TABLE 2. Observed Protease Inhibitor Resistance–Associated Mutations in Each Patient

Patient	Primary Mutation	Secondary Mutation
1	D30N[a]	A71T, V77I, N88D[a]
2	V82A, I84V[a], L90M[a]	L10I[a], I54V, A71V, V77I
3	M46I, I84V[a], L90M	L10I, A71T, G73S
4	D30N, G48V, V82A	L10I, K20M[a], M36I, I54V
5	M46D[a], I84V[a], L90M[a]	L10I, A71V, V77I, N88D
6	M46I, L90M[a]	L10I[a], K20M, A71T[a], G73S[a], V77I[a]
7	M46I, I84V[a], L90M	L10I, K20M, G73A, V77I
8	M46I, L90M	K20M, A71V, G73S, V71I
9	D30N, M46I, I84V, L90M	L10F, A71T[a], V77I, N88D
10	D30N, M46I[a], L90M[a]	L10I, V77I, N88D
11	G48V, V82A	L10I, K20M, M36I, I54V
12	V82A, I84V[a], L90M[a]	L10I[a], K20M, A71V[a], G73S, V77I[a]
13	M46I[a], I84V, L90M	L10I[a], K20M, A71V[a], G73S, V77I
14	D30N[a], M46I	K20R, M36I[a], N88D[a]
15	M46I[a], I84V, L90M	L10I[a], K20M, I54L, A71V, V77I
16	D30N, M46I, I84V[a], L90M[a]	M36I[a], A71T[a], N88D
17	D30N[a], M46I[a]	A71V[a], V77I[a], N88D[a]
18	G48V, I84V[a], L90M[a]	A71V[a], V77I
19	V82A[a]	I54V[a], A71V[a]
20	D30N, M46I[a], V82T[a], L90M	K20M, M36I[a], I54V[a], G73S, V77I
21	D30N[a], M46I[a]	L10F[a], A71V[a], N88D[a]
22	M46I	K20M, M36I[a], A71T[a], N88D

The mutations that are primary for resistance to some protease inhibitors[6] were classified as primary mutations.

[a]The mutations emerging during the study period and the time lag of their emergence in plasma and peripheral blood mononuclear cells were estimated and analyzed.

majority of such mutations (53 of 58 [91.4%]) appeared in plasma viral genotypic assay earlier than in PBMC proviral assay. The other mutations appeared in plasma-derived and PBMC-derived genotypic assays simultaneously. The estimated time lag between the emergence of the mutations in plasma viral and PBMC proviral genotypic assays ranged from 0 to 739 days (mean, 289 days).

To analyze the time lag statistically, the averages of time lag of primary and secondary mutations, respectively, were calculated in each participant. There was no significant difference in time lag between primary (mean, 319 days) and secondary (mean, 223 days) mutations (Mann-Whitney U test, $P = 0.1444$). When the patients were divided by mean viral load, however, the time lag of primary mutation was signifi-

TABLE 3. Number of Protease Inhibitor Resistance–Associated Mutations in Paired Samples of Plasma and Peripheral Blood Mononuclear Cells

Viral Load in Plasma (Copies/mL)	Number of Patients	Mean Number of Mutations in: Plasma	Mean Number of Mutations in: Peripheral Blood Mononuclear Cells	P Value[a]
$<10^4$	10	4.19	1.78	0.0076
$>10^4$	12	5.24	4.02	0.0216
Total	22	4.76	3.00	0.0004

[a]Wilcoxon signed rank test.

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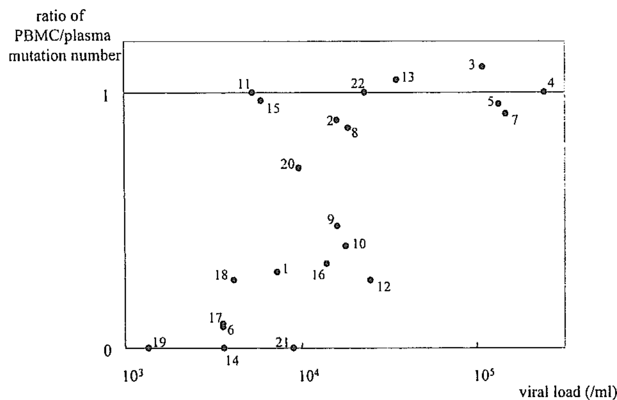

FIGURE 1. Plasma viral load and the ratio of the number of protease inhibitor (PI) resistance–associated mutations in plasma and that in peripheral blood mononuclear cells (PBMCs). Plotted are the ratios of the mean number of PI resistance–associated mutations in PBMCs and that in plasma against the geometric mean of plasma viral load. The patient numbers are also shown beside the dots.

cantly longer in the patients with a viral load $<10^4$ copies/mL (mean, 425 days) than in the patients with a viral load $>10^4$ copies/mL (mean, 225 days) (Fig. 2A). The time lag of secondary mutations was also longer in the patients with a low viral load (mean, 294 days) than in the patients with a high viral load (mean, 152 days), although the difference was not significant (see Fig. 2B), probably because of small sample size.

To delineate the correlation between the time lag and viral load further, the mean time lag in each patient was plotted against the mean viral load during the time lag. In both primary and secondary mutations (Fig. 3), there was a significant correlation between the time lag and viral load. It can be said that PI resistance–associated mutations were first detected in plasma-derived genotypic assay and then in PBMC-derived assay and that the time lag was longer in the patients with a low

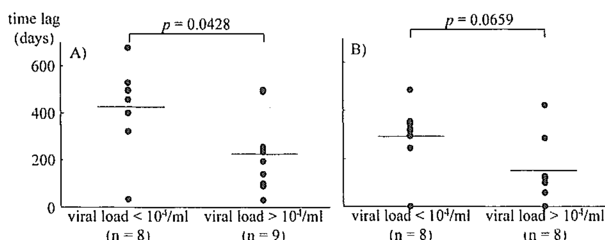

FIGURE 2. Genotypic time lag between plasma viruses and peripheral blood mononuclear cell proviruses. Plotted are means of genotypic time lags of primary (A) and secondary (B) protease inhibitor resistance–associated mutations in each patient classified by geometric means of plasma viral load during the time lags. Patient numbers are also shown. The Mann-Whitney *U* test was used to compare the mean time lags according to stratification groups.

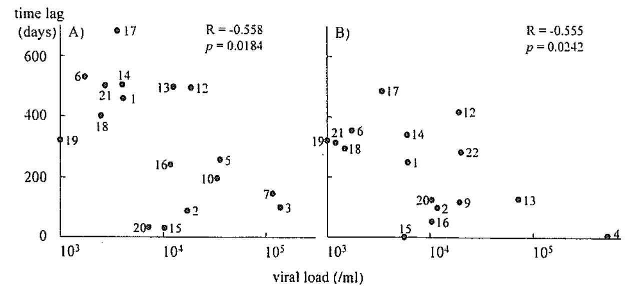

FIGURE 3. Inverse correlation between plasma viral load and genotypic time lag. Plotted are means of genotypic time lags of primary (A) and secondary (B) protease inhibitor resistance–associated mutations in each patient against geometric means of plasma viral load during the time lag. Patient numbers are also shown. The correlation between plasma viral load and time lag was assessed by Pearson correlation analysis.

viral load. That may be the reason why we observed more resistance-associated mutations in plasma genotype than in PBMC genotype during PI treatment, especially when the viral load was low.

DISCUSSION

The major findings of the present study are as follows: (1) more PI resistance–associated mutations observed in plasma viruses than in PBMC proviruses taken at the same time during PI therapy, (2) earlier appearance of such mutations in plasma genotype than in PBMC genotype, and (3) larger and longer genotypic discordance and the time lag of mutation appearance in the patients with a lower viral load.

Our analyses were based on direct sequencing, which could not detect minor genotypic populations. In this respect, Paolucci et al[20] analyzed direct and clonal HIV-1 sequences in plasma and PBMCs and found more resistance-associated mutations in plasma than in PBMCs of the patients during HAART by direct sequencing, which is compatible with our study. Furthermore, they also found multiple minor sequences with resistance-associated mutations in PBMCs by clonal sequences, which could not be detected by direct sequencing. These resistance-associated mutations were probably induced by previous therapy, and they still remained in PBMCs after disappearance from plasma viruses. Resistant genotypes rapidly disappear from plasma after interruption of antiretroviral treatment.[21] Devereux et al[22] detected more resistance-associated mutations in PBMCs than in plasma during the drug-off period of patients with a history of extensive antiretroviral treatment. Venturi et al[23] reported that the drug resistance was greater in plasma viruses than in PBMC proviruses in on-therapy patients and in PBMC proviruses than in plasma viruses in off-therapy patients. These observations, including ours, clearly demonstrate an evolutional change of PBMC proviral genotype later than that of plasma viral genotype.

We estimated the time lag between the emergence of mutations in plasma viral and PBMC proviral genotypes. The

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accuracy of such time lag is dependent on the frequency of blood sampling. Therefore, the exact time lag must be considered with caution. Nevertheless, patients in whom and the time at which resistance-associated mutations will emerge cannot be predicted; it is difficult to collect samples frequently at the appropriate time prospectively. In this study, we enrolled 22 patients who were already known to have PI-resistant HIV-1 from routine genotype assays and whose plasma and PBMC samples had been frequently stocked in our sample bank and analyzed their 275 plasma-derived and 211 PBMC-derived sequences retrospectively. Statistical analyses of such large samples should at least in part average out the inaccuracies that arise from each estimation of the time lag.

Our results showed that the emergence of primary PI resistance–associated mutations in plasma viruses preceded their emergence in PBMC proviruses by about 425 days when the plasma viral load was lower than 10^4 copies/mL and that the time lag between emergence of such mutations in plasma-derived and PBMC-derived genotypes correlated inversely with the plasma viral load. In this regard, Kaye et al[17] analyzed zidovudine (AZT) resistance–associated mutations in plasma viruses and PBMC proviruses in 10 patients receiving AZT therapy and reported that the mean time delay between plasma and PBMC mutations was 25 days in 1995 (before HAART was introduced). Considering that their patients were receiving only AZT and were classified as having Centers for Disease Control and Prevention (CDC) stage IV disease, their plasma viral loads might be around 10^5 copies/mL or higher. Therefore, our results are considered to be compatible with those of Kaye et al.[17]

In summary, PBMC proviral genotype evolves later than plasma viral genotype, and the genetic discordance arising from this latency continues longer in the patients with a lower viral load. Plasma viruses should be the material of choice for early detection of drug resistance during antiretroviral treatment.

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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) ($25.4 \pm 7.5\,\mu M$, $\pm SD$, $n = 2$) was significantly higher than that of patients with genotypes **6* heterozygote ($9.9 \pm 3.3\,\mu M$, $n = 10$) or without alleles **6* ($8.0 \pm 2.6\,\mu M$, $n = 23$) ($p < 0.0001$). 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.
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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.

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 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.

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.

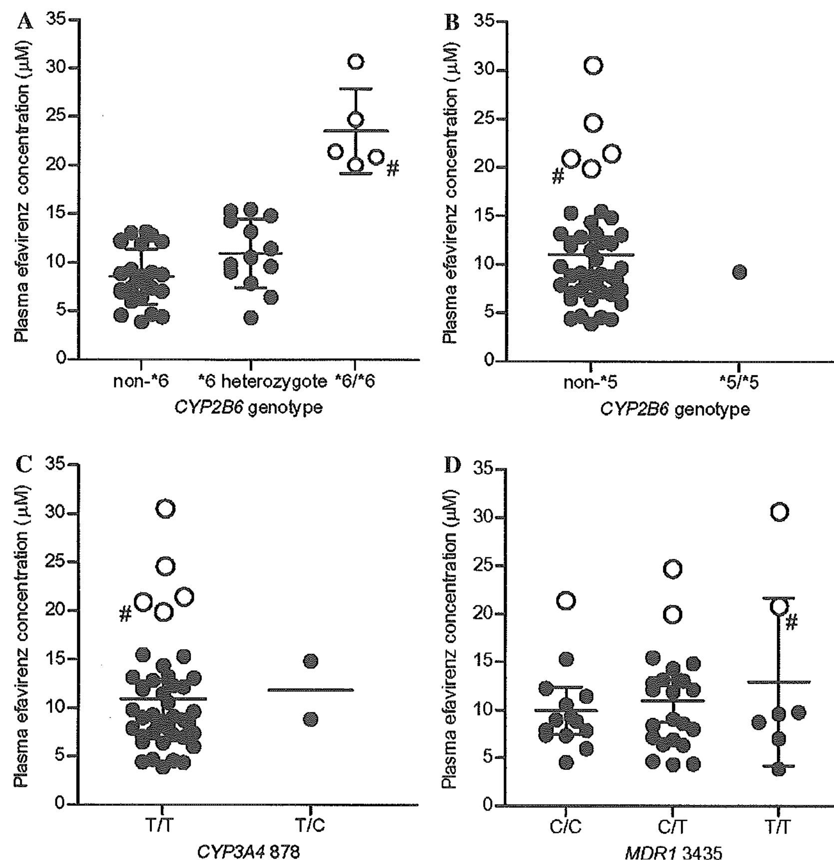

Fig. 1. Correlation between *CYP2B6 *6* genotypes (A), *CYP2B6 *5/*5* genotype (B), *CYP3A4* T878C genotype (C), *MDR1* 3435 genotypes (D), and plasma efavirenz concentrations. A total of 44 HIV-1 patients treated with standard EFV-containing regimens (35 from IMCJ and 9 from ONH) are depicted. Only 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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Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naïve patients

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Abstract

Several reports have recently shown that drug-resistant human immunodeficiency virus type 1 (HIV-1) is often isolated from treatment-naïve patients. We phenotypically analyzed HIV-1 strains isolated from 44 treatment-naïve individuals and found two strains highly resistant (69- and >310-fold) against nevirapine (NVP). Direct sequencing showed these two isolates had a novel mutation, K238S, in reverse transcriptase (RT), but did not have any reported NVP resistance-associated mutation.

A 48-h culture in the presence of NVP, however, selected HIV-1 carrying NVP resistance-associated mutations, V106A, V108I, or both, suggesting that minor viral populations of these two isolates had harbored these mutations. Replication kinetic studies of recombinant HIV-1 clones suggested that K238S conferred a significant resistance against NVP, especially when accompanied with V106A (530-fold) or V108I (56-fold). Our study identified a novel NVP resistance-associated mutation, K238S, which could be persistently detected by genotypic assay longer than V106A and V108I during off-treatment period.
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Keywords: Human immunodeficiency virus type 1; K238S; Drug-naïve; Nevirapine-resistant

Introduction

The emergence of human immunodeficiency virus type 1 (HIV-1) variants resistant to antiretroviral agents is one of the most common causes for therapeutic failure in infected individuals. Fortunately, the availability of drug-resistance testing has substantially improved the ability of clinicians to deal knowledgeably with drug-resistant HIV-1 variants. Various guidelines for therapy of HIV-1 infection recommend that each individual therapy should be optimized by choosing efficient agents based on the results of drug-resistance testing. Numerous studies have recently reported that significant proportion of newly diagnosed HIV-1 infection cases are infected with viral strains resistant to at least one antiretroviral agent, justifying drug-resistance testing even in treatment-naïve patients (Briones et al., 2001; Duwe et al., 2001; Grant et al., 2002; Little et al., 2002; Salomon et al., 2000; Simon et al., 2002). However, it is important to recognize that the ability to detect resistant viruses may decrease as a function of time from initial infection because there is generally a shift to wild type over time during off-treatment (Devereux et al., 1999; Miller et al., 2000; Verhofstede et al., 1999). The usefulness of phenotypic assay could be enhanced if the virus is propagated in the presence of drug, because such a condition could propagate resistant viral strains that might have been outgrown by wild-type viruses in the absence of drug pressure. In this study, we screened 44 clinical strains isolated from newly infected individuals by using phenotypic assay and identified two highly nevirapine (NVP)-resistant isolates that could not be detected by genotypic

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assay. Phylogenetic analyses showed these two isolates were genetically closely related and probably originated from a single patient. Furthermore, we identified a novel NVP resistance-associated mutation, K238S, in these isolates and determined its role in viral replication and resistance using newly generated recombinant HIV-1 clones.

Results

NVP-resistant HIV-1 from treatment-naïve patients

From January 2000 through December 2001, a total of 44 patients, who had had clinical evidence of recent HIV-1 infection (seroconversion or increased bands of western blotting against HIV-1 antigens), visited our clinic and all of them gave written informed consent to the participation in this study. To determine the prevalence of drug resistance in recent HIV-1 infection, susceptibility of HIV-1 isolates derived from these patients to six antiretroviral agents including zidovudine (AZT), lamivudine (3TC), stavudine (d4T), abacavir (ABC), NVP, and efavirenz (EFV) was examined phenotypically by using MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001). As expected, most of such isolates were sensitive to all tested antiretroviral agents. However, two isolates (4.5%; isolated from Cases 33 and 23) showed significantly greater resistance (69-fold and more than 310-fold compared with NL4-3) to NVP, though their resistance to EFV was not high (Fig. 1, Table 1).

To delineate the mechanism of the NVP resistance in the above two isolates, direct sequencing of HIV-1 reverse transcriptase (RT) gene from plasma samples was performed. Both plasma-derived sequences did not have any non-nucleoside RT inhibitor (NNRTI) resistance-associated mutations listed in resistance table of the International AIDS Society (Johnson et al., 2003) but had six-amino-acid substitutions including V35I, R83K, I135T, T200K, R211K, and K238S, compared with HXBII sequences, and the plasma-derived sequence in Case 23 had a mixture of K102 and K102R (Table 2). We postulated that there might be a minor viral population harboring NNRTI resistance-associated mutations in both plasma samples, which could not be detected by direct sequencing. To detect such a minor NVP-resistant viral population, the clinical isolates were propagated in MAGIC-5 cells in the presence of NVP at various concentrations and the RT gene of HIV-1 obtained from a 48-h culture supernatant was analyzed. In the HIV-1 isolated from Case 33, V108I, known as a NVP resistance-associated mutation, emerged in the presence of NVP (1 μM) although it could not be detected in direct sequencing of plasma (Table 2). In the HIV-1 isolated from Case 23, V106A, and V108I emerged in the presence of NVP (1 and 10 μM). Interestingly, clonal sequencing analysis of HIV-1 culture (NVP 1 μM) isolated from Case 23 revealed that K102R and V106A always coexisted on the same molecules and that V108I did not coexist with K102R and V106A (Table 2). All clones analyzed had V35I, R83K, I135T, T200K, R211K, and K238S. Four of these mutations (V35I, R83K, I135T, and R211K) are known as polymorphic mutations and were frequently observed in HIV-1 isolated from treatment-naïve individuals in our cohort (72%, 18%, 27%, and 36%, respectively, $n = 11$). T200K and K238S had not been reported previously and might be associated with NVP resistance.

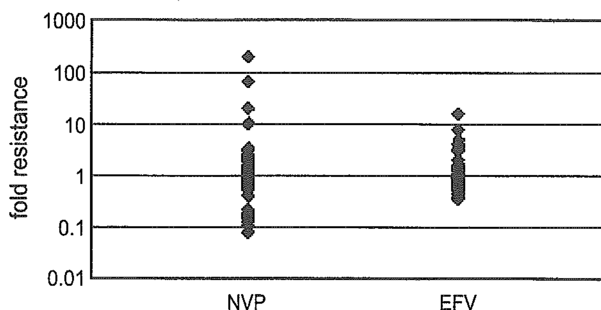

Fig. 1. Results of drug resistance assay using MAGIC-5 cells in treatment-naïve individuals. Fold resistances compared with NL4-3 are shown.

Phylogenetic analysis of clinical HIV-1 isolates

Because the clinical HIV-1 isolates derived from Cases 33 and 23 had unusually high resistance against NVP and had exactly the same amino acid substitutions (V35I, R83K, I135T, T200K, R211K, and K238S), we suspected that these two patients had acquired HIV-1 infection from a single infected patient. Then, we phylogenetically analyzed the direct sequences of *env* region and RT gene of the two HIV-1 isolates derived from Cases 33 and 23, and then compared their sequences with four clinical isolates derived from other patients (Cases 2, 3, 26, and 29) and five referential unrelated strains [U63632, HXB2CG, OY1, RF, and simian immunodeficiency virus (SIV)]. In both phylogenetic trees using the sequences of the *env* region and RT gene, Cases 33- and 23-derived sequences formed a tight cluster separated from other sequences by the tree branch with high bootstrap probabilities (99% and 100%) (Figs. 2A and B), suggesting that the isolates from Cases 33 and 23 were genetically closely related and probably originated from a single source. It was noteworthy that the *env* sequences derived from Cases 33 and 23 were closely related but not identical, which excludes the possibility of contamination during the procedures of viral culture and PCR.

Three-dimensional positions of mutations and NVP-binding pocket in HIV-1 RT

To delineate the positional relationship between NVP-binding pocket and the mutations in RT described above, a structural model of HIV-1 RT complexed with NVP was

Table 1
Results of phenotypic drug resistance testing in two untreated patients

HIV-1	IC_{50}[a] [μM] (fold resistance[b])					
	AZT	3TC	d4T	ABC	NVP	EFV
Case 33	0.04 ± 0.03 (1.2)	0.61 ± 0.20 (1.8)	1.7 ± 0.10 (0.77)	3.6 ± 0.87 (2.3)	2.2 ± 0.29 (69)	0.011 ± 0.003 (4.2)
Case 23	0.12 ± 0.03 (3.5)	0.81 ± 0.15 (2.4)	3.2 ± 0.95 (1.5)	3.3 ± 0.87 (2.1)	>10 (>310)	0.023 ± 0.00002 (8.8)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (AZT, 0.034 μM; 3TC, 0.34 μM; d4T, 2.2 μM; ABC, 1.6 μM; NVP, 0.032 μM; EFV, 0.0026 μM).

prepared based on the published crystal structures (Fig. 3) (Ren et al., 2000). The Val residues at 106 and 108 codons of RT (V106 and V108), the site of NNRTI resistance-associated mutations, V106A and V108I, respectively, were in close contact with NVP, packed in the hydrophobic pocket of the palm domain. Five residues, V35, R83, I135, T200, and R211, were located distantly from the hydrophobic pocket. Two residues, K102 and K238, were located outside the frame of hydrophobic pocket and the substitutions of these amino acids, such as K102R and K238S, were thought to produce some structural changes of the pocket and might be associated with NNRTI resistance.

Role of K102R and K238S in HIV-1 replication and resistance against NVP

Because K238S has not been reported previously and K102 and K238 were located on the frame of NVP-binding pocket, we chose to construct seven recombinant HIV-1 strains, HIV-1_{K102R}, HIV-1_{V106A}, HIV-1_{V108I}, HIV-1_{K238S}, HIV-$1_{V106A/K238S}$, HIV-$1_{V108I/K238S}$, and HIV-$1_{K102R/V106A/K238S}$, to delineate the effects of K102R and K238S on viral susceptibility against NVP. K102R did not confer significant resistance against NVP and EFV (Table 3), while V106A and V108I, both of which were known as NVP resistance-associated mutations, conferred 97- and 3.8-fold resistance against NVP, respectively. However, these mutations did not confer significant resistance against EFV. K238S conferred a significant resistance against NVP as did V108I. Interestingly, the combination of V106A and K238S (V106A/K238S) conferred surprisingly high-level resistance (530-fold) against NVP, and the combination of V108I and K238S (V108I/K238S) also conferred high resistance (56-fold) only against NVP but not against EFV (Table 3). Furthermore, the triple combination of K102R, V106A, and K238S (K102R/V106A/K238S), which were found in 15 of 21 clones derived from Case 23 (Table 2), also conferred high resistance (340-fold) against NVP, although its effect on viral resistance against EFV was not significant (Table 3). These data suggest that K238S is strongly associated with NVP resistance in the combination with V106A or V108I.

We next analyzed the effects of K102R and K238S on viral replication kinetics in the absence or presence of NVP. The addition of K238S to HIV-1WT (HIV-1_{K238S}) reduced the replication of HIV-1_{WT} in the absence of NVP, and the addition of V108I to HIV-1_{K238S} (generating HIV-$1_{V108I/K238S}$) further reduced the viral replication (Fig. 4A). On the other hand, in the presence of NVP (1 μM), K238S made HIV-1 replication-competent (HIV-1_{WT} could not replicate), and HIV-$1_{V108I/K238S}$ had facilitated viral replication (Fig. 4B). The combination of V106A to K238S (HIV-$1_{V106A/K238S}$) severely compromised viral replication and the addition of K102R (HIV-$1_{K102R/V106A/K238S}$) did

Table 2
Sequences of HIV-1 RT-coding region of clinical samples and isolates

	Sequenced sample	Amino acid residue														
		35	83	100[a]	102	103[a]	106[a]	108[a]	135	181[a]	188[a]	190[a]	200	211	230[a]	238
HXBII		V	R	L	K	K	V	V	I	Y	Y	G	T	R	M	K
Case 33	plasma	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	–	–	–	V/I	T	–	–	–	K	K	–	S
Case 23	plasma	I	K	–	K/R	–	–	–	T	–	–	–	K	K	–	S
	1 μM[b]	I	K	–	K/R	–	V/A	V/I	T	–	–	–	K	K	–	S
	10 μM[b]	I	K	–	K/R	–	A	V/I	T	–	–	–	K	K	–	S
	Number of clones															
Isolate from	15	I	K	–	R	–	A	–	T	–	–	–	K	K	–	S
Case 23	3	I	K	–	–	–	–	–	T	–	–	–	K	K	–	S
(1 μMb)	2	I	K	–	–	–	–	I	T	–	–	–	K	K	–	S
	1	I	K	–	–	R	–	–	T	–	–	–	K	K	–	S

Amino acids identical to HXBII (top column) are indicated with dashes.
[a] Reported residue associated with NVP resistance (Johnson et al., 2003).
[b] Cultured HIV-1 isolate in the presence of NVP at the indicated concentrations.

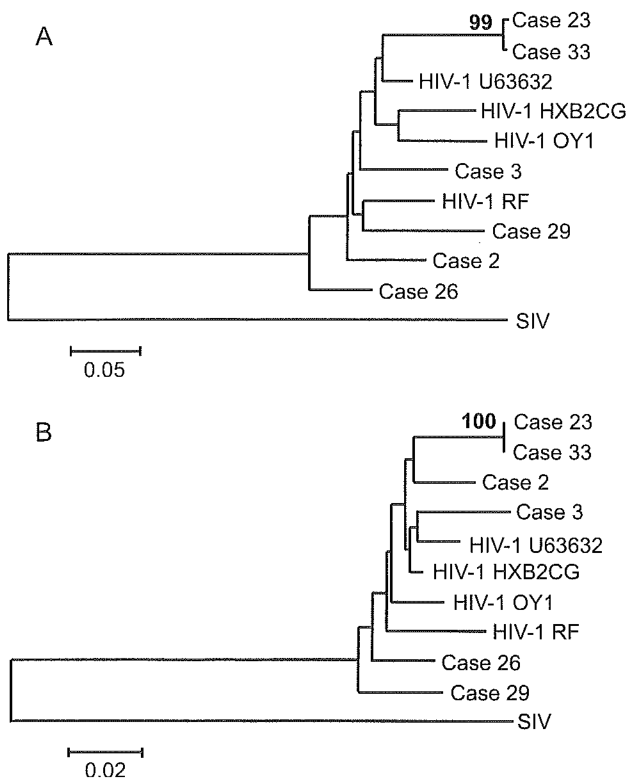

Fig. 2. Phylogenetic analysis of *env* sequences (A) and RT genes (B) of six clinical isolates and five referential strains. Bootstrap probabilities of important tree branches are shown as percentages.

not seem to rescue its replicability in the absence of drugs (Fig. 4C). In the presence of NVP (1 μM), the combination of V106A and K238S (HIV-$1_{V106A/K238S}$) rendered HIV-1 replicable, and the addition of K102R to V106A/K238S (HIV-$1_{K102R/V106A/K238S}$) did not significantly alter the replication (Fig. 4D). These data showed that V108I/K238S and V106A/K238S can confer viral replicability in the presence of NVP, although the role of K102R when combined with V106A/K238S remained to be determined.

Table 3
Drug resistance of recombinant HIV-1s

Recombinant HIV-1	IC_{50}[a] [μM] (fold resistance[b])	
	NVP	EFV
K102R	0.047 ± 0.02 (1.5)	0.002 ± 0.001 (0.77)
V106A	3.1 ± 0.36 (97)	0.002 ± 0.0003 (0.77)
V108I	0.12 ± 0.03 (3.8)	0.001 ± 0.0001 (0.38)
K238S	0.14 ± 0.04 (4.4)	0.002 ± 0.0003 (0.77)
V106A/K238S	17 ± 4.6 (530)	0.004 ± 0.001 (1.5)
V108I/K238S	1.8 ± 0.66 (56)	0.001 ± 0.0001 (0.38)
K102R/V106A/K238S	11 ± 3.1 (340)	0.002 ± 0.0001 (0.77)

Data are mean ± SD. Numbers in parentheses represent fold resistance.
[a] Phenotypic drug resistance assay was performed using MAGIC-5 cells.
[b] Fold resistance was calculated by dividing IC_{50} of clinical isolate by that of NL4-3 (NVP; 0.032 μM, EFV; 0.0026 μM).

To further define the significance of each mutation, the viral replicability was compared among molecular infectious HIV-1 clones described above in the presence and absence of NVP using competitive HIV-1 replication assay (CHRA) (Kosalaraksa et al., 1999). Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells, and their population changes through passages were determined by the relative peak height on sequencing electrogram. In the absence of NVP, HIV-1_{WT} readily outgrew HIV-

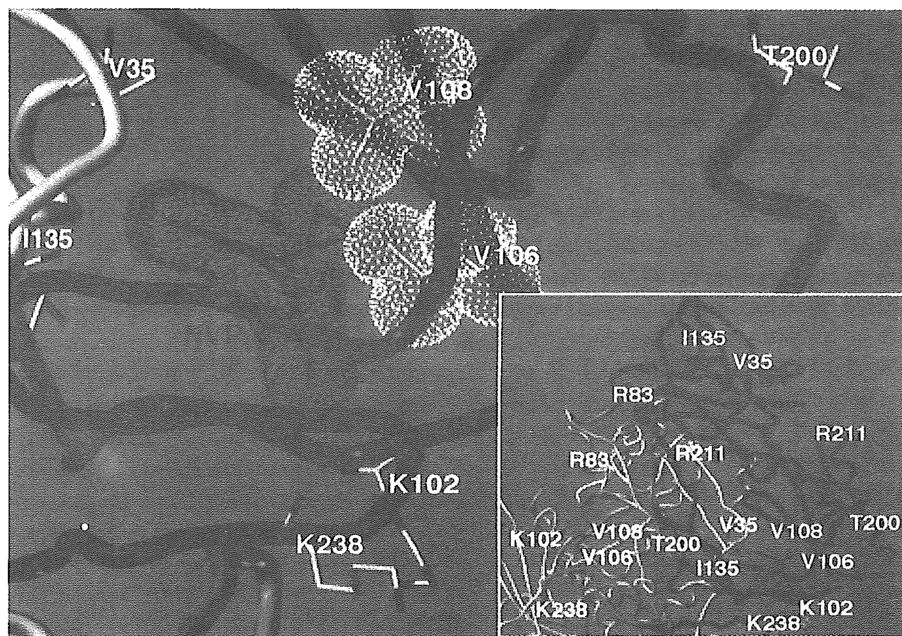

Fig. 3. The structure of the HIV-1 RT complexed with NVP. A view of the RT complexed with NVP, generated using SYBYL 6.7 and the coordinate set 1FKP.pdb obtained from the Protein Data Bank (Ren et al., 2000). The fingers, palm, thumb, and connection subdomains of the p66 subunit are colored blue, red, green, and yellow, respectively. The p51 subunit is colored white. Residues of the p66 subunit are yellow-colored, while those of the p51 subunit are white-colored. The van der Waals volumes of side chain of V106 and V108 (white) are shown to interact with NVP (orange).

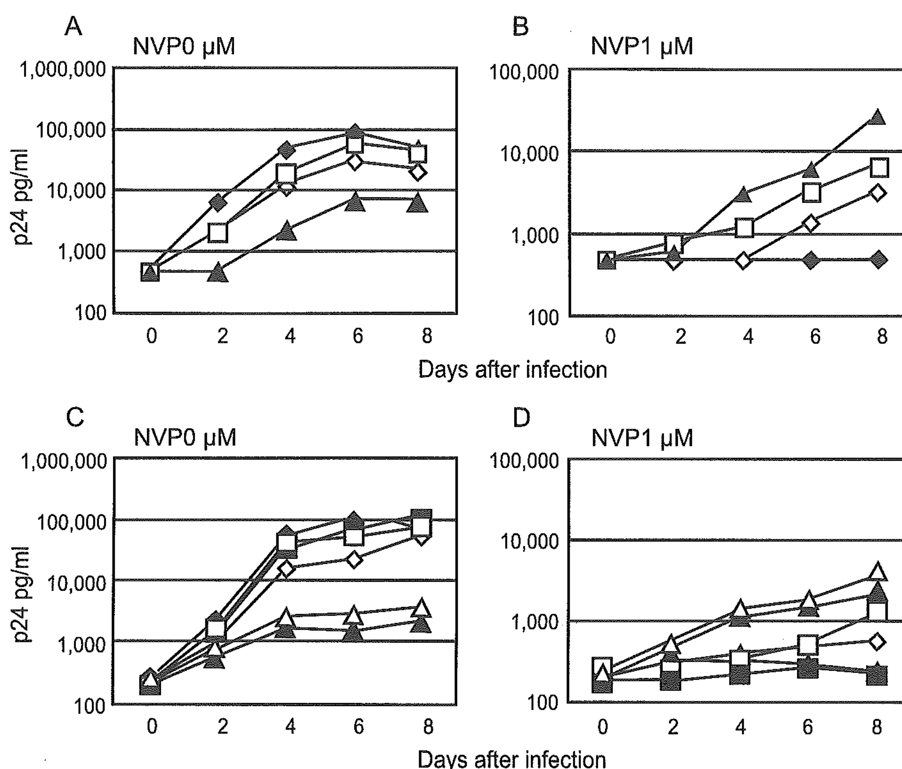

Fig. 4. Replication kinetics of recombinant HIV-1s. Graphs A and B show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{V108I} (open squares), HIV-1_{K238S} (open diamonds), and HIV-$1_{V108I/K238S}$ (solid triangles) in the absence and presence of NVP (1 μM), respectively. Graphs C and D show replication kinetics of HIV-1_{WT} (solid diamonds), HIV-1_{K102R} (solid squares), HIV-1_{V106A} (open squares), HIV-1_{K238S} (open diamonds), HIV-$1_{V106A/K238S}$ (solid triangles), and HIV-$1_{K102R/V106A/K238S}$ (open triangles) in the absence and presence of NVP (1 μM), respectively. The results shown are representative of three independent experiments.

1_{K238S} (Fig. 5A). In the presence of NVP (0.1 μM), however, HIV-1_{K238S} predominated over HIV-1_{WT} (Fig. 5B), suggesting that K238S compromised the viral replication in the absence of NVP but conferred resistance against NVP. Next, we analyzed the effect of V106A and V108I on HIV-1_{K238S} replication. In comparison between HIV-1_{K238S} and HIV-$1_{V106A/K238S}$, HIV-1_{K238S} predominated over HIV-$1V_{106A/K238S}$ in the absence of NVP (Fig. 5C), but was outgrown by HIV-$1_{V106A/K238S}$ in the presence of NVP (1 μM) (Fig. 5D). In comparison between HIV-1_{K238S} and HIV-$1_{V108I/K238S}$, HIV-1_{K238S} predominated over HIV-$1_{V108I/K238S}$ in the absence of NVP (Fig. 5E), but was outgrown by HIV-$1_{V108I/K238S}$ in the presence of NVP (1 μM) (Fig. 5F). Taken together, these data suggest that each of V106A and V108I compromised the viral replication in the absence of NVP but conferred resistance against NVP. Finally, we analyzed the effect of K102R on replication of HIV-1_{WT} and HIV-$1_{V106A/K238S}$. HIV-1_{K102R} was slowly overcome by HIV-1_{WT} in the absence of NVP (Fig. 5G). In comparison between HIV-$1_{V106A/K238S}$ and HIV-$1_{K102R/V106A/K238S}$, HIV-$1_{K102R/V106A/K238S}$ predominated over HIV-$1_{V106A/K238S}$ both in the absence (Fig. 5H) and presence (1 μM) (Fig. 5I) of NVP. Considered together, these results suggest that K102R compromised the replication of HIV-1_{WT} but compensated the replication ability of HIV-$1_{V106A/K238S}$ regardless of the presence of NVP.

Discussion

Several studies reported the recent spread of drug-resistant HIV-1 in the developed countries and among treatment-naïve individuals as well, which has been a menace for clinicians. Therefore, for certain treatment-naïve patients, such as cases of acute or recent HIV-1 infection, drug-resistance assay is recommended (Hirsch et al., 2003). However, resistant HIV-1 can be replaced with wild-type HIV-1 rapidly in plasma in the absence of treatment, and it may be difficult to detect resistant HIV-1 in the treatment-naïve patients even if they harbored resistant HIV-1. In this regard, phenotypic assay may be superior to genotypic assay using direct sequencing, because in phenotypic assay, resistant viruses could be propagated during culture in the presence of antiretroviral agents, while direct sequencing can only detect relatively major viral population in plasma. In fact, in the present study, the phenotypic assay using MAGIC-5 cells detected two highly NVP-resistant isolates in treatment-naïve patients, in whom direct sequencing failed to detect the presence of minor population of V106A and V108I.

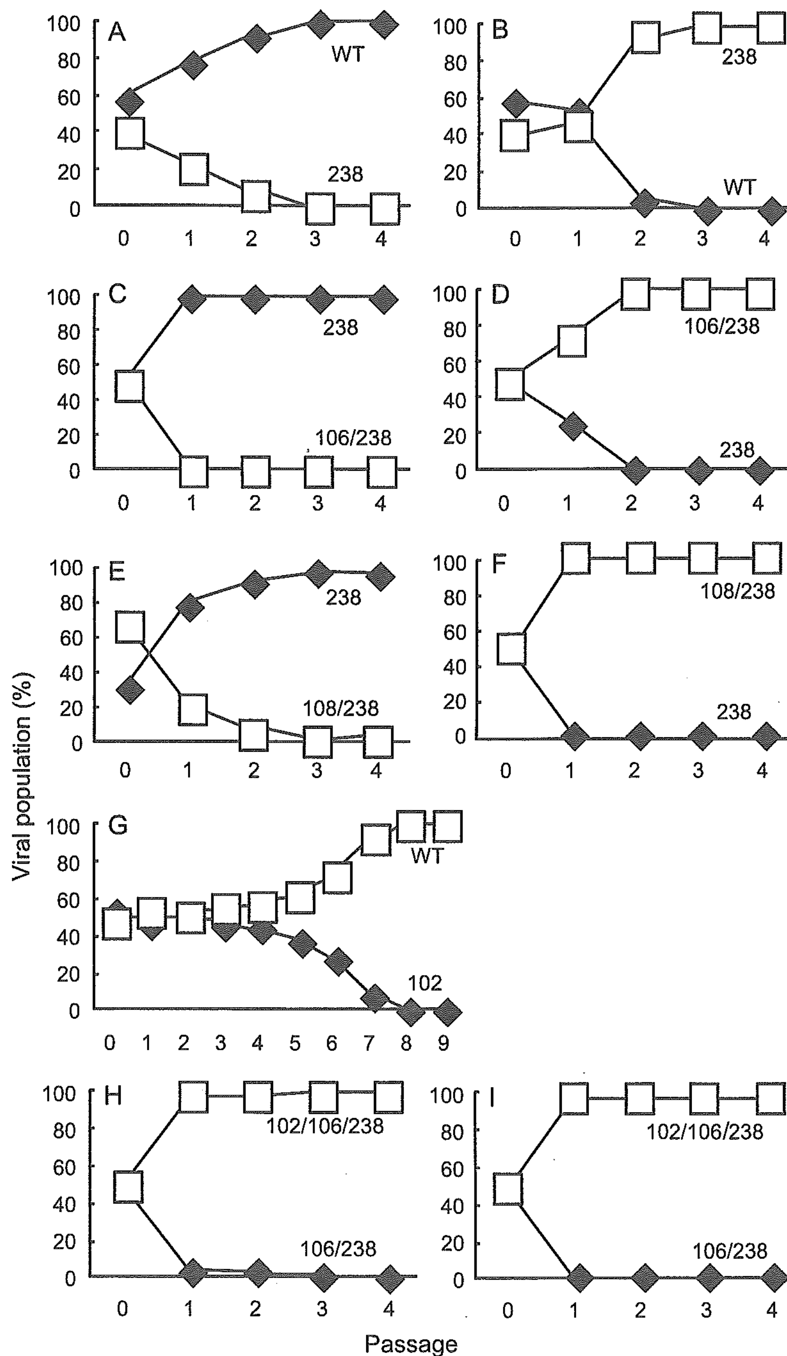

Fig. 5. Competitive HIV-1 replication assay for recombinant HIV-1s. Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells in the absence (A, C, E, G, and H) and presence of NVP (B: 0.1 μM; D, F, and I: 1 μM). The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells at the end of each passage were subjected to nucleotide sequencing, and proportion of Lys and Ser at position 238 (Graphs A and B), those of Val and Ala at position 106 (Graphs C and D), those of Val and Ile at position 108 (Graphs E and F), and those of Lys and Arg at position 102 (Graphs G–I) were determined.

The use of NVP has markedly increased worldwide because a single formulation of NVP combined with d4T and 3TC is a generic drug widely used in resource-limited situations (Cohen, 2003; Penzak et al., 2003). The use of NVP in pregnant women is also one of the main strategies to prevent mother-to-child transmission of HIV-1 (Guay et al., 1999; Marseille et al., 1999). Therefore, understanding of the mechanism of NVP resistance development is urgently needed.

In the present study, we identified a novel mutation, K238S, which was associated with a high multitude of NVP resistance when it was coupled with V106A or V108I. Interestingly, direct sequencing identified only K238S in the two patients infected with highly NVP-resistant HIV-1.

These results showed that V106A and V108I were reverting to their wild-type V106 and V108, respectively, in major viral populations in the plasma. Furthermore, the results indicate that K238S can remain longer than V106A and V108I in the absence of NVP treatment, partly because at least two nucleotide substitutions are required for K238S while V106A and V108I can occur with only a single nucleotide substitution.

M230 is located on the opposite side of Q151 in relation to the incoming dNTP, forming the primer grip of RT and contacting the primer strand (Huang et al., 1998; Sarafianos et al., 1999), and several mutations around this site were reported to occur in patients during NNRTI treatment. Parkin et al. (2000) observed the emergence of F227L and M230L during NVP-containing combination therapy and it was associated with the loss of NVP susceptibility of their clinical isolates. Demeter et al. (2000) reported that P236L occurred in subjects receiving delavirdine monotherapy. With regard to K238, Demeter et al. (1998) observed the emergence of K238T in one patient treated with atevirdine (an NNRTI) and AZT. Considering these reports, the region containing M230 and K238 is important for drug susceptibility and there may be more resistance-associated mutations around these sites.

Phylogenetic analysis showed a close relation between the isolates from Cases 33 and 23 (Fig. 2), suggesting that the two patients had acquired HIV-1 infection from the same source, although they did not know each other and interviewing them could not identify the source patient. This source patient presumably had been under antiretroviral treatment including NVP, which had failed to suppress his viral load because of the sets of mutations, K102R/V106A/K238S and V108I/K238S. It remains unknown whether K238S had existed as a polymorphism before the introduction of antiretroviral treatment, or it had emerged during NVP treatment in the source patient. Considering that a single dose of NVP to prevent mother-to-child transmission can induce resistant strains (Morris et al., 2003; Sullivan, 2003), some polymorphic mutations in treatment-naïve population could be associated with NVP resistance.

Materials and methods

Reagents and cells

AZT, d4T, and ABC were purchased from Sigma Co. (Tokyo, Japan). 3TC, NVP, and EFV were generously provided by Nippon Glaxo-Smithkline (Tokyo, Japan), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Merck Co. Inc. (Rahway, NJ), respectively.

H9 cells and COS-7 cells were grown in RPMI 1640 and Dulbecco's modified eagle medium (DMEM), respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001, 2003) were grown in DMEM supplemented with 10% FCS, hygromycin B (100 μg/ml) (Invitrogen Co., Carlsbad, CA), and blasticidin (1 μg/ml) (Funakoshi, Tokyo). Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were stimulated by phytohemagglutinin (PHA) in RPMI 1640-based medium containing interleukin-2 (20 U/ml) (R & D Systems, Minneapolis, MN) for 2 days before HIV-1 exposure.

Patients and clinical isolates

Clinical strains were isolated from fresh plasma of the study participants attending the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan, by using MAGIC-5 cells as described previously (Hachiya et al., 2001). The obtained isolates were stored at −80 °C until use and the infectivity was measured as blue cell-forming unit (BFU) of MAGIC-5 cells (Hachiya et al., 2001). The Institutional Review Board approved this study (IMCJ-H13-80) and a written informed consent was obtained from all the participants.

Case 33 was a 33-year-old homosexual man. He suffered from high fever and severe headache, probably symptoms of primary HIV-1 infection, in April 2000. On May 15, Western blotting showed faint bands against HIV-1 gp160, p68, p55, p25, and p18 antigens. One week after, these bands became bold and new bands against HIV-1 gp110, p40, and p34 antigens appeared. The CD4 count was 401/μl and HIV-1 viral load was 2.0×10^5/ml.

Case 23 was a 23-year-old homosexual man. He also suffered from severe headache and was hospitalized in January, 2001. Western blotting showed one faint band against HIV-1 gp160 antigen. In April, 2001, the band became bold and new bands against gp110, p68, p55, p52, gp41, p40, p34, p25, and p18 antigens appeared. The CD4 count was 210/μl and HIV-1 viral load was 7.1×10^4/ml.

Sequence analyses of HIV-1 RT and V3 region

Viral RNA was extracted from plasma and clinical isolates with Smi-test nucleic acid extraction kit (Genome Science, Fukushima, Japan). The HIV-1 RT gene was amplified by RT-PCR using One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan). For plasma-derived RNA, nested PCR was conducted subsequently to amplify enough DNA for direct sequencing. The sequences of primer sets for the first PCR (T1 and T4) and the second PCR (T12 and T15) were as follows (Gatanaga et al., 1999; Hachiya et al., 2001, 2003): T1, 5′-AGGGGGAATTGGAGGTTT (RF positions, 1910 to 1927); T4, 5′-TTCTGTTAGTGCTTTGGTT (RF positions, 2939–2921); T12, 5′-CCAGTAAAATTAAAGCCAG (RF positions, 2091–2109); and T15, 5′-TCCCACTAACTTCTGTATGTC (RF positions, 2852–2832). The gp120 V3 domain of several HIV-1 isolates was amplified by RT-

PCR and nested PCR. The sequences of primer sets for the first PCR (ES1 and EA1) and the second PCR (ES2 and EA2) were as follows (Ida et al., 1997): ES1, 5′-AATGTCAGCACAGTACAATGTACAC (RF positions, 6502–6526); EA1, 5′-ACAATTTCTGGGTCCCCTCCT-GAGGA (RF positions, 6890–6865); ES2, 5′-ATGGAAT-TAGGCCAGTAGTG (RF positions, 6527–6546); EA2, 5′-CTCCTAATTTTGTAACTAC (RF positions, 6829–6811). Specific precautions, including physical separation of processing areas, were taken to avoid template and amplified product carryover. Stringent quality control to prevent PCR contamination was employed to protect against cross-contamination of product DNA. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) and model 3700 automated DNA sequencer (Applied Biosystems). Amino acid sequences were deduced with the Genetyx-Win program version 4.1 (Software Development, Tokyo). For some PCR products, molecular cloning was performed with pT7 Blue T-Vector (Novagen, CA) and their sequences were analyzed.

For the phylogenetic analysis, RT gene and V3 sequences were aligned by Clustal-W program with reference sequences from the Los Alamos sequence database. The results of the alignment were then analyzed by the neighbor-joining method and the distance matrixes were generated with Kimura two-parameter model.

Bootstrap resampling (1000 data sets) of the multiple alignments was performed to test the statistical robustness of the tree.

Construction of recombinant HIV-1 clones

Recombinant infectious HIV-1 clones carrying various mutations in RT were constructed with a site-directed mutagenesis. Briefly, the desired mutations were introduced into *Xma*I–*Nhe*I region (759 bp) of pTZNX1, which encoded Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (Kodama et al., 2001). The *Xma*I–*Nhe*I fragment was inserted into pNL101-based plasmid (a kind gift from Jeang Kuan-The, NIAID/NIH, Bethesda, MD), generating various molecular clones with the desired mutations. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each molecular clone (10 μg/ml as DNA) was transfected into COS-7 cells (4×10^5 cells/100-mm-diameter dish) by Fugene transfection reagent (Roche Diagnosis, Basal, Switzerland). After 48 h, culture supernatants were harvested and stored at −80 °C until use. The infectivity of the viruses was determined as BFU in MAGIC-5 cells.

Drug susceptibility assay with MAGIC-5 cells

HIV-1 drug susceptibility to various RTIs was determined in triplicate by using MAGIC-5 cells as described previously (Hachiya et al., 2001). Briefly, MAGIC-5 cells were infected with adjusted virus stock (300 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Takara Shuzo). The blue-stained cells were counted under a light microscope. Drug concentrations that inhibited 50% of the stained cells of drug-free control (IC_{50}) were determined by referring to the dose–response curve.

Viral replication kinetic assays

PHA-stimulated PBMCs were exposed to each infectious virus preparation (300 BFU) in a final volume of 200 μl for 2 h. Infected cells were then washed and cultured in a volume of 200 μl in the absence or presence of NVP (1 μM). Assays were performed in triplicate and repeated at least three times using independently generated virus preparations. On days 0, 2, 4, 6, and 8 of infection, 100-μl aliquots of culture supernatants were filtered and stocked for measurements of p24 antigen concentration and replaced with equal amount of fresh medium with or without NVP. The concentrations of p24 in the supernatants were determined by chemiluminescence enzyme immunoassay (CLEIA) kit (Fuji-Rebio, Tokyo, Japan).

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed to virus preparations (300 BFU) to be examined for their replication ability and cultured in the presence or absence of NVP as described previously (Gatanaga et al., 2002; Kosalaraksa et al., 1999; Yoshimura et al., 1999). On day 1 in culture, one-third of the infected H9 cells were harvested and washed twice with PBS, and cellular DNA was extracted with Smi-test nucleic acid extraction kit. Purified DNAs were subjected to nested PCR for sequencing HIV-1 RT gene. Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at each passage were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Structural analysis of mutations in HIV-1 RT

To examine the interaction of HIV-1 RT with NVP, the SYBYL 6.7 (http://www.tripos.com/, Tripos Associates, St. Louis, MO) on a Silicon Graphics Octane2 workstation was employed. The site of the enzyme ligand complex was constructed based on the previously reported X-ray structure of the covalently trapped catalytic complex of HIV-1 RT with NVP (Protein Data Bank entry 1FKP) (Ren et al., 2000).

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Efficacy and Immunologic Responses to Influenza Vaccine in HIV-1–Infected Patients

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Summary: Influenza vaccine is recommended for HIV-1–infected patients. The present prospective study was conducted to evaluate the clinical efficacy and immunologic responses to the vaccine. From November 1 to December 27, 2002, 262 HIV-1–infected patients received a trivalent influenza subunit vaccine, whereas 66 did not. Influenza illness occurred in 16 vaccinated and 14 nonvaccinated patients (incidence = 6.1% [95% confidence interval (CI): 4%–10%] in vaccinated vs. 21.2% [CI: 13%–35%] in nonvaccinated persons, $P < 0.001$; relative risk = 0.29 [CI: 0.14–0.55]). Influenza vaccine provided clinically effective protection against influenza illness in HIV-1–infected patients. In baseline antibody-negative patients, anti-H1 and anti-H3 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 ($P < 0.05$). In contrast, in baseline antibody-positive patients, good antibody responses were observed irrespective of CD4 counts, like the healthy controls. Based on these results, annual vaccination is recommended. Specific CD4 responses correlated with HIV-1 viral load (VL), especially in patients treated with highly active antiretroviral therapy (HAART) compared with those without HAART ($P < 0.01$), although the clinical efficacy did not correlate with HIV-1 VL. HAART may enhance the immunologic efficacy of influenza vaccine.

Key Words: HIV-1, influenza, vaccination, antibody response, specific CD4

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After the recent approval of various anti-influenza drugs and rapid diagnosis kits for influenza infection by the Ministry of Health, Labor, and Welfare of Japan, it has become easier to diagnose this infection. Along with the developments in diagnostic methods and treatment of the infection, influenza vaccination programs have been actively applied in HIV-1–infected individuals. Influenza virus infection may be more prolonged in individuals with immunodeficiency[1] and can cause a transient increase in plasma HIV-1 viral load (VL)[2] that might become relevant to the clinical course of HIV-1 infection.[2,3] Therefore, influenza vaccine has been generally recommended for HIV-1–infected patients,[4–6] as is already stated in the guidelines of the Advisory Committee on Immunization Practices.[7] Few studies have reported the protective effect of such vaccination in patients with HIV-1 infection, however. Previous studies demonstrated that the number of CD4 T cells (CD4 count) could predict the efficacy of and/or antibody response to the vaccine but did not clearly demonstrate the correlation between the vaccine efficacy and HIV VL.[1,8–15]

Activated memory $CD4^+$ T cells are the predominant target of HIV-1,[16] and the antibody response to hemagglutinin (HA) is T-cell dependent.[17–19] Therefore, highly active antiretroviral therapy (HAART) may reconstitute the immune function of not only the antibody responses but T helper (Th)–cell responses. In this large prospective clinical study, we investigated the clinical efficacy of influenza vaccine in HIV-1–infected patients and correlated it with the immune response to the vaccine as determined by increased antibody titer and/or HA-specific CD4 T cells.

MATERIALS AND METHODS

Study Design and Participants

A 0.5-mL dose of single-shot trivalent influenza subunit vaccine, which contains 15 μg of influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Shanton/7/87, was prepared for adults in the 2002 through 2003 winter season in Japan. All HIV-1–infected patients who consulted the outpatient clinic of the AIDS Clinical Center at the International Medical Center of Japan from November 1 to December 27, 2002 were advised to receive the vaccine, although the final decision was left to the individual. In previous seasons, nearly half of HIV-1–infected patients received influenza vaccine in our clinic. This study was designed to be prospective in nature but nonrandomized. Only individuals, vaccinated and nonvaccinated, who understood the purpose of the study were enrolled, without any incentives. To keep selective bias to a minimum, all vaccinated and consecutive first-come 100 nonvaccinated patients were asked to participate in this study. All study participants gave

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informed consent, and the institutional ethical committee approved this study (protocol IMCJ-141). Twenty-six hospital staff members who were vaccinated with the same vaccine batch were enrolled as healthy immunized controls after consenting to participate in this study. Among them, 4 had no anti-influenza antibodies before vaccination. All participants were asked to visit to our clinic at least at week 0, 8, and/or 16 after enrollment to allow the withdrawal of 17 mL of blood at each visit for analysis of immunologic responses and routine examinations, including CD4 count and HIV VL.

Definition and Diagnosis of Influenza Virus Infection

In this study, influenza infection (illness) was defined if the patient had flulike symptoms associated with at least 1 adjunct diagnosis such as a serologic or virologic diagnosis. Flulike symptoms were defined as a fever of ≥38.0°C combined with 2 of the following 5 clinical symptoms: cough, rhinitis, myalgia, sore throat, and headache. All participants were asked to visit the clinic if they developed flulike symptoms. To avoid a bias in the clinical diagnosis, a history of influenza vaccination was written out on a separate colored sheet, which was removed from medical records before the outpatient clinic physician attended and examined the patient. The serologic diagnosis was defined as a >4-fold rise in anti-influenza antibody titer compared with before and 4 weeks after the symptoms. In addition, a change of the antibody titer from <10 to 40 U was defined as a 4-fold rise. Patients who had only the antibody rise but no flulike symptoms were not considered to have influenza-related illness. The virologic diagnosis was made by means of viral culture and/or a Rapidvue influenza test kit (Quidel, San Diego, CA) using a nasal or throat swab.

Laboratory Investigations

At each visit, CD4 T cells were enumerated by standard flow cytometry and HIV VL was measured using the Roche Amplicor assay kit, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ). Antibody responses to each of the 3 individual vaccine components were examined by the standard hemagglutinin inhibition (HAI) assay.[20] Titers ≥40 U were defined as protective, and a >4-fold rise in the antibody titer was considered an adequate response in previously antibody-negative patients.

For assessment of HA-specific CD4 T-cell responses, intracellular γ-interferon (IFN) production was examined by flow cytometry using the method described previously.[21,22] Because of the limited availability of peripheral blood mononuclear cells (PBMCs), we analyzed the H1-specific CD4 T cells only. Because fresh PBMCs must be used for this assay, as a result of a labor limitation, only the first 10 participants per day were examined on any particular day. Briefly, HA was purified from influenza virus strain, A/New Caledonia/20/99 (H1N1), as described previously.[23] PBMCs were isolated from the fresh heparinized blood and cultured (2×10^6 cells/mL) with diluted H1 plus anti-CD28 antibody (1 μg/mL) or medium alone for 16 hours at 37°C. Brefeldin A (10 μg/mL) was added to each sample in the final 5 hours of incubation. After 16 hours of stimulation, the cells were collected and stained with anti-CD4 allophycocyanin antibody (Beckman Coulter, Fullerton, CA) and anti-CD69–fluorescent isothiocyanate antibody (Becton Dickinson). Subsequently, the cells were fixed and permeabilized to examine for the intracellular production of γ-IFN as described previously.[21,22] The flow cytometry analysis was performed by means of the FACSCalibur fluorescence-activated cell sorter with CellQuest software (BD Biosciences, San Jose, CA), and 10,000 CD4 T cells were collected for each analysis.

Statistical Analysis

The data on HA-specific CD4 T cells are presented as the arithmetic mean ± SEM. The data on anti-HA antibody titer are presented as the geometric mean. Statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA). Differences in the proportion of influenza virus infection between vaccinated and nonvaccinated groups were analyzed by the χ^2 test. Multiple logistic regression analysis was used to identify factors that contributed to protection against influenza illness. For the analyses of immune responses, participants were stratified by their CD4 count or HIV VL. Changes in antibody titer and HA-specific CD4 T cells were analyzed using the Kruskal-Wallis test or the Mann-Whitney U test. In all tests, a P value <0.05 was considered significant.

RESULTS

Subjects

During the period of vaccination, 626 HIV-1–infected patients visited our clinic, and 332 of these received the vaccine, whereas 294 did not. Among them, 317 of those vaccinated and 87 of 100 approached to participate as nonvaccinated patients agreed to participate in the present study. Consequently, 76 patients dropped out of the study (55 of 317 vaccinated patients and 21 of 87 nonvaccinated patients). There were no characteristic differences at baseline between the analyzed and drop-out patients (data not shown). None of the patients dropped out from the study because of HIV-1 disease progression, and none received anticancer or immunosuppressive agents during this study. The final composition of the study group based on compliance with the study protocol, including visits on the fixed dates, was 262 vaccinated (82.6%) and 66 nonvaccinated (75.9%) patients (Fig. 1). Table 1 summarizes the baseline characteristics of the participants.

Efficacy of Influenza Vaccine

The peak of the influenza epidemic of the 2002 through 2003 winter season in Japan was documented during the fourth week of January 2003 and was predominantly caused by influenza A/H3N2. The prevalence of influenza infection in this season was the third highest in the last decade.[24] In this study, 30 participants were diagnosed as having definitive influenza illness (5 patients with A/H1N1 strain, 16 with A/H3N2 strain, and 9 with B strain). Six patients were confirmed to have an influenza illness by flulike symptoms, positive viral cultures, positive influenza test kit results, and a >4-fold rise in antibody titer (1 with H1N1 strain, 1 with H3N2 strain, and

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