI-resistant mutations (**A**), NNRTI-resistant mutations (**B**), PI-resistant mutations (**C**), and 215-revertant mutations (**D**). Major drug-resistant mutations and 215-revertant mutations are shown in bold and italic characters, respectively. *1, five variants detected in non-Japanese patients. *2, a variant simultaneously possessing M41L, D67N, and T215C mutations in the RT and V32I, M46I, I47V, and L90M mutations in the PR. *3, a variant possessing the K103N mutation in the RT and the M46L mutation in the PR.

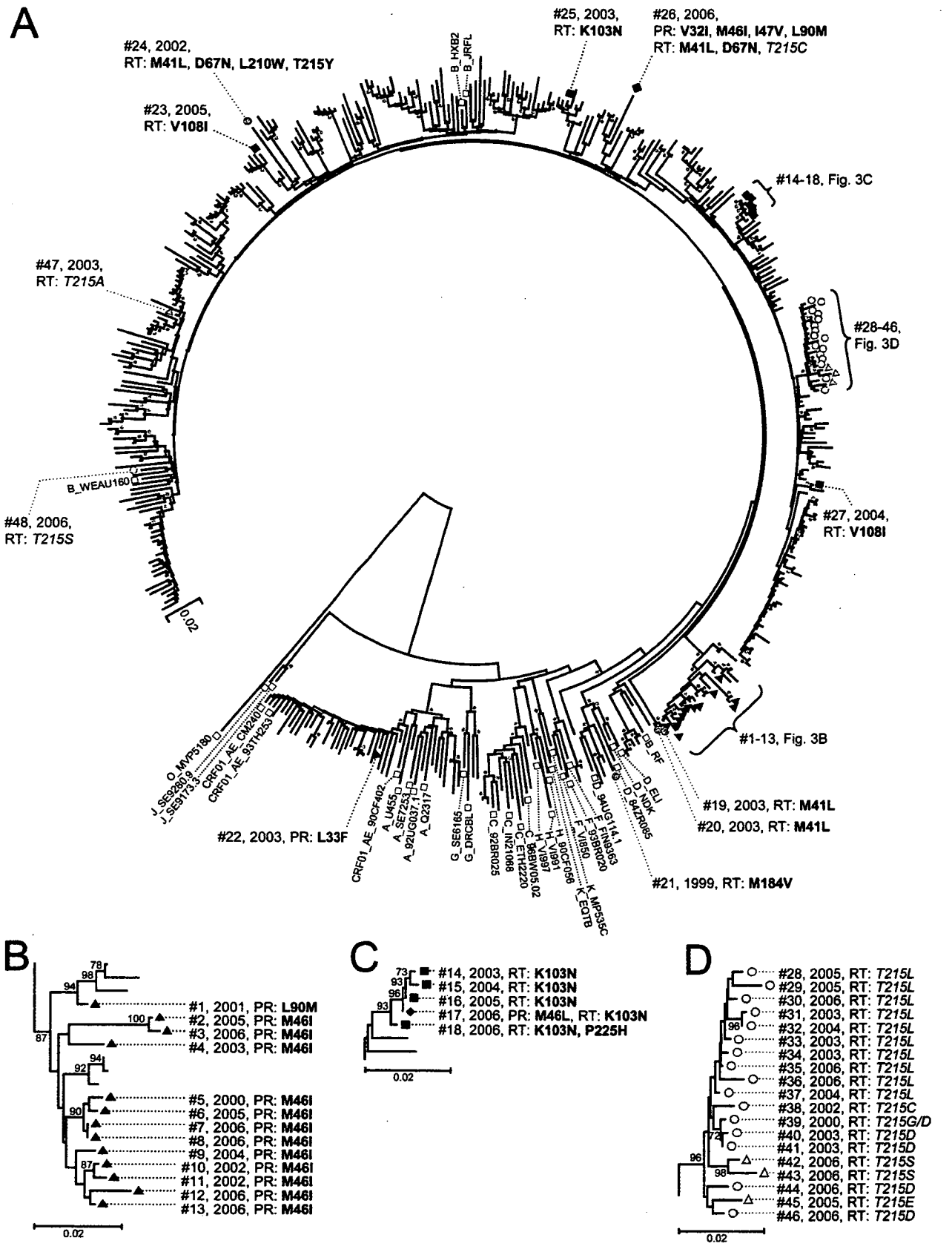
A
#24, 2002,
RT: M41L, D67N, L210W, T215Y
#23, 2005,
RT: V108I
#25, 2003,
RT: K103N
#26, 2006,
PR: V32I, M46I, I47V, L90M
RT: M41L, D67N, T215C
#14-18, Fig. 3C
#28-46,
Fig. 3D
#27, 2004,
RT: V108I
#1-13, Fig. 3B
#19, 2003, RT: M41L
#20, 2003, RT: M41L
#21, 1999, RT: M184V
#22, 2003, PR: L33F
#47, 2003,
RT: T215A
#48, 2006,
RT: T215S
B_HXB2
B_JRFL
B_WEAU160
0.02
B
#1, 2001, PR: L90M
#2, 2005, PR: M46I
#3, 2006, PR: M46I
#4, 2003, PR: M46I
#5, 2000, PR: M46I
#6, 2005, PR: M46I
#7, 2006, PR: M46I
#8, 2006, PR: M46I
#9, 2004, PR: M46I
#10, 2002, PR: M46I
#11, 2002, PR: M46I
#12, 2006, PR: M46I
#13, 2006, PR: M46I
0.02
C
#14, 2003, RT: K103N
#15, 2004, RT: K103N
#16, 2005, RT: K103N
#17, 2006, PR: M46L, RT: K103N
#18, 2006, RT: K103N, P225H
0.02
D
#28, 2005, RT: T215L
#29, 2005, RT: T215L
#30, 2006, RT: T215L
#31, 2003, RT: T215L
#32, 2004, RT: T215L
#33, 2003, RT: T215L
#34, 2003, RT: T215L
#35, 2006, RT: T215L
#36, 2006, RT: T215L
#37, 2004, RT: T215L
#38, 2002, RT: T215C
#39, 2000, RT: T215G/D
#40, 2003, RT: T215D
#41, 2003, RT: T215D
#42, 2006, RT: T215S
#43, 2006, RT: T215S
#44, 2006, RT: T215D
#45, 2005, RT: T215E
#46, 2006, RT: T215D
0.02

FIG. 3. Phylogenetic analysis of HIV-1 strains from 402 therapy-naive patients. (**A**) A phylogenetic tree was constructed by the neighbor-joining method using nucleotide sequences (1005 bases) containing both the PR (codons 1–99) and RT (codons 1–236) genes. Bootstrap analysis was performed with 1000 replicates, and values greater than 70 were shown as orange dots at the nodes of the tree. The scale bars represent nucleotide substitutions per site. Green closed circles, NRTI-resistant variants; blue closed squares, NNRTI-resistant variants; red closed triangles, PI-resistant variants; brown closed diamonds, two-class-resistant variants. Green open symbols indicate HIV-1 variants with a 215-revertant mutation that can reconvert to the T215Y/F NRTI-resistant mutation by acquiring a single nucleotide mutation (green open circles) or more than two nucleotide mutations (green open triangles). Black open squares indicate reference HIV-1 strains. Group O_MVP5180 was used as the outgroup. Each cluster containing 13 variants with the M46I or L90M mutation in the PR (**B**), 5 variants with the K103N mutation in the RT (**C**), or 19 variants with the 215-revertant mutation in the RT (**D**) is shown as an enlarged figure. Major drug-resistant mutations and 215-revertant mutations are shown in bold and italics, respectively. PR, protease; RT, reverse transcriptase.

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Beneficial Effect of GB Virus C Co-Infection in Human Immunodeficiency Virus Type 1-Infected Individuals

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Abstract: **Several reports have documented a better prognosis for HIV-1-infected patients co-infected with GBV-C, while other reports have contradicted such findings with the result that this issue remains controversial. We attempted to clarify the complicated status of the effect of GBV-C co-infection on HIV-1-infected patients. GBV-C RNA was detected in 37 samples in 182 HIV-1-infected patients (20.3%) using RT/nested PCR. Of these, 3 were determined to be GBV-C genotype 1, 12 were genotype 2, and the remaining 22 were genotype 3. The GBV-C viral load quantified by real-time PCR ranged from 7.8×10^3 to 3.3×10^6 copies/ml. Weakly negative correlation was observed between GBV-C viral load and HIV-1 viral load in 19 HAART-naïve patients, indicating that a higher GBV-C viral load is associated with a greater suppression of HIV-1 replication. A previously published *in vitro* study suggested that GBV-C infection would induce up-regulation of RANTES, leading to suppression of HIV-1 replication. However, in our present study, the blood RANTES level was significantly lower in the GBV-C co-infected group than in the uninfected group (190–9,959 vs. 264–31,038 pg/ml, $P=0.004$). Our results suggested that a suppression of HIV-1 replication by GBV-C co-infection is not mediated by up-regulated RANTES, and thus call for another as yet unknown factor.**

Key words: **GBV-C, HIV-1, RANTES, Co-infection**

GB virus C (GBV-C) is a single-stranded RNA virus belonging to the Flaviviridae family (10, 12, 20). It is distributed worldwide, and five major genotypes that have been recognized are prevalent in various geographical regions (15, 16, 24). GBV-C is transmitted parenterally, and a high prevalence rate of GBV-C infection is observed among hemophiliacs (22.9–38.6%), hemodialysis patients (10.2–16.4%) and intravenous drug users (IVDU) (32.1–34.9%) (2, 4, 17, 23, 27, 28). GBV-C is also transmitted through sexual contacts (5, 11). For example, 12.5% of homosexual men were determined to be infected with GBV-C in our previous report (6).

Due to its close relationship to hepatitis C virus, also a member of the Flaviviridae family, GBV-C was thought to be the cause of hepatitis in humans at the time of discovery in 1995 (12). As it became clear that GBV-C by itself is not a causative agent of hepatitis or any other disease, however, studies shifted toward the influence of GBV-C co-infection.

In 2001, two reports that appeared in the New England Journal of Medicine showed a beneficial effect of the GBV-C co-infection in HIV-1 infection in so far as human immunodeficiency virus type 1 (HIV-1)-infected patients who are co-infected with GBV-C had an improved mortality rate (22, 26). In addition, subsequent studies by Xiang et al. and Jung et al. showed that the co-infection with GBV-C resulted in increased cytokine levels including RANTES (CCL5), SDF-1, and MIP-1, whereby HIV-1 replication is inhibited (7,

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; GBV-C, GB virus C; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; IVDU, intravenous drug users; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RANTES, regulated upon activation, normal T-cell expressed and secreted; RT, reverse transcription; VL, viral load.

25).

In contrast, several other studies have found no beneficial effect of the GBV-C co-infection and one that showed that an increased mortality rate was observed in GBV-C co-infected individuals (8, 18, 19).

We conducted the present study to clarify the complex status of the effect of GBV-C co-infection on HIV-1-infected patients.

Materials and Methods

Patients. HIV-1-infected patients ($n=182$) who underwent medical examinations at Nagoya Medical Center, Japan, and from whom informed consent was obtained, were enrolled in the study. Of the 182, 151 were male. One hundred forty-three patients were infected with HIV-1 through sexual contacts (78 homosexuals, 53 heterosexuals, and 12 bisexuals). Other routes included via blood or blood products in 13 patients, and IVDU and accidental needle-stick in 1 patient each. One hundred forty-seven patients were Japanese, 19 were from Brazil, 5 from Thailand, 2 each from Peru, America, and Korea, and 1 each from China, Bangladesh, Uganda, Myanmar, and Ukraine.

Samples. Plasma samples of the individuals mentioned above were collected, and stored at −80 C until analyzed. In addition, approximately 1 year after the date of the sample collection, plasma samples were obtained again from the same patients to conduct a follow-up study.

Detection of GBV-C RNA. Purification of RNA from the plasma samples and the reverse transcription-polymerase chain reaction (RT-PCR) method to amplify GBV-C RNA were performed as described in our previous report (6). Briefly, RNA was extracted from the thawed samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo). The extracted RNA was used in a RT reaction followed by a first-round PCR (RT-PCR) using a Superscript One-Step RT-PCR for long templates (Invitrogen, Tokyo). Subsequently, cDNA was further amplified in a second-round PCR using a TaKaRa LA *Taq* (TaKaRa Shuzo, Tokyo). The same primers and the parameter settings as in our previous report were used for the RT/nested PCR. The PCR products were detected on a 1.2% agarose gel containing ethidium bromide. The expected size of the PCR product was 252 base pairs.

Determination of nucleotide sequences. PCR products of approximately 250 base pairs in size were purified from the agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), and labeled using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Tokyo). The nucleotide sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Determination of GBV-C genotypes. The obtained nucleotide sequences were compared against the reference sequences of 5 GBV-C genotypes: genotype 1 (accession #U36380), genotype 2 (U44402), genotype 3 (D90601), genotype 4 (AB013188), and genotype 5 (AF131112) in the GenBank of NCBI. The genotype for each GBV-C-positive sample was determined with an UPGMA method using Genetyx-Mac (version 10.1) system (Software Development, Tokyo).

Quantification of plasma GBV-C load. Total RNA from each GBV-C-positive patient was purified from 200 μl plasma samples using Trizol LS Reagent (Gibco BRL Life Technologies, Md., U.S.A.) according to the manufacturer's instructions. The obtained RNA was dissolved in 10 μl DEPC-treated water, and a half of the volume was reverse transcribed by Superscript II RNase H− Reverse Transcriptase (Invitrogen) at 42 C for 50 min. Subsequently, 2 μl of the 20 μl RT reaction mixture was applied to quantify the GBV-C copy numbers in triplicate by real-time PCR using a TaqMan probe. The same primer/probe set was used as in our previous report (6).

Determination of plasma RANTES concentrations. Concentrations of regulated upon activation, normal T-cell expressed and secreted (RANTES) in the plasma samples were measured using the Human RANTES enzyme-linked immunosorbent assay (ELISA) Kit (Biosource, Calif., U.S.A.) according to the manufacturer's protocol.

Measurement of plasma HIV-1 viral loads and CD4 cell counts. Plasma HIV-1 viral loads were measured using an Amplicor HIV-1 monitor version 1.5 system (Roche, Tokyo). CD4 cell counts were flow-cytometrically measured by FACSCalibur (Becton Dickinson, Tokyo).

Statistical analysis. The means of GBV-C VL among 3 GBV-C genotypes were compared by the Kruskal-Wallis test. The Mann-Whitney U test was used to compare the means of HIV-1 VL, or RANTES concentration between GBV-C co-infected group and uninfected group. Linear regression analysis was used to evaluate the relationship between GBV-C VL and HIV-1 VL. The significance level was set at 0.05, and all P values were two-sided.

Results

Prevalence of GBV-C Infection among HIV-1-Infected Patients

RT/nested PCR was performed to detect GBV-C RNA from the plasma samples of HIV-1-infected

patients. PCR products of approximately 250 bp were detected in 37 of the 182 samples (20.3%) by agarose gel electrophoresis. The characteristics of these patients are summarized in Table 1.

GBV-C Genotypes

Following the determination of the GBV-C prevalence, the nucleotide sequences of the PCR products were analyzed to classify the 37 samples into the 5 GBV-C genotypes. Of the 37 samples, 22 samples were GBV-C genotype 3, which is abundant in Asia. Twelve were classified as genotype 2 found in Europe and the U.S., and the remaining 3 were genotype 1 found in Africa. None were classified as either genotype 4 or 5 (Fig. 1).

Plasma GBV-C RNA Load

The GBV-C copy number in each of the 37 plasma samples was quantified with a real-time PCR method using a Roche LightCycler. Viral loads ranged from 7.8×10^3 to 3.3×10^6 copies/ml. The distribution of the viral loads among GBV-C genotypes 1, 2 and 3 were 7.3×10^5–3.3×10^6 copies/ml (2.4×10^6 copies/ml av.), 7.8×10^3–1.4×10^6 copies/ml (3.4×10^5 copies/ml av.), and 2.1×10^4–2.3×10^6 copies/ml (3.0×10^5 copies/ml av.), respectively (Fig. 2). The average viral loads of the 3 genotypes were compared, and it was found that the viral loads among genotype 1 were significantly higher than for the remaining genotypes ($P=0.04$). However, in the follow-up study, the GBV-C viral loads, which ranged from 2.4×10^1 to 5.1×10^6 copies/ml, did not differ among the 3 genotypes ($P=0.57$) (data not shown). Thus, a difference in viral load among genotypes may not exist.

Table 1. Characteristics of 182 patients with HIV-1 infection, grouped by GBV-C status

	$n=182$	
GBV-C status	+	−
	37	145
Mean CD4+ cell count (cells/ml)	376.8	412.3
HAART received	18	80
Mode of HIV-1 transmission		
Sexual contact	31	112
Homosexual	22	56
Heterosexual	7	46
Bisexual	2	10
Blood or blood products	3	10
IVDU	0	1
Needle-stick	0	1
Unknown	3	21
Sex		
Male	34	117
Female	3	28
Nationality		
Japan	33	114
South America	2	19
East Asia	0	7
South/Southeast Asia	2	1
North America	0	2
Africa	0	1
Easterm Europe	0	1

HAART, highly active antiretroviral therapy; IVDU, intravenous drug use.

HIV-1 VL among HAART-Naïve Patients and Its Correlation with GBV-C VL

To evaluate the effect of GBV-C co-infection on HIV-1-infected patients, we analyzed the HIV-1 viral load of highly active antiretroviral therapy (HAART)-naïve patients. The HIV-1 viral load for 19 GBV-C-infected and 65 GBV-C–uninfected patients ranged from 7.8×10^3 to 3.2×10^6 copies/ml, and from 4.7×10^2 to 1.1×10^6 copies/ml, respectively, and there was a statistically significant difference between the two groups ($P=0.01$) (Fig. 3). In addition, a weakly inverse correlation was observed between the GBV-C viral load and HIV-1 viral load in 19 HAART-naïve GBV-C-co-infected patients (Fig. 4).

Plasma RANTES Concentrations

Plasma RANTES concentrations of the HAART-naïve HIV-1-infected patients with or without GBV-C viremia were measured using a commercially available ELISA kit. The RANTES concentrations of those who showed a GBV-C viremia ranged from 190 to 9,959 pg/ml (3,856 pg/ml av.). Unexpectedly, in those without GBV-C viremia, the RANTES concentration ranged from 264 to 31,038 pg/ml (9,233 pg/ml av.), which was significantly higher than in those with viremia ($P=0.004$) (Fig. 5A).

In the follow-up study, the number of the patients decreased to 47 (11 GBV-C-co-infected, and 36 GBV-C-uninfected patients) since approximately half of the patients had started receiving HAART. The RANTES concentration in those with GBV-C viremia ranged from 72 to 14,007 pg/ml (5,369 pg/ml av.), and in those without viremia ranged from 241 to 22,670 pg/ml (5,973 pg/ml av.). The RANTES levels of the 2 groups were not significantly different ($P=0.86$) (Fig. 5B).

Discussion

We have performed RT/nested PCR to detect GBV-C RNA in the plasma samples of 182 HIV-1-infected patients, who were seen at the Nagoya Medical Center. The prevalence rate was determined to be 20.3%, and

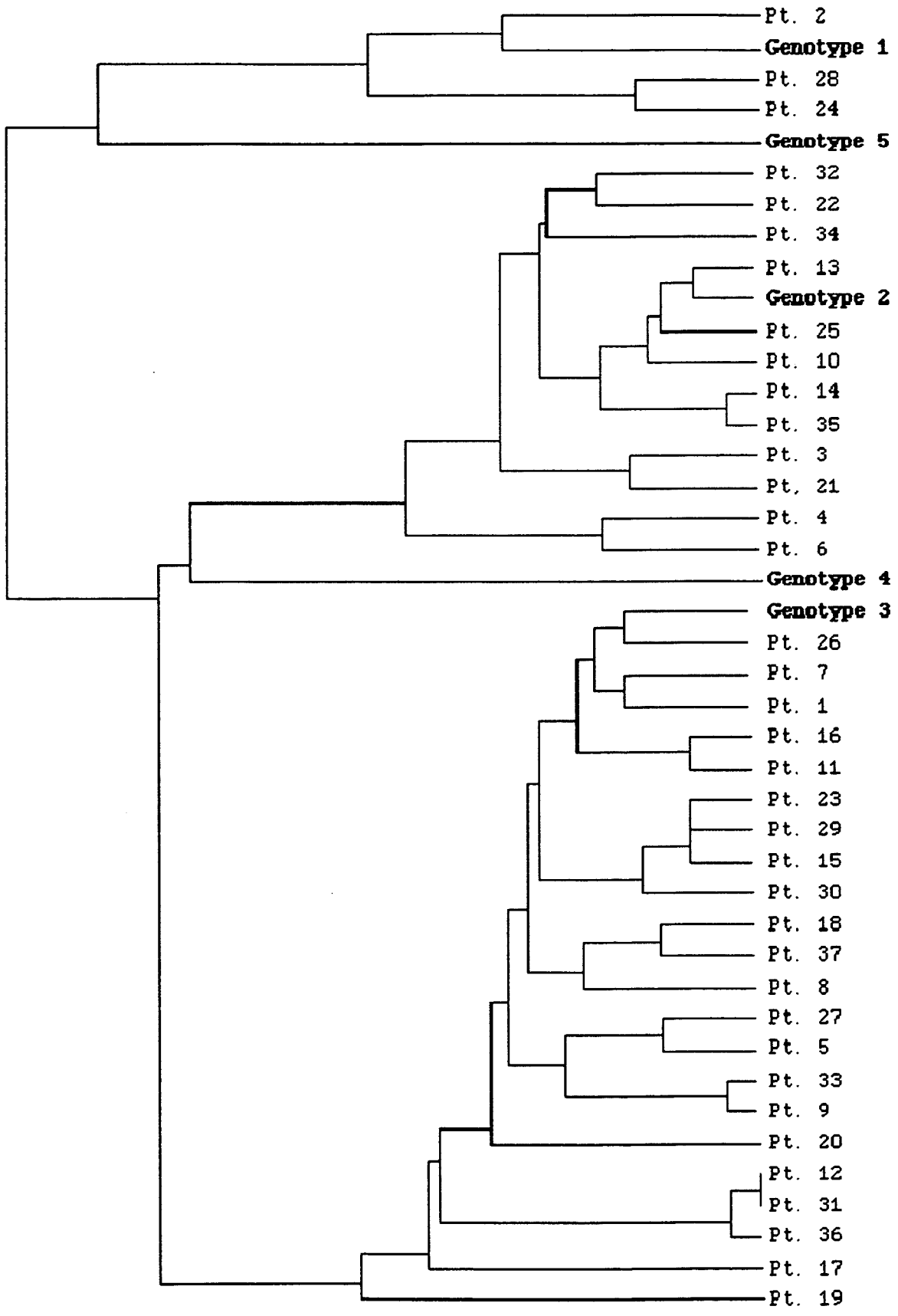

Fig. 1. Phylogenetic tree of GBV-C co-infected patients. The 37 GBV-C-infected patients (Pt.) are numbered 1 through 37. The reference sequences of the 5 GBV-C genotypes are shown in bold letters.

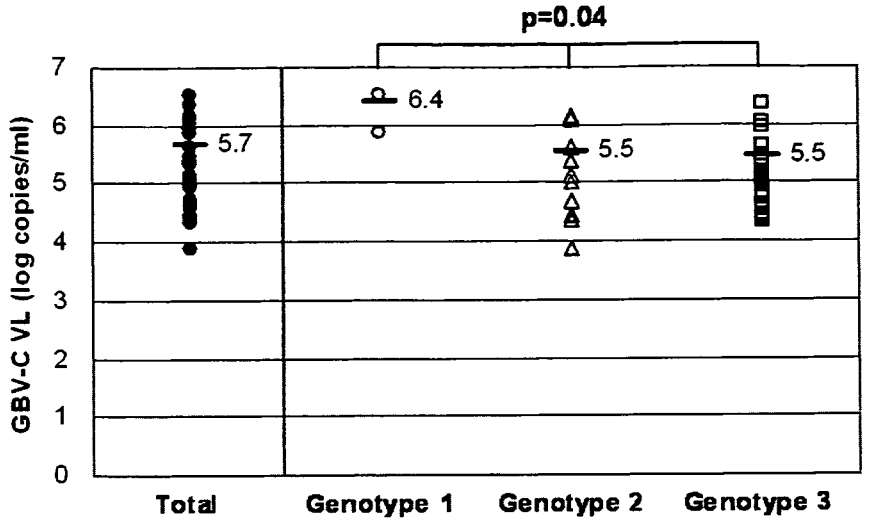

Fig. 2. Distribution of GBV-C viral load among 37 patients (●), and according to the genotypes 1 (○), 2 (△), and 3 (□). The bars indicate the mean viral load.

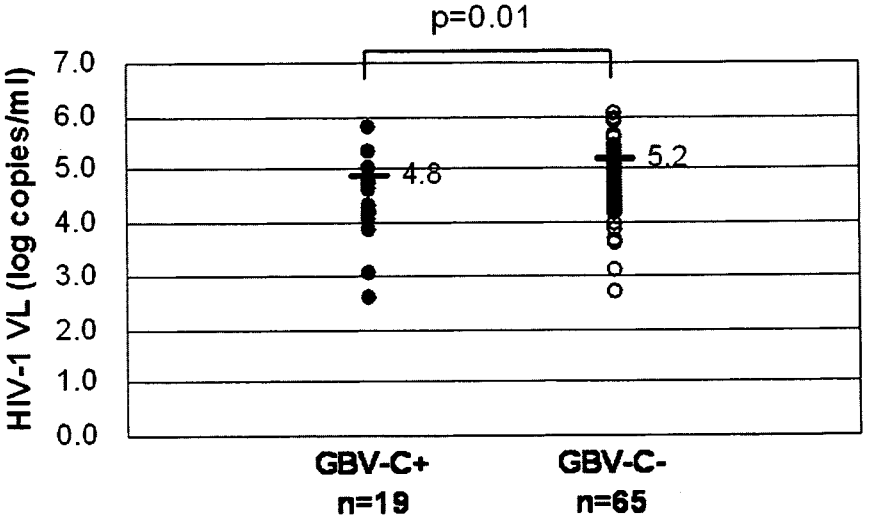

Fig. 3. Distribution of HIV-1 viral load among HAART-naïve GBV-C-co-infected (●) and GBV-C-uninfected (○) patients. The bars indicate the mean viral load.

was similar to the results of HIV-1-infected patients obtained by others (3, 21, 22). This fairly high value seems reasonable as the prevalence seen among homosexual men (12.5%) in our previous report was higher than that of healthy blood donors, and the majority of subjects in this study were infected with HIV-1 through homosexual contacts.

GBV-C genotypic analysis showed that 59.5% (22/37) of the GBV-C-infected individuals were classified as genotype 3, 32.4% (12/37) as genotype 2, and 8.1% (3/37) as genotype 1. None were classified as genotype 4 or 5. Our results agreed with reports by others in that genotype 3 is prevalent in Japan (1, 14). Two of the 3 individuals with GBV-C genotype 1 were hemophiliacs, agreeing with a previous report by Liu et al. (13).

To study the effect of GBV-C co-infection on HIV-1-infected patients, plasma HIV-1 viral loads of antiretroviral therapy-naïve GBV-C-infected and -uninfected groups were compared. Statistical analysis showed that the HIV-1 viral loads of the GBV-C co-infected group were significantly lower than those of the singly-infected group (P=0.01). Consequently, we looked into the relationship between GBV-C viral load and HIV-1 viral

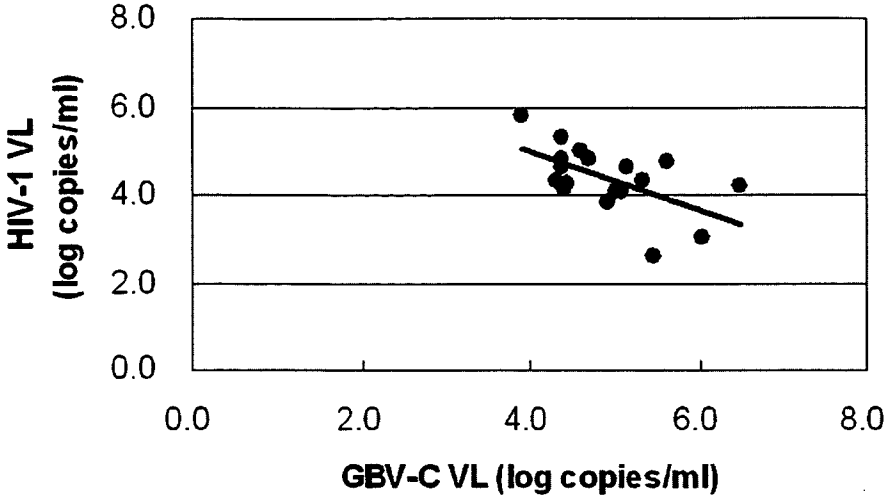

Fig. 4. Correlation between the GBV-C viral load and HIV-1 viral load (R^2=0.34).

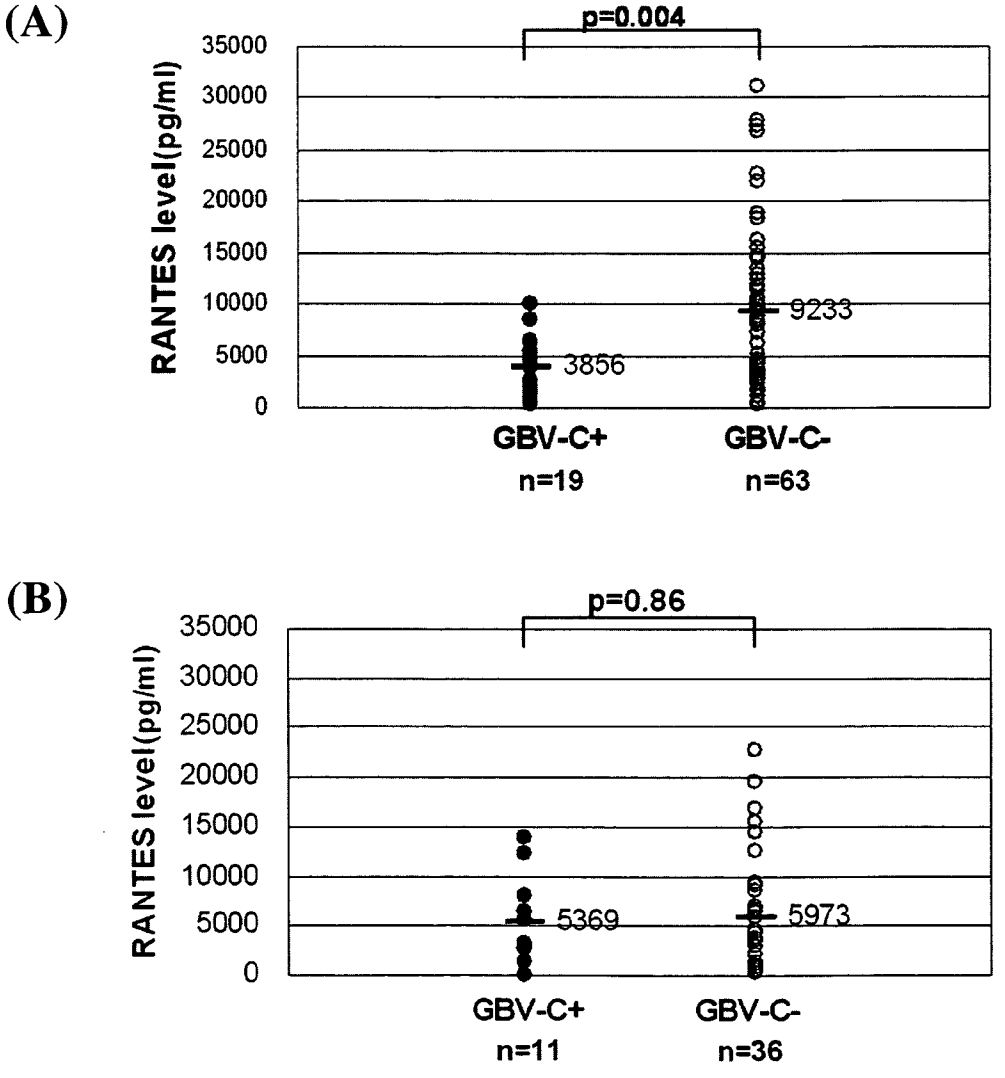

Fig. 5. Distribution of RANTES concentrations among HAART-naïve GBV-C-co-infected (●) and GBV-C-uninfected (○) patients in (A)the initial study, and (B)the follow-up study. The bars represent the mean RANTES concentrations.

load of the same study groups, and observed an inverse correlation in that individuals with higher GBV-C viral loads tended to have lower HIV-1 viral loads (R^2=0.34). Although the suppression level shown here was weak and partial, it is clear that the GBV-C infection is associated with the suppression of HIV-1 replication. Our results do not directly support the findings by Xiang et al. (26) or Tillmann et al. (22), but we believe that the HIV-1 replication suppressive effect by GBV-C possibly leads to a better prognosis and a low mortality rate. The question of why some research groups found a beneficial effect of GBV-C co-infection while others did not still remains. The discrepancy may occur from the differences in race, HIV-1 clade, or GBV-C genotype. However, divergent results were observed even when the studies were composed of mostly Caucasian subjects who were presumably infected with HIV-1 subtype B (18, 19, 22, 26). In addition, the differences in GBV-C genotype do not seem to account for the contrasting findings since the HIV-1 VLs between GBV-C genotypes 2 and 3 were similar as observed in the present study. Thus, other reasons may exist for the conflicting observations.

One possible mechanism of the suppression of HIV-1 replication is the inhibition of HIV-1 entry into target cells. As Xiang et al. reported that the co-infection of GBV-C suppressed the HIV-1 replication through up-regulation of RANTES, a natural CCR5 ligand, and also known as CCL5, in an *in vitro* study (25), we assessed the scenario by measuring the RANTES concentration in patients' plasma. In contrast to the *in vitro* study, however, much higher RANTES concentrations were observed in GBV-C-uninfected individuals. In addition, a mean RANTES level of 3,856 pg/ml in HAART-naïve GBV-C-infected patients at the first medical examination increased to 5,369 pg/ml after 1 year, whereas that of 9,233 pg/ml in HAART-naïve GBV-C-uninfected patients decreased to 5,973 pg/ml. This increase in the RANTES level in the GBV-C co-infected group, and in contrast, the decrease in the uninfected group, was unexpected and is inexplicable. Kwofie et al. reported a transient decrease in RANTES level at the early stage of HIV-1 infection in macaques (9). A similar fluctuation in RANTES level might occur in humans as well. Regrettably, we were unable to conclude that the HIV-1 replication was suppressed by up-regulation of RANTES by GBV-C, and the inhibition of HIV-1 replication might be supported through other mechanisms induced by GBV-C co-infection.

We can conclude that GBV-C co-infection affects the clinical status of HIV-1-infected patients, and that the co-infection confers a beneficial effect on the patient.

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＜症例報告＞

HIV・HCV重複感染に対するリバビリン併用ペグ・インターフェロン療法によりCD4，CD8陽性リンパ球数が減少した1症例

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はじめに

多剤併用療法（highly active antiretroviral therapy：HAART）導入後のHIV感染者の死亡原因は，AIDS関連と非AIDS関連が半々で，非AIDS関連死の約9割が慢性HCV感染症によるといわれている[1]。HIVとHCVの主な感染経路は血液を媒介するもので，静注薬物使用者や血液製剤による感染例で重複感染が多いのが実状である。HIV・HCV重複感染例におけるHCV治療は，HCV単独感染の標準ガイドラインに従いインターフェロンを基本とし，近年ではリバビリンを併用することが多い。

今回，HAART導入によりHIV-RNA量が検出限界（50copies/mL）以下，CD4陽性リンパ球数が200/μL以上と良好なコントロールを得たHIV・HCV重複感染患者に対してリバビリン併用ペグ・インターフェロン療法を行ったところ，CD4，CD8陽性リンパ球数が200/μL以下まで減少した症例を経験したので報告する。

I 症例提示

【症例】 49歳，男性

既往歴：血友病B，C型慢性肝炎

臨床経過：1985年にHIV感染が判明し，91年1月よりジドブジン（AZT，レトロビル®）の内服を開始，93年12月からジダノシン（ddI，ヴァイデックス）の内服に変更した。その後，97年4月よりAZT＋ラミブジン（3TC，エピビル®）＋インジナビル（IDV，クリキシバン®）の3剤の内服（HAART）を開始した。2004年9月からAZT＋3TC＋ロピナビル・リトナビル配合剤（LPV/r，カレトラ®）の組み合わせに変更となった。この間，HIV-RNA量は検出限界以下を維持しており，CD4陽性リンパ球数は平均397/μL，CD8陽性リンパ球数は平均910/μLと安定していた。

一方でHCV-RNA量は，04年11月時点で190×10⁴IU/mL，05年7月時点で86×10⁴IU/mLと高値を示した。そのため，抗HCV療法と

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して，05年８月から06年８月までの１年間，リバビリン併用ペグ・インターフェロン療法を行った。その結果HCV-RNA量は05年10月から陰性となったが，$68\times10^2/\mu$Lあった白血球数は，最も少ない時で$29\times10^2/\mu$Lまで減少した。なかでもCD4陽性リンパ球数は，05年９月で315/μL，12月で194/μL，06年２月で116/μL，４月で134/μL，８月で141/μLと，免疫不全状態の指標である200/μL以下まで減少し，その状態は８カ月にわたり継続した。またCD8陽性リンパ球数も05年９月で656/μL，12月で334/μL，06年２月で171/μL，４月で176/μL，８月で137/μLまで減少した。なおこの間，赤血球数は$23\times10^5/\mu$L以上，血小板数は$9\times10^4/\mu$L以上を維持していたが，ヘモグロビン量については13g/dLから徐々に減少し，06年４月には10g/dL以下となった。そのため，エポエチンアルファ（エスポー®）12000IUの皮下注射を予防投与も含めて４回行い造血の活性化を促した。その効果は残念ながら判然としなかったが，抗HCV療法終了後，赤血球数，ヘモグロビン量および白血球数は増加し，CD4陽性リンパ球数は06年９月で389/μL，10月で291/μLに，CD8陽性リンパ球数もそれぞれ613/μL，442/μLと増加した（**図１**）。

なお抗HCV療法を行うにあたり，リバビリンとAZTの併用による副作用と考えられる貧血を防止するため，HAARTの組み合わせを05年８月からテノホビル（TDF，ビリアード®）＋3TC＋LPV/rに変更した。TDF変更後もHIV-RNA量は検出限界以下を維持していた。

一方でTDFには，副作用として重度の腎機能障害が報告[1]されている。そこでTDF内服開始後のTDF血中濃度（内服３時間後）と血中クレアチニンの推移について検討した。TDF内服前（05年７月）の血中クレアチニンは1.1 mg/dLであったが，内服後徐々に上昇し，06年４月以降は1.4mg/dLを超えていた。同様にTDF血中濃度も05年９月での0.45μg/mLから徐々に高くなり，内服１年後の06年９月では0.67μg/mLとなった。通常TDFの最高血中濃度は0.30～0.38μg/mLとされており，本症例では２倍近い高い値を示した（**図２**）。

Ⅱ　考　　察

HIV・HCV重複感染患者に対する抗HCV療法は，HIV-RNA量が良好にコントロールされ，CD4陽性リンパ球数が200/μLを超える安定した患者を対象に行われている[1]。しかしHIV非感染者に比べてHIV・HCV重複感染患者に対しては，インターフェロン投与に伴う白血球数減少やリバビリン投与に伴う貧血などの副作用について特に注意が必要である。なかでもAZTとリバビリンの併用は重度の貧血を起こすことが知られているので[2]，本症例もリバビリン投与開始時にHAARTの組み合わせをAZTからTDFに変更した。またHIV・HCV重複感染患者では，インターフェロンによるCD4陽性リンパ球数の一時的減少が報告[3)～5)]されている。本症例においてもリバビリン併用ペグ・インターフェロン療法を開始した直後から白血球数の減少が認められた。なかでもCD4，CD8陽性リンパ球数は免疫不全状態の指標である200/μL以下まで減少した。本症例ではCD4陽性リンパ球数の減少が緩やかであり日和見感染もみられなかったため，抗HCV療法を継続したが，CD4陽性リンパ球数の減少が重度である場合にはインターフェロンの中止が必要と考える。

一方，リバビリン投与開始時にAZTから変更したTDFについても好中球減少等の副作用が知られており，本症例でもその副作用は顕著に観察された。また本症例では徐々に腎機能が悪化しており，血中クレアチニンの上昇と共にTDF血中濃度も上昇していた。TDFは腎排泄型の薬剤であり，腎機能が悪化すればその代謝も遅延し，血中濃度が上昇すると考えられる。本症例における高いTDF血中濃度と白血球数等の減少との関連は否定できないが，抗HCV療法終了後に再び白血球数が増加

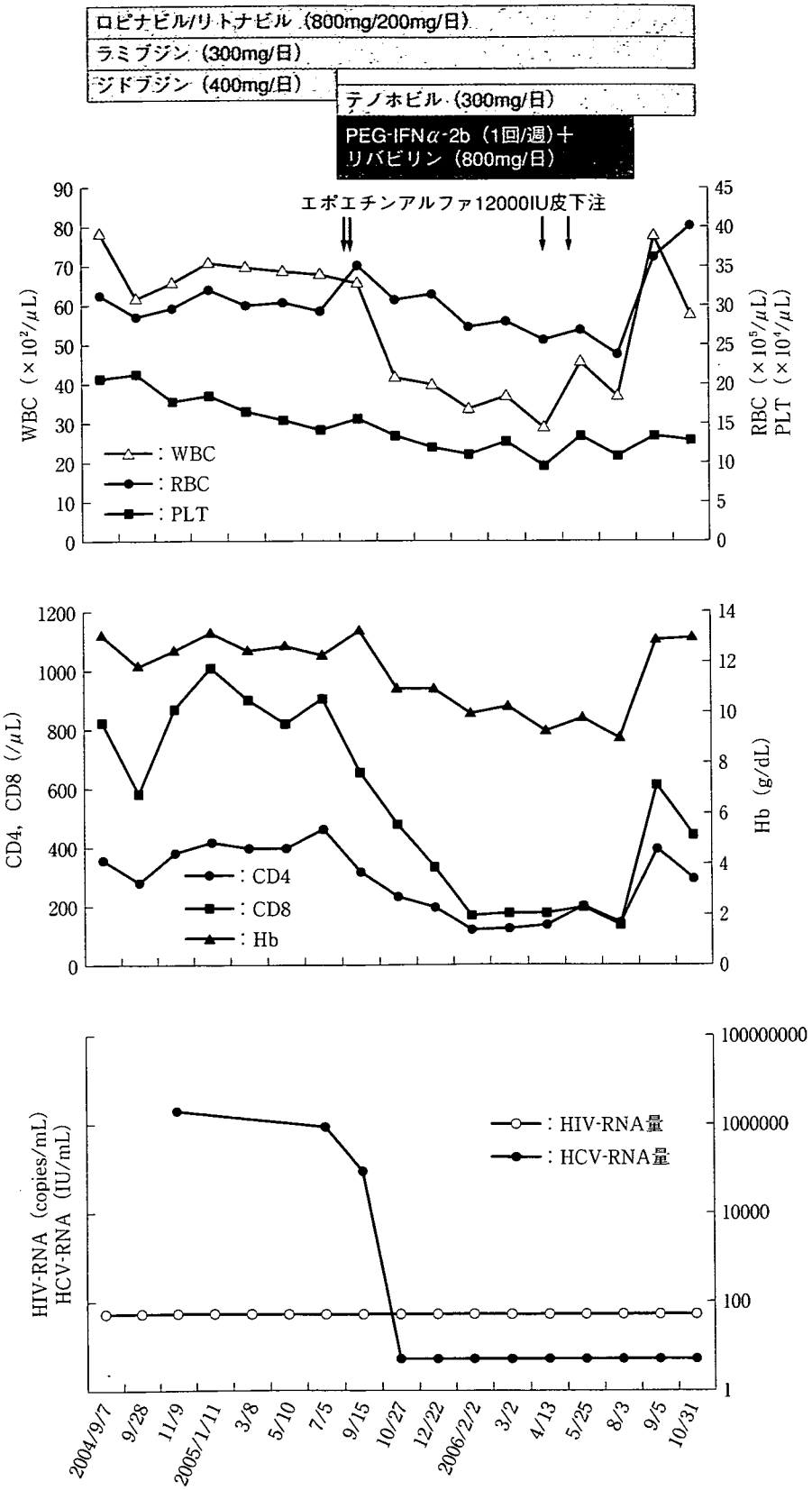
ロピナビル/リトナビル（800mg/200mg/日）
ラミブジン（300mg/日）
ジドブジン（400mg/日）
テノホビル（300mg/日）
PEG-IFNα-2b（1回/週）＋
リバビリン（800mg/日）
エポエチンアルファ12000IU皮下注
WBC（×10²/μL）
RBC（×10⁵/μL）
PLT（×10⁴/μL）
：WBC
：RBC
：PLT
CD4, CD8（/μL）
Hb（g/dL）
：CD4
：CD8
：Hb
HIV-RNA（copies/mL）
HCV-RNA（IU/mL）
：HIV-RNA量
：HCV-RNA量
2004/9/7
9/28
11/9
2005/1/11
3/8
5/10
7/5
9/15
10/27
12/22
2006/2/2
3/2
4/13
5/25
8/3
9/5
10/31

図1　臨床経過

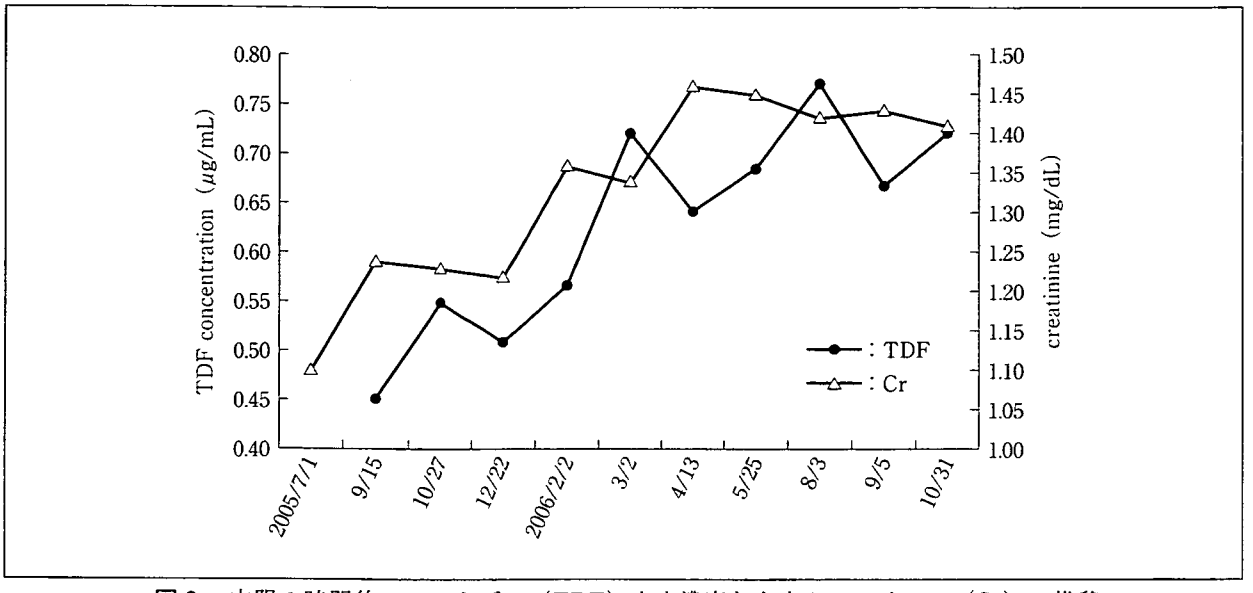

図2　内服3時間後のテノホビル（TDF）血中濃度と血中クレアチニン（Cr）の推移

したことからTDFの関与は小さいと考える。

HIV・HCV重複感染患者に対して抗HCV療法を行う場合には，日和見感染を予防する上でCD4陽性リンパ球数の減少に十分に注意を払う必要がある。加えてHAARTにおいてTDFを選択した場合には腎機能低下に注意し，腎障害時には投与間隔の調節を考慮することが望ましい。

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Original Article

Performance and Quality Assurance of Genotypic Drug-Resistance Testing for Human Immunodeficiency Virus Type 1 in Japan

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SUMMARY: Highly active antiretroviral therapy (HAART) can suppress human immunodeficiency virus type 1 (HIV-1) replication and plasma HIV-1 to below detectable levels. However, HAART becomes ineffective when drug-resistant viruses emerge during HAART. Monitoring drug-resistance mutations in viruses is necessary for selecting new drugs or therapies effective at inhibiting such HIV-1 variants. Most laboratories in Japan perform the tests using in-house protocols. However, the quality of these tests has never been assessed. Our study assessing the accuracy and reliability of HIV-1 genotypic drug-resistance testing in 15 laboratories in Japan revealed that the quality was very high (97.3% accurate). The errors, though rare, were caused by human errors, poor electropherograms, and the use of inadequate primers. Here, we propose troubleshooting procedures to improve testing accuracy and reliability in Japan.

INTRODUCTION

Genotypic drug-resistance testing for human immunodeficiency virus type 1 (HIV-1) is clinically useful for successful antiretroviral treatment (1-5). In Japan, the test, which could initially be performed at only a few virological laboratories in 1996, is now conducted at more than 15 laboratories. The cost of testing is almost entirely covered by government research funds. The laboratories use in-house protocols, the main advantage of which is their low cost relative to that of commercial kits. As the quality of these protocols had not previously been evaluated, we conducted an assessment of in-house protocol reliability by sending HIV-1 RNA samples to the relevant laboratories.

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MATERIALS AND METHODS

Laboratories participating in the quality assurance assessment of genotypic drug-resistance testing protocols: Eight HIV/AIDS clinical centers, five local government institutes of public health, one commercial laboratory, and the National Institute of Infectious Diseases (NIID) were enrolled in this study on voluntary basis.

Construction of drug-resistant HIV-1 clones: We chose two plasma specimens, i.e., viruses well-characterized for harboring multidrug-resistance mutations. The HIV-1 of one specimen (case 1) had eight drug-resistance mutations: L63P, A71T, and V77I in the protease (PR), and A62V, V75I, F77L, F116Y, and Q151M in the reverse transcriptase (RT); and 10 mutations unrelated to drug-resistance: V3I, E35D, S37N, R41K, and K70R in the PR, and S68G, T69V, E122K, Q197E, and R211K in the RT. The HIV-1 of the other specimen (case 2) had nine drug-resistance mutations: L10I, L63P, V77I, and L90M in the PR, and M41L, T69S-SG insertion, G190A, L210W, and T215Y in the RT; and 15 mutations unrelated to drug-resistance: V3I, I15V, S37D, and I93L in the PR, and