Fig. 4. Longitudinal analysis of drug-resistance-associated amino acid mutations and the existence of insertion mutations in the p6^{gag} and p6^{pol} genes. Drug-resistance-associated amino acid mutations and the types of the p6^{gag} and p6^{pol} genes are shown in the boxes. W, wild p6^{gag}/p6^{pol} type alone; I, inserted p6^{gag}/p6^{pol}type alone; W/I, mixed type. Drug resistance-mutations against prescribed drugs are shown in italic type, and primary mutations are shown with underlines. The graph shows the time course of anti-HIV-1 therapy (the horizontal axis) and viral load (the vertical axis). Anti-HIV-1 drugs used on the therapy are shown below the graph; AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; NFV, nelfinavir; IDV, indinavir. A) PTH#1, B) PTH#4, C) PTH#5, D) PTH#6, and E) PTH#16.

ed amino acid sequences were located within or nearby a "PTAPP" sequence (codon 7–11) which was conserved among the major group of HIV-1. The inserted amino acid sequences and their adjacent sequences were identical in seven cases (PTH#4, PTH#5, PTH#6, PTH#16, PTH#18, PTH#21, and PTH#22), and they were also similar in the other case (PTH#1). Figure 2 C shows the amino acid sequence of p6^{pol} protein in each case. Inserted amino acid sequences were located within or nearby a conserved sequence, "RANSP", at codon 14–18. The inserted sequences and adjacent sequences were identical in three cases (PTH#16, PTH#21, and PTH#22), and they were only similar in the other five cases (PTH#1, PTH#4, PTH#5, PTH#6, and PTH#18).

The inserted-type HIV-1 was also detected in four of thirty-nine (10.3%) therapy-naive patients (NP#9, NP#11, NP#28, and NP#38). The insertion mutations were identical or similar to those of the inserted-type HIV-1 detected in the patients treated with HAART (Fig. 3). However, the frequency of inserted-type HIV-1 detection in the HAART-receiving patients is significantly higher than that in the therapy-naive patients ($P=0.02$).

Longitudinal Analysis of the Insertion Mutations in the p6^{gag} and p6^{pol} Genes

To clarify whether the nucleotide insertions in the p6^{gag} and p6^{pol} genes of HIV-1 were induced or selected by the antiretroviral therapy, we retrospectively analyzed the appearance of the insertion mutations using stocked plasma samples of PTH#1, PTH#4, PTH#5, PTH#6, and PTH#16.

Figure 4A shows the result for the case of PTH#1. The patient had never received any antiretroviral drugs until the beginning of the anti-HIV-1 therapy. HIV-1 with an insertion mutation in the p6^{gag} and p6^{pol} genes (inserted p6^{gag}/p6^{pol} type HIV-1) alone was detected from samples before the antiretroviral therapy began. This variant was found to contain a secondary mutation against some PR inhibitors, V77I, in the PR gene. After the beginning of HAART with AZT, 3TC, and NFV, the viral load had been suppressed and controlled below 10^3 copies/ml. However, it increased above the order of 10^3 at the 32nd month. HIV-1 detected from samples at the 32nd, 34th, and 35th months were also the inserted p6^{gag}/p6^{pol} type, and the primary and other secondary mutations appeared in the PR and RT genes (Fig. 4A).

PTH#4 had previously undergone an antiretroviral therapy with AZT at another hospital. Both types of HIV-1, inserted p6^{gag}/p6^{pol} type and wild p6^{gag}/p6^{pol} type, coexisted before the beginning of HAART. Primary and secondary mutations against AZT, T215Y and L210W, respectively, were detected in the RT gene.

Viral load was immediately decreased below the detectable level by HAART with d4T, 3TC, and NFV, however, it started increasing at the 4th month. HIV-1 detected at this point was the wild p6^{gag}/p6^{pol} type alone, and primary mutations against NFV and 3TC, D30N and M184V, were additionally detected in the PR and RT genes, respectively. Although HIV-1 detected at the 7th and 8th months were also the wild p6^{gag}/p6^{pol} type alone, another primary mutation against NFV, L90M, was detected in the PR gene. Inserted p6^{gag}/p6^{pol} type HIV-1 appeared again at the 9th month. The inserted p6^{gag}/p6^{pol} type HIV-1 alone has continuously been detected since the 9th month (Fig. 4B).

PTH#5 had never received any antiretroviral drugs until the beginning of the anti-HIV-1 therapy. HIV-1 detected before the beginning of HAART was only the inserted p6^{gag}/p6^{pol} type, and no drug-resistance-associated mutation was detected in the PR and RT genes. At the 4th month after HAART with AZT, 3TC, and NFV, the viral load of the patient decreased below the detectable level, however, it rebounded at the 12th month. HIV-1 detected at the 12th and 16th months was also the inserted p6^{gag}/p6^{pol} type alone, and drug-resistance-associated mutations against the prescribed drugs were detected in the PR and RT genes and in the cleavage site of gag polyprotein (Fig. 4C).

PTH#6 had never received any antiretroviral drugs until the beginning of the anti-HIV-1 therapy. Both inserted and wild p6^{gag}/p6^{pol} types coexisted before the beginning of the antiretroviral therapy. Viral load rebounded to above the order of 10^4 copies/ml at the 29th month after the start of HAART with AZT, 3TC, and NFV. HIV-1 surviving at the 34th and 39th months was the inserted p6^{gag}/p6^{pol} type alone, and the wild p6^{gag}/p6^{pol} type disappeared. The inserted-type HIV-1 contained primary mutations against the prescribed drugs, L90M and M184V, in the PR and RT genes, respectively (Fig. 4D).

PTH#16 had the experience of an antiretroviral therapy with AZT at another hospital. Both inserted and wild p6^{gag}/p6^{pol} type viruses coexisted before the beginning of an antiretroviral therapy with 3TC. A primary mutation against AZT, K70R, was detected in the RT gene at the time. At the 6th month when viral load rebounded, the inserted p6^{gag}/p6^{pol} type HIV-1 alone survived, and the wild p6^{gag}/p6^{pol} type disappeared. The inserted-type HIV-1 contained a primary mutation against 3TC, M184V, in the RT gene. HIV-1 detected at the 18th, 31st, and 46th months were also the inserted p6^{gag}/p6^{pol} type alone (Fig. 4E).

We can summarize the data as follows; 1) inserted p6^{gag}/p6^{pol} type viruses had already existed in three patients (PTH#1, PTH#5, and PTH#6) when they were

still therapy-naive, 2) inserted $p6^{gag}/p6^{pol}$ type viruses were selected over the wild $p6^{gag}/p6^{pol}$ type during HAART in all three cases in which both types of HIV-1 coexisted in the beginning of the therapy (PTH#4, PTH#6, and PTH#16), 3) in two cases in which the inserted-type HIV-1 alone was detected before the beginning of HAART, the inserted-type HIV-1 alone was continuously detected during the therapy (PTH#1 and PTH#5).

Discussion

Characteristics of the Insertion Mutations Detected in the $p6^{gag}$ and $p6^{pol}$ Genes

There are two characteristics of the insertion mutations. First, the number of inserted nucleotides was a multiple of three in each case, and thus, the frameshift of codons did not occur. Second, the same or similar nucleotide sequences originally existed near the inserted nucleotide sequences.

Concerning the mechanism of how such insertion mutations occur in the $p6^{gag}$ and $p6^{pol}$ genes, they are probably produced by the mechanism that nucleotide sequences once synthesized are successively repeated, as proposed in previous reports (1, 2). As similar types of insertion mutations were reported in the RT gene (6, 20, 21), insertion mutations might easily happen in the HIV-1 genome. The finding that the inserted $p6^{gag}/p6^{pol}$ type viruses were detected in four of thirty-nine (10.3%) therapy-naive patients suggested this type of insertion mutation exists as a polymorphism in the $p6^{gag}$ and $p6^{pol}$ genes.

The Effects of the Insertion Mutation in the $p6^{gag}$ and $p6^{pol}$ Genes on Proliferation and/or Survival of the HIV-1 Variant

Retrospective and longitudinal analysis clarified which type of HIV-1 between inserted and wild types can survive in the presence of antiretroviral drugs. Both types of HIV-1, inserted and wild $p6^{gag}/p6^{pol}$ types, coexisted in PTH#4, PTH#6, and PTH#16 at the beginning of HAART, and then the inserted-type viruses were selected during the therapy in all patients. Other findings that the inserted-type variants once appeared did not change to the wild $p6^{gag}/p6^{pol}$ type during the therapy in all cases were obtained. Taken together, we suggest that this type of insertion in the $p6^{gag}$ and $p6^{pol}$ genes enhances the proliferation and/or the survival of the HIV-1 variant in the presence of antiretroviral drugs.

As for the relationship between drug resistance and the acquisition of insertion mutation, it seems unlikely that the insertion mutations directly confer drug resistance to HIV-1 since both $p6^{gag}$ and $p6^{pol}$ proteins are not the target

molecules of the anti-HIV-1 drugs. Peters et al. (18) reported that HIV-1 with exactly the same insertion mutation detected in PTH#21, NP#11, NP#28, and NP#38 exhibited about two-fold resistance to nucleoside RT inhibitors. However, since the resistance level was fairly low, whether the insertions within these genes participate in the development of drug resistance to antiretroviral drugs is still unclear.

Concerning the assembly and budding of HIV-1 particles, it was reported that the conserved "PTAPP" sequence of $p6^{gag}$ protein played an important role (8, 9, 11, 17, 22). The finding that the "PTAPP" motif was completely or partially duplicated in $p6^{gag}$ protein suggests that the inserted $p6^{gag}/p6^{pol}$ type HIV-1 enhances budding in the presence of antiretroviral drugs. We are currently analyzing the influence of the insertions within the $p6^{gag}$ and $p6^{pol}$ genes using infectious clones *in vitro*.

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A Simple HPLC Method for Simultaneous Determination of Lopinavir, Ritonavir and Efavirenz

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We developed a simple HPLC method for the simultaneous determination of lopinavir (LPV), ritonavir (RTV) and efavirenz (EFV) to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese patients enrolled in a clinical study. The monitoring of LPV plasma concentration is important because co-administration of LPV/RTV with EFV sometimes decreases plasma concentrations of LPV caused by EFV activation of cytochrome P-450 3A. A solution of acetonitrile, methanol and tetramethylammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (TFA) has been used as the mobile phase in a HPLC method to elute LPV and RTV. We found that a solvent ratio of 45 : 5 : 50 (v/v/v) of acetonitrile/methanol/0.02 M TMAP in 0.2% TFA optimized separation of LPV, RTV and EFV. A column temperature of 30 °C was necessary for the reproducibility of the analyses. Standard curves were linear in the range 0.060 to 24.06 μg/ml for LPV, 0.010 to 4.16 μg/ml for RTV, and 0.047 to 37.44 μg/ml for EFV. Coefficients of variation (CVs) of LPV, RTV and EFV in intraday and interday assays ranged from 1.5 to 4.0%, 2.5 to 16.8% and 1.0 to 7.7%, respectively. Accuracies ranged from 100 to 110%, 101 to 116% and 99 to 106% for LPV, RTV and EFV, respectively. The extraction recoveries were 77—87, 77—83 and 81—91% for LPV, RTV and EFV, respectively.

Key words   human immunodeficiency virus (HIV)-1; HPLC; therapeutic drug monitoring; lopinavir; ritonavir; efavirenz

In recent years, the treatment of human immunodeficiency virus (HIV)-1 infection and AIDS has been advanced by the development of highly active antiretroviral therapy (HAART). HAART reduces plasma HIV-RNA below detectable limits in most cases. However, some patients become nonresponsive to HAART even after experiencing a decrease in plasma HIV-RNA, because of the rapid development of drug-resistant variants of HIV-1. This undesirable outcome may result from a failure to achieve effective plasma concentrations of the anti-retroviral drugs. Therefore, monitoring plasma drug concentrations is important to ensure efficacious levels are achieved during HAART.

Combination therapy with the HIV protease inhibitors lopinavir (LPV)/ritonavir (RTV) (Kaletra®), and the non-nucleoside reverse transcriptase inhibitor efavirenz (EFV), has been shown to be effective against drug-resistant HIV-1.[1,2] These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver.[3—5] When LPV is administered with RTV as Kaletra®, RTV inhibits the CYP3A-mediated metabolism of LPV, thereby providing increased plasma levels of LPV. In contrast to RTV, however, EFV enhances CYP3A. Therefore, co-administration of LPV/RTV and EFV can result in the decrease of LPV plasma concentrations.[6] To counteract this effect, the administration of an increased dose of LPV/RTV is required when used with EFV[6]; Clumeck *et al.*[1] also recommended use of an increased dose. Kaletra® was approved for use by the US Food and Drug Administration in September 2000 and became available for use in Japan in December 2000, but a Japanese clinical trial was not conducted due to a strong and urgent demand for the drug. However, an understanding of the pharmacokinetics of these drugs in Japanese patients remains essential.

A clinical study to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese HIV-1-infected patients and determine the correlation between plasma concentrations of these drugs and their efficacy is planned. Prior to the onset of the clinical study, however, development of a simple and convenient method to monitor plasma concentrations of these drugs was necessary.

The aim of this study is to develop a simple and rapid HPLC method for the simultaneous determination of LPV, RTV and EFV.

Experimental

Chemicals   LPV, RTV and the internal standard (IS), (5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). EFV was kindly provided by Merck & Co., Inc. (Rahway, NJ, U.S.A.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Katayama Chemical, Osaka, Japan) were HPLC grade. L-Consera was purchased from Nissui Pharmaceutical Co., LTD. (Tokyo, Japan). Trifluoroacetic acid (TFA) and tetramethylammonium perchlorate (TMAP) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan).

Chromatography   The HPLC system consisted of a Waters pump model 6000A, 484 tunable absorbance detector, 741 data module, and WISP 710B auto sample processor. The analytical column was a Radial-Pak Nova-Pak C_{18} column (4 μm, 8×100 mm, Waters) protected by a Guard-Pak Inserts Nova-Pak C_{18} precolumn. Absorbance was measured at 205 nm. Drugs were quantified by measuring peak areas of chromatograms.

Standard Solutions   A LPV/RTV stock solution was prepared at concentrations of 120.3/20.8 μg/ml in water/ethanol (50 : 50, v/v). An EFV stock solution of 93.6 μg/ml was prepared by dissolving the drug in water/ethanol (50 : 50, v/v). These stock solutions were stored at −80 °C and thawed on the day of analysis.

Each stock solution was diluted in drug-free serum to yield concentrations of 0.060, 0.120, 0.241, 0.602, 1.203, 2.406, 6.015, 12.03 and 24.06 μg/ml for LPV, 0.010, 0.021, 0.042, 0.104, 0.208, 0.416, 1.04, 2.08 and 4.16 μg/ml for RTV, and 0.047, 0.094, 0.187, 0.468, 0.936, 1.872, 4.68, 9.36, 18.72 and 37.44 μg/ml for EFV.

Sample Preparation   Two milliliters of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (2.024 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to 500 μl of serum sample. The mixture was vortexed and centrifuged at 2800×*g* for 5 min. The organic layer was separated and evaporated to dryness. The dried material was dissolved in 100 μl of mobile phase

solution and centrifuged at $13000 \times g$ for 5 min. Then $25 \mu l$ of the upper solution was injected into the HPLC.

Validation Intraday and interday precision values for the method were estimated by assaying control sera containing five different concentrations of LPV, RTV and EFV five times on the same day and on three separate days to obtain the coefficient of variation (CV).

Accuracy was determined as the percentage of the nominal concentration.

Recovery from serum was evaluated by analyzing triplicate samples with or without extraction.

Results

Selection of Mobile Phase When acetonitrile/methanol/ 0.01 M TMAP in 0.1% TFA (50 : 5 : 45, v/v/v) was used as the mobile phase solution at a flow rate of 1.5 ml/min, complete separation of LPV from RTV and the IS was achieved (Fig. 1a). HPLC performed with the same mobile phase solution using a standard solution containing LPV, RTV and EFV (see Experimental) resulted in incomplete separation; EFV eluted after LPV with a very similar retention time (Fig. 1b). As a first trial, the acetonitrile/methanol/0.01 M TMAP in 0.1% TFA ratio was changed to 45 : 5 : 50. EFV then eluted earlier than LPV, but separation was still incomplete (Fig. 1c). In a second trial, the concentration of TFA was increased to 0.2%, but the separation of LPV and EFV was not complete (Fig. 1d). When the concentration of TMAP was increased to 0.02 M in 0.1% TFA, separation of EFV from LPV was considerably improved; however, then the IS eluted close to EFV (Fig. 1e). Finally, we chose conditions of 0.02 M TMAP and 0.2% TFA, which resulted in separation of LPV, RTV, EFV and the IS (Fig. 1f).

Temperature can affect peak separation in samples containing ions. To obtain better separation of LPV and EFV, the effect of temperature was investigated. Complete separation of LPV and EFV was achieved when the column temperature was increased from room temperature to 30 °C (Fig. 1g).

Validation: Linearity, Precision, Accuracy, and Recovery Standard curves of LPV, RTV and EFV showed linearity in the concentration range of 0.060 to 24.06 $\mu g/ml$ for LPV, 0.010 to 4.16 $\mu g/ml$ for RTV, and 0.047 to 37.44 $\mu g/ml$ for EFV, with correlation coefficients of 1.000, 0.9998, and 0.9994, respectively.

Precision, accuracy, and extraction recovery of the method are shown in Table 1. The selected concentrations of each drug cover the expected plasma concentrations found in the patients.

The CVs calculated for LPV in the intraday and interday assays ranged from 1.5 to 3.1% and 2.3 to 4.0%, respectively, which are similar to those reported by Marzolini et al.,[7] Poirier et al.,[8] Titier et al.,[9] and Tribut et al.[10] For RTV, CVs in the intraday and interday assays ranged from 2.5 to 12.5% and 2.8 to 16.8%, respectively, which are comparable to values reported by others.[8—10] A slightly high CV value of greater than 10% was obtained at the RTV concentrations of 0.021 and 0.208 $\mu g/ml$. Intraday and interday CVs of EFV ranged from 1.0 to 4.2% and 3.4 to 7.7%, respectively, which are similar to or much lower than previously reported values.[8—10]

Accuracies ranged from 100 to 110% and 99 to 106% for LPV and EFV, respectively. For RTV, accuracy values at concentrations of 0.208 $\mu g/ml$ and higher ranged from 101 to 116%, but the value at the low concentration of 0.021 $\mu g/ml$ was somewhat high at 146.7%.

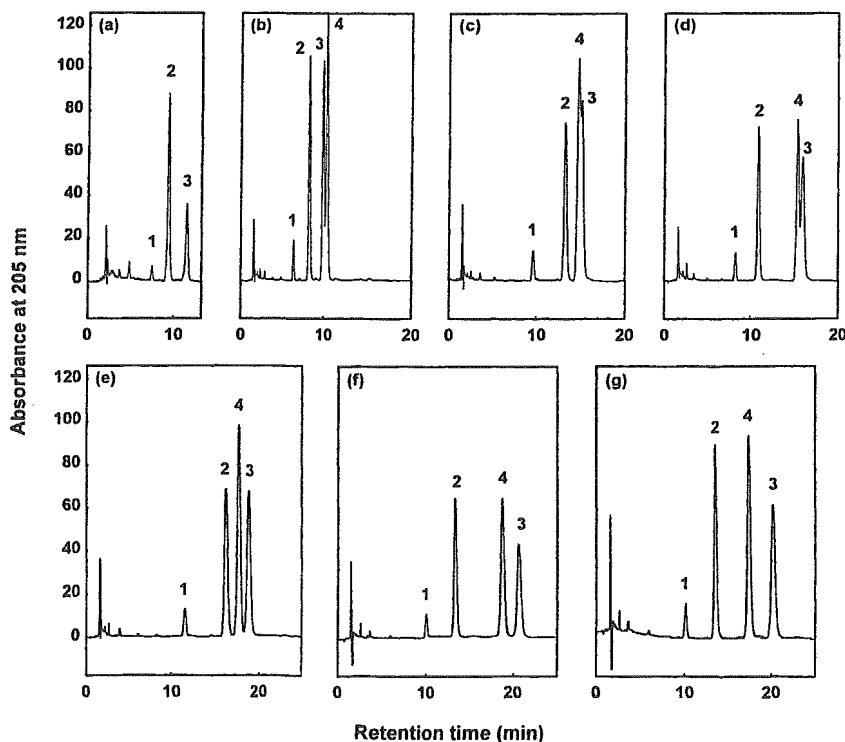Recoveries from serum ranged from 77 to 87%, 77 to 83%

Fig. 1. Chromatograms under Different Mobile Phase Solution Conditions

Peak 1, RTV; 2, IS; 3, EFV; 4, LPV. (a) LPV, RTV and the IS were separated with a mobile phase of 50 : 5 : 45 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA. LPV, RTV, EFV and the IS were separated with mobile phase solutions of (b) 50 : 5 : 45 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA, (c) 45 : 5 : 50 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA, (d) 45 : 5 : 50 acetonitrile/methanol/0.01 M TMAP in 0.2% TFA, (e) 45 : 5 : 50 acetonitrile/methanol/0.02 M TMAP in 0.1% TFA, and (f) 45 : 5 : 50 acetonitrile/methanol/0.02 M TMAP in 0.2% TFA at room temperature. (g) LPV, RTV, EFV and the IS were separated with the same mobile phase solution as (f) at 30 °C.

Table 1. Intraday and Interday Precision and Accuracy for LPV, RTV and EFV

	Expected (μg/ml)	Intraday (n=5)		Interday (n=15)		Accuracy (%)	Recovery (%)
		Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)		
LPV	0.060	0.064±0.002	2.8	0.066±0.002	3.2	109.4±3.5	77.6±1.9
	0.120	0.129±0.002	1.3	0.128±0.004	3.0	106.0±3.1	86.4±0.7
	1.203	1.240±0.038	3.1	1.258±0.029	2.3	104.6±2.4	78.8±0.5
	6.015	6.068±0.138	2.3	6.314±0.250	4.0	105.0±4.2	81.2±2.1
	24.060	23.331±0.351	1.5	24.288±0.837	3.4	100.9±3.5	84.5±3.3
RTV	0.021	0.034±0.004	11.7	0.031±0.005	16.8	146.7±24.7	83.0±9.6
	0.208	0.253±0.032	12.5	0.240±0.024	10.0	115.4±11.6	77.8±2.5
	1.040	1.050±0.037	3.5	1.058±0.033	3.1	101.8±3.2	77.2±2.3
	4.160	4.136±0.102	2.5	4.218±0.119	2.8	101.4±2.6	82.8±0.6
EFV	0.047	0.048±0.002	4.0	0.049±0.004	7.7	105.2±8.1	86.3±3.9
	0.094	0.095±0.004	4.2	0.097±0.006	5.9	103.7±6.1	90.8±1.0
	0.936	0.966±0.036	3.7	0.991±0.033	3.4	105.9±3.6	87.3±2.2
	4.680	4.669±0.115	2.5	4.951±0.265	5.4	105.8±5.7	81.6±2.3
	18.720	17.389±0.167	1.0	18.595±0.917	4.9	99.3±4.9	84.6±2.6

and 81 to 91% for LPV, RTV and EFV, respectively. Extraction recovery of the IS was 100%.

These results indicate that the method developed in this study achieves a high degree of reproducibility and accuracy.

Chromatograms of Serum or Plasma Samples Figure 2 shows chromatograms obtained after extraction of control sera containing (a) 6.015 μg/ml of LPV, 1.04 μg/ml of RTV and 4.68 μg/ml of EFV and (b) plasma from an HIV-1-infected patient treated with Kaletra® and EFV. There was no significant drift of the baseline and no interfering peaks affected quantification of LPV, RTV and EFV. In the case of the HIV-1-infected patient (b), RTV could not be determined because RTV plasma concentration was below the limits of detection.

Discussion

Using HPLC, we now can measure plasma concentrations of protease inhibitors routinely in HIV-1-infected patients treated with HAART. Measurement of plasma concentrations of LPV/RTV in an HIV-1-infected patient treated with Kaletra® and EFV by HPLC revealed that EFV eluted very closely to LPV. Combination therapy with Kaletra® and EFV is effective, and its use is expected to increase. A pharmacokinetic clinical study on the combination therapy in Japanese patients necessitated the development of a method for simultaneous quantification of the concentrations of LPV, RTV and EFV. The method needed to be convenient and time-efficient because of the need for frequent measurement of the drugs in patient plasma samples. The method described here requires only 90 min for one sample measurement, including the time for drug extraction. The isocratic condition of our method contributes to the convenience of serial runs.

When EFV is administered at the recommended dose of 600 mg once daily postprandial, plasma concentrations are expected in the 1 to 5 μg/ml range.[11] When LPV/RTV is administered at a dose of 400/100 mg twice daily postprandial, plasma concentrations are expected in the 1 to 10 or 0.5 to 1 μg/ml range, respectively.[6] Our method successfully covers these regions with good precision and accuracy.

This simple and timesaving HPLC method can be performed using conventional instruments and will be useful for
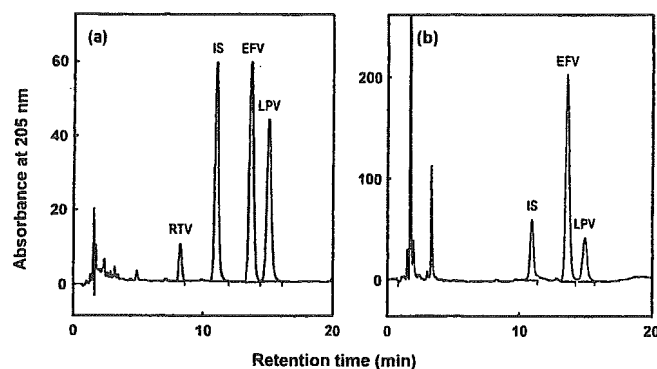

Fig. 2. Chromatograms Obtained after Extraction of (a) Control Serum Sample Containing 6.015 μg/ml of LPV, 1.04 μg/ml of RTV and 4.68 μg/ml of EFV and (b) Plasma Sample from an HIV-1-Infected Patient Treated with Kaletra® and EFV

monitoring combination therapy of LPV/RTV and EFV in HIV-1-infected patients to prevent treatment failure.

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Prevalence of Drug-Resistant Human Immunodeficiency Virus Type 1 in Therapy-Naive Patients and Usefulness of Genotype Testing

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Abstract: In the present study, we performed genotypic drug-resistance testing in 116 therapy-naive human immunodeficiency virus type 1 (HIV-1)-infected patients between 1999 and 2002 at Nagoya National Hospital, Japan. The prevalence of drug-resistant HIV-1 with one or more major mutations significantly increased from 5.3% (4/75) in 1999–2001 to 17.1% (7/41) in 2002 ($P=0.05$), suggesting the spread of drug-resistant HIV-1. We identified a patient who possessed a protease (PR) inhibitor-resistant HIV-1 with a major mutation consisting of L90M before the initiation of therapy. The patient was administered zidovudine, lamivudine, and efavirenz as highly active antiretroviral therapy (HAART), as PR inhibitors were excluded based on the result of the drug-resistance testing. The treatment succeeded in strongly suppressing the proliferation of drug-resistant HIV-1 and concomitantly increased CD4 cell counts. Thus, we conclude that drug-resistance testing prior to the initiation of therapy is important for therapy-naive patients to devise the optimum therapy regimen for each individual.

Key words: Drug-resistant HIV-1, Therapy-naive patients, Genotype testing

Antiretroviral drugs against human immunodeficiency virus type 1 (HIV-1), protease (PR) inhibitors and reverse transcriptase (RT) inhibitors, are used for treatment of HIV-1-infected patients. Combination therapy with three or more antiretroviral drugs (highly active antiretroviral therapy, HAART) can strongly suppress viral replication and maintain the plasma HIV-1 RNA of patients below the detectable level (6, 11). However, when variants with decreased susceptibility to the antiretroviral drugs become a major population among quasi-species of HIV-1, the result is therapy-failure (16). The level of decreased susceptibility depends on the acquisition of major or minor amino acid mutations that confer drug-resistance (5, 7, 19). We routinely examine the existence of drug-resistance-associated mutations in the gag, PR, and RT genes of HIV-1 when the emergence of drug-resistant variants is clinically suspected in therapy-receiving patients. The genotypic drug-resistance testing of HIV-1 can clarify the emergence of drug-resistant variants, and the resulting data is invaluable in formulating an effective antiretroviral drug regimen against the variants.

Drug-resistant HIV-1 causes therapeutic problems not only in therapy-receiving patients but also in therapy-naive patients, as such cases in which patients already possess the drug-resistant variants before the initiation of therapy (2, 18). In the present study, we performed genotypic drug-resistance testing in 116 therapy-naive HIV-1-infected patients between 1999 and 2002 at Nagoya National Hospital, Japan, and estimated the prevalence of drug-resistant amino acid mutations. We also report the therapy-success case of a patient who had a PR inhibitor-resistant HIV-1 with a major mutation, consisting of L90M, where the choice of antiretroviral drugs was guided by the result of the drug-resistance testing.

Materials and Methods

Patients. All 116 therapy-naive HIV-1-infected patients

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; PR, protease; RT, reverse transcriptase; RT-PCR, reverse transcription and polymerase chain reaction.

who underwent their initial consultation at Nagoya National Hospital, Japan, from January 1, 1999 to December 31, 2002 were enrolled in this study. The characteristics of the patients are shown in Table 1. The genotype testing of HIV-1 was performed following patient consent. Plasma samples obtained at initial consultation were used for the drug-resistance testing.

Measurement of viral load and CD4 cell counts. The amount of plasma HIV-1 RNA (viral load) was measured using an Amplicor HIV-1 monitor v1.5 system (Roche, Tokyo). CD4 cell counts were measured by flow cytometry (FACSCalibur, Becton Dickinson) using an anti-CD4 antibody (DAKO, Kyoto, Japan).

Primers. We designed four primers for an amplification of regions (1,539 base pairs) containing codons 425–500 of the gag gene, codons 1–99 of the PR gene, and codons 1–349 of the RT gene of HIV-1 (8). The sense and antisense primers for reverse transcription and polymerase chain reaction (RT-PCR) were K1 and U13, respectively, while the sense and antisense primers for nested PCR were K4 and U12, respectively (8). Four other primers were used for the sequencing reactions. C1, (5'-GTCCTATTGAAACTGTACCA-3'); Z2, (5'-GTAGCATGACAAAATCTTAG-3'); NB, (5'-AAGTCAGATCCTACATACAA-3'); F1, (5'-AGTATTG-

TATGGATTTTCAGGC-3'). The primers reported by Ou et al. (12) were used for the determination of HIV-1 subtypes.

Genotypic drug-resistance testing of HIV-1. HIV-1 RNA was purified from plasma of ethylenediaminetetraacetic acid (EDTA)-containing peripheral blood using a QIAamp viral RNA mini kit (QIAGEN, Tokyo). The target region was amplified by RT-PCR using the Superscript one-step RT-PCR for long templates kit (Invitrogen, Tokyo) and successive nested PCR with LA Taq polymerase (TaKaRa, Osaka, Japan). The RT-PCR and PCR were performed as previously reported (8). The products of RT-nested PCR were separated through 1.0% agarose gel, and purified with the QIAquick gel extraction kit (QIAGEN). Sequencing was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Tokyo). DNA sequences were determined by the direct sequencing method using the ABI PRISM 310 system (Applied Biosystems). Both DNA and amino acid sequences were compared with those of the HXB2D strain belonging to subtype B (Accession No. K03455).

Criteria for drug-resistant amino acid mutations. The grouping of drug-resistant amino acid mutations into major or minor mutations was performed according to the consensus guidelines for drug-resistance testing (7).

Table 1. Characteristics of therapy-naive HIV-1-infected patients

Year	1999	2000	2001	2002	1999–2002
Age, years					
Median	33	28	32	31	32
Range	20–58	22–45	21–69	21–65	20–69
Sex					
Male	14	15	37	34	100
Female	1	2	6	7	16
Nationality					
Japanese	10	14	32	30	86
Foreign	5	3	11	11	30
Risk factor for infection					
Homosexual	9	13	20	15	57
Heterosexual	3	2	12	11	28
Bisexual	0	0	5	4	9
Unknown	3	2	6	11	22
CD4 cell count, cells/μl					
Median	324	376	261	266	283.5
Range	43–893	15–943	4–1,107	9–1,508	4–1,508
Viral load, $\log_{10}$ copies/ml					
Median	4.52	4.49	4.65	5.04	4.66
Range	3.23–5.58	2.79–5.89	2.45–6.11	2.67–6.46	2.45–6.46
Subtype					
B	14	12	27	34	87
AE	0	3	8	4	15
A	0	1	4	2	7
C	0	1	2	0	3
D	1	0	0	1	2
F	0	0	2	0	2

The drug-resistant mutations of A431V and F449L in the gag cleavage sites were judged as minor mutations against PR inhibitors according to other reports (5, 19).

Determination of HIV-1 subtypes. The subtyping was performed by the neighbor-joining method using the Genetyx-Mac system (Software Development, Tokyo) after determining the nucleotide sequence of the V3 region of the env gene. Reference sequences reported by Leitner et al. (9) were used.

Statistics. The Fisher exact test and the Mann-Whit-

ney test were used for analysis of statistical significance between categorical variables and between quantitative variables, respectively. A *P* value of 0.05 or less was considered significant.

Results

Prevalence of Drug-Resistant HIV-1 with a Major Mutation(s) in Therapy-Naive Patients

Genotypic drug-resistance testing was successful in all

Table 2. Drug-resistant amino acid mutations detected in therapy-naive patients

Year	Number of patients	Drug-resistant mutations[a]	
		PR region	RT region
1999	1	—	**M184V**
	1	A71T, V77I	—
	2	A71T	—
	7	V77I	—
	4	—	—
2000	1	**M46I**	—
	1	K20R, A71T, V77I	—
	1	A71T, V77I	—
	1	L10I	—
	2	M36I	—
	3	V77I	—
	8	—	—
2001	1	**L90M**	—
	1	—	**E44D**
	1	L10V, K20R, M36I	—
	1	K20R, M36I, V77I	—
	1	A431V[b], M36I	—
	1	L10V/I, M36I	—
	1	L10I, M36I	—
	1	M36I, V77I	—
	1	L10I	—
	7	M36I	—
	10	V77I	—
	17	—	—
2002	1	L10I, V77I	L41M, **E44D**, D67N, **V118I**, L210W, **T215Y**
	2	**M46I**, V77I	—
	4	A71V, V77I	**V118I**
	1	K20R, M36I, A71T, V77I	—
	1	M36I, A71T, V77I	—
	3	L10I, M36I	—
	1	K20R, M36I	—
	3	A71V, V77I	—
	1	A71T, V77I	—
	2	L10I	—
	6	M36I	—
	1	A71V	—
	5	V77I	—
	10	—	—

[a] Major mutations are shown in bold type. —, not detected.
[b] A431V is a gag cleavage site mutation.

Table 3. Characteristics of patients having HIV-1 with a major mutation.

Patient No.	Year[a]	Sex[b]	Age, years	Nationality	Risk factor for infection[c]	CD4 cell count (cells/μl)	Viral load (copies/ml)	Subtype	Drug-resistant mutations[d] PR region	RT region
88	1999	M	32	Ugandan	hetero	418	25,000	D	—	M184V
117	2000	M	22	Brazilian	homo	385	52,000	B	M46I	—
127	2001	M	36	Ugandan	hetero	199	72,000	A	M36I	E44D
132	01	M	30	Japanese	homo	98	98,500	B	L90M	—
179	2002	M	63	Japanese	bi	34	180,000	B	A71V, V77I	V118I
186	02	M	65	Japanese	hetero	186	26,000	B	A71V, V77I	V118I
194	02	M	25	Japanese	homo	479	12,000	B	M46I, V77I	—
197	02	M	48	Japanese	unknown	602	1,200	B	M46I, V77I	—
203	02	F	31	Russian	hetero	716	530	A	L10I, V77I	L41M, E44D, D67N, V118I, L210W, T215Y
212	02	M	56	Japanese	unknown	329	30,000	B	A71V, V77I	V118I
218	02	M	61	Japanese	homo	151	27,000	B	A71V, V77I	V118I

a) Year in which patients underwent initial consultation at Nagoya National Hospital.
b) M, male; F, female.
c) hetero, heterosexual; homo, homosexual; bi, bisexual.
d) Major mutations are shown in bold type. —, not detected.

116 patients (Table 2). In 1999, a drug-resistant HIV-1 with a major mutation was found in 1 of 15 patients (6.7%). The virus possessed an M184V mutation in the RT gene, which conferred a high degree of resistance to the RT inhibitors, abacavir, lamivudine, and zalcitabine. In 2000, a drug-resistant HIV-1 with a major mutation was found in 1 of 17 patients (5.9%). The virus possessed an M46I mutation in the PR gene, which conferred a high degree of resistance to the PR inhibitor, indinavir, and exhibited cross-resistance to the other PR inhibitors. In 2001, drug-resistant viruses with a major mutation were found in 2 of 43 patients (4.7%). In one case, a virus possessed an L90M mutation, which conferred a high degree of resistance to both the PR inhibitors, nelfinavir and saquinavir, and exhibited cross-resistance to the other PR inhibitors. In another case, a virus possessed an E44D mutation in the RT gene, which was a major mutation conferring resistance to the RT inhibitor, lamivudine. In 2002, drug-resistant viruses with one or more major mutations were found in 7 of 41 patients (17.1%). In four cases, viruses contained a V118I mutation in the RT gene, which was a major mutation conferring resistance to lamivudine. In two cases, viruses contained an M46I mutation in the PR gene. In the remaining case, the virus contained three major mutations in the RT gene, which were E44D and V118I mutations conferring resistance to lamivudine and a T215Y mutation conferring resistance to zidovudine.

The prevalence of drug-resistant HIV-1 with one or more major mutations significantly increased from 5.3% (4/75) in 1999–2001 to 17.1% (7/41) in 2002 (P=0.05).

Characteristics of Patients Having Drug-Resistant HIV-1 with a Major Mutation(s)

The characteristics of patients having drug-resistant HIV-1 with a major mutation(s) are shown in Table 3. From 1999 to 2001, the drug-resistant HIV-1 was more frequently found among patients of foreign nationality (15.8%, 3/19) than among Japanese patients (1.8%, 1/58) (P=0.05). However, drug-resistant HIV-1 with a major mutation was identified in 6 of 30 Japanese patients (20.0%) in 2002. The prevalence among Japanese patients significantly increased from 1.8% in 1999–2001 to 20.0% in 2002 (P=0.01). There was no significant correlation between the prevalence of drug-resistant HIV-1 and other factors, including age, sex, risk factor, CD4 cell count, viral load, or subtype.

Successful Response of Patient #132 to Drugs Selected Based on Genotypic Drug-Resistance Testing

We identified patient #132 as possessing a PR inhibitor-resistant HIV-1 containing the major mutation
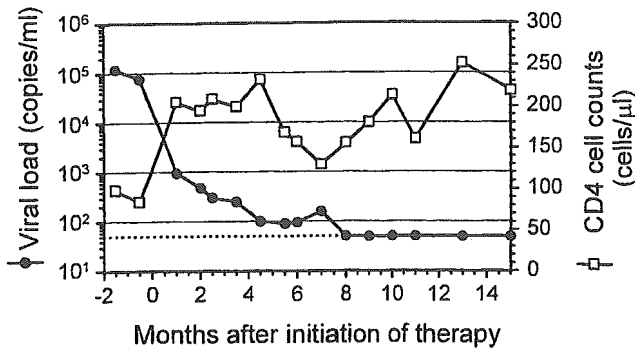
Fig. 1. Virological response of patient #132 to HAART with zidovudine, lamivudine, and efavirenz. Dotted line indicates the detectable level of viral load (50 copies/ml). Viral load below the detectable level is represented as 50 copies/ml.

L90M by genotypic testing before the initiation of therapy. Based on the results of the testing, we abandoned the administration of PR inhibitors and initiated HAART with three RT inhibitors; zidovudine, lamivudine, and efavirenz. The viral load of the patient sharply decreased from 7.7×10^4 to 9.6×10^2 copies/ml at the 1st month after the initiation of therapy, after which the viral load gradually decreased. When we performed drug-resistance testing 5.5 months after the initiation of therapy, the HIV-1 still had only the major L90M mutation in the PR gene. The viral load dipped below the detectable level at the 8th month, after which it continued to be well controlled below the detectable level at 15th months after the start of therapy. CD4 cells concomitantly recovered from <100 to >200 cells/μl in the course of the HAART (Fig. 1).

Discussion

Prevalence of Drug-Resistant HIV-1 with a Major Mutation(s) in Therapy-Naive Patients

We performed genotypic drug-resistance testing in 116 therapy-naive HIV-1-infected patients who underwent their first medical consultation at Nagoya National Hospital between 1999 and 2002. The prevalence of drug-resistant HIV-1 with a major mutation did not significantly change from 1999 to 2001; 6.7% in 1999, 5.9% in 2000, and 4.7% in 2001. Almost all patients who possessed drug-resistant HIV-1 with a major mutation were foreigners in Japan during this period. However, the prevalence dramatically increased to 17.1% in 2002, and importantly, 6 of the 7 patients having drug-resistant HIV-1 with a major mutation were Japanese patients. This result strongly suggests the rapid spread of drug resistant HIV-1 in Japan.

We correlated the prevalence of drug-resistant HIV-1 against the three classes of antiretroviral drug and com-

Table 4. Articles on the prevalence of drug-resistant HIV-1 with a major mutation(s) in therapy-naive patients

References	Country	Duration of sampling	Sample number	Prevalence of HIV-1 with a major mutation(s) (%)[a]			
				NRTIs	NNRTIs	PIs	Any drug
Yerly et al. (18)	Switzerland	1996.1–1998.7	70 (PR), 82 (RT)	8.5	2.4	4.3	8.5–9.8
Boden et al. (2)	U.S.A.	1995.7–1999.4	80	10.0	3.8	2.5	12.5
Tamalet et al. (17)	France	1995–1998	48	16.7	0.0	2.1	16.7
Perno et al. (13)	Italy	1997.3–1999.5	347	3.2–4.6	0.3–0.6	0.3	3.2–5.5
Little et al. (10)	U.S.A. and Canada	1995.5–1998.12	213 (PR), 176 (RT)	8.5[b]]P=0.09	1.7]P=0.03	0.9]P=0.001	8.0[b]]P<0.001
		1999.1–2000.6	88 (PR), 82 (RT)	15.9[b]	7.3	9.1	22.7[b]
Present study	Japan	1999.1–2001.12	75	2.7]P=0.02	0.0	2.7]P=0.61	5.3]P=0.05
		2002.1–2002.12	41	12.2	0.0	4.9	17.1

[a] Prevalence was estimated according to the criteria of drug-resistance mutations used in this study. NRTIs, nucleoside RT inhibitors; NNRTIs, non-nucleoside RT inhibitors; PIs, PR inhibitors.
[b] T215D/S/E were included as major drug-resistant mutations.

pared them with previous reports from European and North American countries (Table 4). The prevalence of nucleoside RT inhibitor (NRTI)-resistant HIV-1 significantly increased in 2002. The prevalence in Japan of each drug-resistant HIV-1 resistant to NRTIs or PR inhibitors (PIs) reached the levels of the European and North American countries by 2002.

Minor Mutations Detected in the PR and Gag Genes of HIV-1

The drug-resistant minor amino acid mutations, L10I/V, K20R, M36I, A71V/T, and V77I, were frequently found in the PR gene. As these minor mutations have also been found in therapy-naive patients at high frequency in other studies (13, 18), these mutations are considered to be polymorphisms in the PR gene. It is still unclear whether the accumulation of minor mutations is related to the failure of HAART containing PR inhibitors (1, 3, 14). A p7/p1 gag cleavage site mutation of A431V, which is related to PR inhibitor-resistance, was found in one patient in 2001. To our knowledge, this is the first report of HIV-1 having a drug-resistant amino acid mutation within the gag cleavage site in therapy-naive patients.

Usefulness of Drug-Resistance Testing before the Initiation of Therapy

As patient #132 possessed HIV-1 with the major and cross-resistant L90M mutation in the PR gene, we abandoned the administration of PR inhibitors and designed HAART using three RT inhibitors; zidovudine, lamivudine, and efavirenz. The treatment succeeded in strongly suppressing the replication of the drug-resistant HIV-1 and in maintaining the viral load of the patient below the detectable level. The CD4 cells concomitantly recovered from <100 to >200 cells/μl during the therapy. Thus, the therapy of this patient was optimized by utilizing drug-resistance testing prior to the initiation of therapy. A recent retrospective study reported that the time to viral suppression after the initiation of therapy was significantly longer among therapy-naive patients having HIV-1 with a major mutation than among those having viruses with no major mutation (10). This also suggests the importance of selecting an effective therapy according to drug-resistance testing before therapy begins.

Controversy exists concerning the implementation of drug-resistance testing before the initiation of therapy, with some reports recommended testing (15, 18), while others do not since the prevalence of drug-resistant variants with major mutations is low (4, 7). A recent economic study reported that genotypic testing before therapy initiation is cost-effective when the prevalence of drug-resistant HIV-1 with a major mutation among ther-

apy-naive patients is more than 4% (Sax et al., abstract #3129 in XIV International AIDS Conference 2002). If the prevalence at our hospital is maintained at more than 10% in the future, genotypic drug-resistance testing before the initiation of therapy will have merit from the viewpoint of economics. Therefore, we plan to continue testing of therapy-naive patients for surveillance of the prevalence of drug-resistant HIV-1. .

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Prevalence of Infection and Genotypes of GBV-C/HGV among Homosexual Men

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Abstract: Since the discovery of GB virus-C (GBV-C) and hepatitis G virus (HGV), many studies have been performed. These viruses are now known to be parenterally, as well as sexually transmitted. A phylogenetic analysis also revealed that GBV-C has five major genotypes: type 1 predominates in West Africa, type 2 in Europe and the United States, type 3 in parts of Asia, type 4 in Southeast Asia, and type 5 in South Africa. Despite the number of reports so far, there have been few large-scale surveys of homosexual men to determine the prevalence of the GBV-C/HGV infections. We examined the levels of GBV-C/HGV viremia in 297 homosexual men who attended the Nagoya Lesbian and Gay Revolution held in Nagoya, Japan. Reverse transcription-polymerase chain reaction (RT-PCR)/nested PCR of the GBV-C/HGV 5'-non-coding region (NCR), and base sequence analyses showed that the infection rate was 12.5%, and genotypes in this population were classified into type 2 (32%) and type 3 (68%). None were classified as types 1, 4, or 5 in this study. Our results indicate that the GBV-C/HGV type 2 seen mainly in Europe and the US is spreading widely in Japan, especially in the Nagoya district.

Key words: GBV-C/HGV, Homosexual men, Prevalence, Genotype

Hepatitis virus types A, B, C, D, and E are known to induce hepatitis. In addition to these viruses, GB virus was identified as a hepatitis-causing agent in tamarins in 1967 from the serum of a surgeon (whose initials were GB) exhibiting symptoms of jaundice (4). Simons et al. (19) found two exogenous, distinct RNA molecules in the plasma of a tamarin infected with GB agent, and named them GB virus type A (GBV-A) and type B (GBV-B). Following that experiment, another virus with 50–60% nucleotide homology was isolated from the serum of a West African individual, and was named GBV-C (18).

Meanwhile, hepatitis G virus (HGV) was isolated from the serum of a patient who had chronic non-A, non-B hepatitis by Linnen et al. (10) in 1996. HGV has homology with GBV-C of over 95% at the nucleotide level and over 86% at the amino acid level. GBV-C and HGV are now considered to be different isolates of the same virus (12, 26). GBV-C/HGV can be classified into five major genotypes, types 1 through 5 (11, 17, 23). The genotypes tend to follow geographical distrib-

ution patterns (5, 12–14, 24): type 1 (or GB type) predominates in West Africa, type 2 (or HG type) in Europe and the United States, type 3 in parts of Asia, type 4 in Southeast Asia, and type 5 in South Africa.

GBV-C/HGV is transmitted parenterally (10, 26). In fact, although the rate of infection is 1–4% among blood donors in developed countries (1, 6, 7, 20), blood transfusion recipients, hemophiliacs, and intravenous drug users (IVDU) have higher rates at 12–18%, 14–18%, and 33%, respectively (9, 10, 15, 20). In addition, there have been a few reports on the prevalence of infection among prostitutes and homosexual men, which are also higher than blood donors at 18% (16), and 12–17% (9, 16), respectively. These data suggest that GBV-C/HGV can be transmitted sexually.

There have been few large-scale studies on the prevalence of GBV-C/HGV infection among homosexual men without HIV-1 infection. In this study, we examined the rate and level of GBV-C/HGV viremia among 297 homosexuals, and also identified the GBV-C/HGV geno-

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types that are present in Japan.

Materials and Methods

Subjects. Homosexual men ($n=297$) who attended the "Nagoya Lesbian and Gay Revolution: NLGR 2002" held in Nagoya, Japan, in June 2002, and from whom informed consent was obtained, were enrolled in the study.

Purification of RNA and RT-PCR. Blood from each individual was drawn into a vacuum tube with EDTA, and plasma was separated by centrifugation and stored at -80 C until analyzed. RNA was isolated from the thawed plasma samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo). RNA was converted to cDNA using the Superscript One-Step RT-PCR (reverse transcription-polymerase chain reaction) for long templates (Gibco BRL Life Technologies, Md., U.S.A.) at 55 C for 30 min. Subsequently, cDNA was amplified using a denaturation step at 94 C for 2 min, followed by 35 cycles of denaturing at 94 C for 15 sec, annealing at 53 C for 30 sec, and elongating at.68 C for 30 sec. RT-PCR products were further amplified by nested PCR (94 C for 2 min, followed by 35 cycles of 94 C for 15 sec, 57 C for 30 sec, and 70 C for 30 sec) using TaKaRa LA Taq (Takara Shuzo, Tokyo). The primers used in the RT-PCR/nested PCR were as follows: GBV-C1 (antisense), 5'-ATGCCACCCGCCCTCACCCGAA-3'; GBV-C2 (sense), 5'-AAAGGTGGTGGATGGGTGATG-3'; GBV-C3 (antisense), 5'-CCCCACTGGTCYTTGYCAACTC-3'; GBV-C4 (sense), 5'-AATCCCGGTCAYAYTG-GTAGCCACT-3'(22).

Identification of the PCR products and determination of the base sequences. PCR products were electrophoresed on a 1.2% agarose gel. The 252-base pair (bp) PCR products judged to represent a portion of the GBV-C/HGV were used as positive controls. PCR products of approximately 250 bp in size were extracted from the gel using a QIAquick Gel Extraction kit (QIAGEN, Tokyo), and labeled using a BigDye terminator cycle sequencing ready reaction kit (ABI, Tokyo). The base sequences were determined using an ABI PRISM 310 Genetic Analyzer. BLAST searches of the NCBI (National Center for Biotechnology Information) database were used to detect any sequences other than that of GBV-C/HGV.

Genotypic and phylogenetic analyses. GENETYX-MAC version 10.1 was used to perform the genotypic and phylogenetic analyses. Reference sequences of GBV-C/HGV type 1 (accession #U36380), type 2 (#U44402), type 3 (#D90601), type 4 (#AB013188), and type 5 (#AF131112) in the GenBank of NCBI were used for the homology comparisons. In the same com-

puter program, all the determined sequences were aligned, and a phylogenetic tree was generated using the UPGMA method.

The sequences of the GBV-C/HGV-positive samples were deposited in the DNA Data Bank of Japan (accession numbers AB102905 through AB102941).

Quantification of GBV-C/HGV load. Total RNA from each individual was extracted from 200 μl plasma samples using Trizol LS Reagent (Gibco BRL Life Technologies) according to the manufacturer's instructions. RNA samples were dissolved in 10 μl of DEPC-treated water, and 5 μl of the extracted RNA samples were incubated with SuperScript II RNase H⁻ Reverse Transcriptase at 42 C for 50 min in RT reactions (Invitrogen, Tokyo). Subsequently, the GBV-C/HGV levels were quantified, in duplicate, by adding 2 μl of the 20 μl reaction mixture to a TaqMan PCR assay in a LightCycler Quick System 350S (Roche Diagnostics, Tokyo).

A primer/probe set was designed from the conserved region of the base sequences of 37 samples obtained as described above: primers, GBV-C F, 5'-ACGGTC-CACAGGTGTTG-3' and GBV-C R, 5'-GACATTGAA-GGGCGACG-3'; and the GBV-C TaqMan Probe, 5'-FAM-CCGACGTCAGGCTCGTCGTTAAAC-TAMRA-3'.

A 252-bp PCR product of GBV-C/HGV obtained from the RT-PCR/nested PCR was cloned into the pT7Blue-2 plasmid. A standard curve was generated by using the serial tenfold dilution of the 10^8 to 10^1 copies of the plasmid.

Detection of anti-HIV-1 antibody. Anti-HIV-1 antibody was detected using a LAV BLOT 1 kit according to the manufacturer's instructions (BioRad, Tokyo).

Results

RNA was purified from 297 plasma samples, and a portion of the 5'-non coding region (NCR) of the GBV-C/HGV genome was amplified by the RT-PCR/nested PCR method. PCR products of approximately 250-bp were tentatively judged as being related to GBV-C/HGV. These products were detected in 37 of the 297 samples; the incidence rate was 12.5%. All 37 PCR products were confirmed to have the GBV-C/HGV base sequences by BLAST, indicating that there were no false positive PCR products in this study.

Each of the 37 sequences was compared with the five GBV-C/HGV genotype reference sequences. Twenty-five samples were shown to have high homology (94.5–98.0%) with type 3, while the other 12 samples had high homology (94.5–98.5%) with type 2, and none were classified as types 1, 4, or 5 (Table 1). A phylogenetic tree generated using the UPGMA method also

Table 1. Homologies (%) between sequences of GBV-C/HGV-positive individuals and reference sequences of genotypes 1-5

indiv. #	Type 1	Type 2	Type 3	Type 4	Type 5
H004-02	85.4	97.5	90.5	90.5	88.4
H006-02	88.9	93.0	96.0	92.5	91.5
H013-02	85.8	97.0	89.3	89.8	88.8
H018-02	86.4	98.0	90.5	90.5	88.9
H021-02	87.4	90.5	94.5	91.5	90.0
H050-02	85.4	96.5	88.9	89.4	89.4
H052-02	86.4	98.5	92.5	93.5	90.5
H053-02	88.4	92.3	97.1	92.5	90.5
H066-02	85.9	98.0	89.9	91.0	88.9
H073-02	89.9	92.0	95.5	93.0	92.5
H090-02	86.6	91.1	96.6	92.7	88.8
H097-02	89.4	91.5	96.5	91.1	90.5
H098-02	87.4	91.5	97.0	93.0	90.0
H109-02	87.9	92.0	98.0	93.0	90.5
H110-02	87.9	92.0	98.0	93.0	90.5
H123-02	88.9	93.0	98.0	93.0	91.0
H125-02	85.4	94.5	91.0	91.0	88.4
H138-02	87.9	92.0	95.5	93.0	90.0
H145-02	87.4	91.5	95.5	93.0	89.4
H152-02	84.4	96.5	89.9	92.5	88.9
H160-02	87.4	91.5	96.5	92.5	89.4
H162-02	87.4	91.5	97.5	92.5	90.0
H172-02	87.9	92.0	96.0	93.0	90.0
H182-02	87.4	91.5	96.5	92.5	89.4
H183-02	88.4	89.9	96.0	90.0	91.5
H190-02	85.4	96.5	88.9	89.4	89.4
H197-02	88.4	90.5	97.5	91.5	90.5
H199-02	87.4	91.5	97.5	92.5	89.4
H201-02	86.9	90.7	97.1	93.0	88.9
H232-02	85.9	98.0	91.0	91.0	88.9
H252-02	87.9	92.5	98.0	92.0	90.0
H255-02	87.4	91.6	96.9	93.2	89.5
H261-02	87.4	98.5	91.0	91.5	90.5
H262-02	87.9	92.0	98.0	92.0	90.0
H271-02	89.9	91.5	95.0	91.5	92.0
H291-02	88.4	92.3	97.1	93.5	90.5
H296-02	86.4	95.5	92.0	91.0	88.4

The highest homologies of the five genotypes are shaded.

Fig. 1. Phylogenetic tree of GBV-C/HGV-positive individuals. The reference sequences of the five genotypes are shown in bold.

shows the grouping of 12 individuals into type 2 and 25 individuals into type 3 (Fig. 1). This result agreed completely with that of the homology analysis.

The GBV-C/HGV level in each of the 37 virus-positive individuals was quantified except for one case where the plasma sample was unavailable. The distribution of the viral loads among genotypes 2 and 3 is shown in Fig. 2. The GBV-C/HGV levels ranged from 7.0×10^2 to 1.35×10^6 copies/ml (median: 1.24×10^5) for genotype 2, and 9.2×10^2 to 2.17×10^6 copies/ml (median: 3.12×10^5) for genotype 3. While the GBV-C/HGV level of genotype 3 seemed higher than that of genotype 2, the difference was not statistically significant ($P=0.08$) in this study. The average viral load for all the GBV-C/HGV-positive samples (4.06×10^5 copies/ml) corresponded to that reported for patients with immune deficiency (8).

Concerning HIV-1 prevalence, seven (2.4%) of 297 individuals were HIV-1-positive. Among the seven
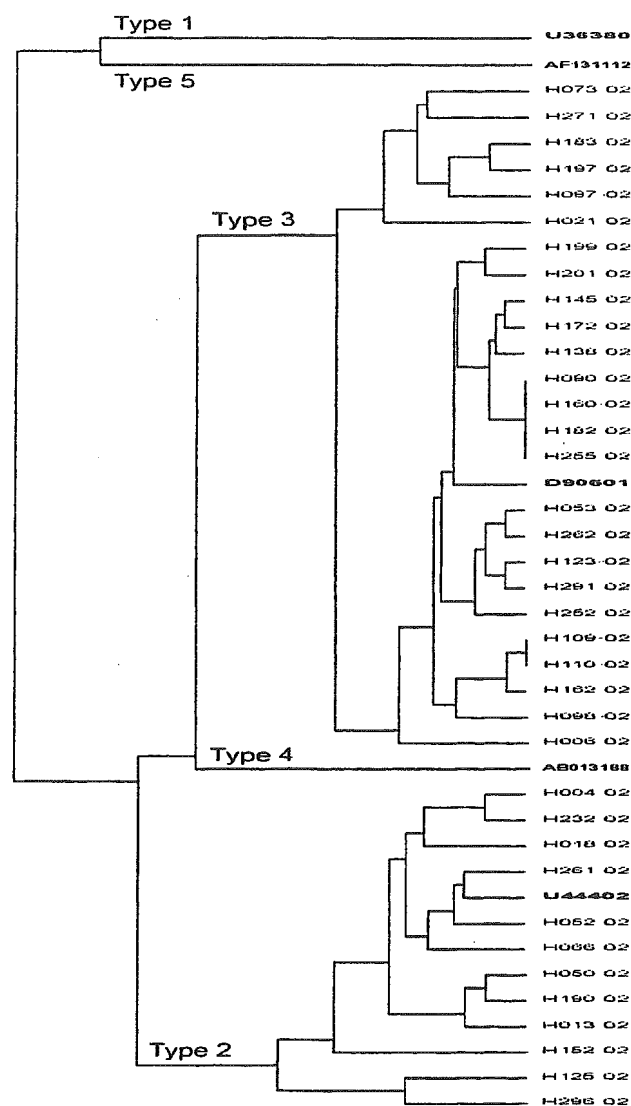
HIV-1-infected individuals, two (28.6%) were co-infected with GBV-C/HGV and had GBV-C/HGV loads of 3.17×10^5 and 8.4×10^3 copies/ml. The prevalence of GBV-C/HGV among HIV-1-negative individuals was 12.1% (35/290).

Discussion

The aim of this study was to determine the prevalence of infection and viral load of GBV-C/HGV, and the distribution of GBV-C/HGV genotypes among homosexual men residing in Japan. A total of 297 homosexual men who participated in the "Nagoya Lesbian and Gay Revolution: NLGR 2002" held in Nagoya, Japan, in June 2002, gave informed consent and participated in this study. PCR-based GBV-C/HGV viral detection tests
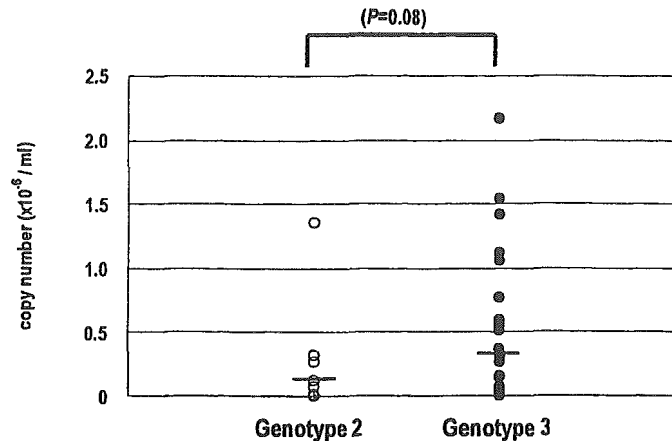
Fig. 2. Distribution of GBV-C/HGV viral load in individuals with genotypes 2 and 3. The
bars represent the median viral load for each genotype.

showed that 37 subjects out of 297 were positive. Sub-
sequent base sequence analyses confirmed that all the
positive PCR products contained GBV-C/HGV-specific
sequences. The prevalence of GBV-C/HGV infection
was 12.5%. Since this type of study among homosexu-
al men has never been performed in Japan, data obtained
in this study cannot be directly compared. While the
infection rate among this group is similar to those
(12–17%) reported in other countries (9, 16), the rate is
extremely high (approximately 10 times) compared to
that of blood donors (1–4%, 1, 6, 7, 20). This result also
suggests that GBV-C/HGV is sexually transmitted, and
sexual contact is included among the routes of GBV-
C/HGV transmission.

GBV-C/HGV genotypic analyses showed that of the
37 positive cases, 25 (68%) were classified as type 3,
common to parts of Asia, and the remaining 12 (32%)
were type 2, seen in Europe and the US. None were clas-
sified as types 1, 4, or 5. There have been reports that
GBV-C/HGV types 1 and 2 are rarely detected in Japan,
and only in low frequency (1, 11, 21). However, accord-
ing to this study, GBV-C/HGV type 2 was detected in a
significant portion (32%) of the GBV-C/HGV-infected
individuals, suggesting that type 2 is widely spreading in
Japan, especially in the Nagoya district. The GBV-
C/HGV levels in individuals with genotypes 2 and 3
were measured. Although the viral load of genotype 3
seemed higher than that of genotype 2, the difference was
not statistically significant (Fig. 2).

Although GBV-C/HGV was characterized as an agent
that induces hepatitis at the time of its discovery, recent
studies have reported that there is no pathogenicity (2, 3).
However, GBV-C/HGV has been of interest from a dif-
ferent point of view; individuals infected with both
GBV-C/HGV and HIV-1 tend to have later onset to
AIDS as compared to GBV-C/HGV-uninfected HIV-1-

infected patients (22, 25).

The prevalence of GBV-C/HGV among HIV-1-posi-
tive individuals was 28.6% (2/7), while that among HIV-
1-negative individuals was 12.1% (35/290). The differ-
ence between these prevalence rates was not statistical-
ly significant ($P=0.21$). However, this suggests that
HIV-1 infection tends to increase the risk of GBV-
C/HGV infection. As studies to clarify the clinical sig-
nificance of GBV-C/HGV superinfection in HIV-1-
infected patients are important, we are now engaged in
such activities.

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