Jpn. J. Infect. Dis., 59, 122-125, 2006

Short Communication

Analysis of a Long-Term Discrepancy in Drug-Targeted Genes in Plasma HIV-1 RNA and PBMC HIV-1 DNA in the Same Patient

Shuzo Usuku*, Yuzo Noguchi, Mitsuo Sakamoto[1], Takuya Adachi[2], Hiroko Sagara[2], Koji Sudo[3], Masako Nishizawa[4], Makiko Kondo[3], Osamu Tochikubo[5] and Mitsunobu Imai[3]

Department of Testing and Research, Yokohama City Institute of Health, Kanagawa 235-0012;
[1]The Jikei University School of Medicine, Tokyo 105-8461;
[2]Department of Infectious Diseases, Yokohama Municipal Citizen's Hospital, Kanagawa 240-8555;
[3]Microbiology Division, Kanagawa Prefectural Institute of Public Health, Kanagawa 253-0087;
[4]AIDS Research Center, National Institute of Infectious Diseases, Tokyo 208-0011; and
[5]Yokohama City University School of Medicine, Kanagawa 236-0004, Japan

(Received March 14, 2005. Accepted January 30, 2006)

SUMMARY: Drug-resistance genotypes were investigated in a patient under treatment with anti-HIV drugs. Since the drug resistance-associated mutations in plasma HIV-1 RNA and proviral DNA in peripheral blood mononuclear cells (PBMCs) were inconsistent, changes were followed over time, and the discrepancy was shown to persist for a long period. In plasma HIV-1 RNA, D67N, K70R, T215Y, and Y188L were present in the reverse transcriptase (RT) region, and two primary mutations, I84V and L90M, were noted in the protease (Pro) region. In contrast, in proviral DNA, no drug resistance-associated mutations were found in the RT region, and mutations such as L90L/M were only infrequently present in the Pro region. This situation persisted for more than 3 years. In addition, sequencing analysis of the V3 loop in the envelope gene showed that non-syncytium-inducing/macrophage-tropic viruses contribute to acquisition of drug resistance. In this study, drug-resistant viruses were produced primarily at macrophages, and drug-sensitive viruses were maintained in PBMCs as a reservoir.

Smith et al. noted a discrepancy between peripheral blood mononuclear cell (PBMC) proviral DNA and HIV-1 RNA isolated from plasma by culture in an AIDS patient under zidovudine (AZT) monotherapy (1), and Kaye et al. reported detecting AZT resistance mutations in plasma HIV-1 RNA earlier than in PBMC proviral DNA in patients under AZT monotherapy (2). After detection of drug-resistance mutations in plasma HIV-1 RNA, 25 days were required, on average, to detect the same mutations in proviral DNA (2). There have been several reports of a transient discrepancy in resistance mutations between different test specimens (3-7), but the mechanism and frequency of such discrepancies are unclear. In the patient we encountered, drug-resistance mutations occurred primarily in plasma HIV-1 RNA during highly active antiretroviral therapy (HAART) given for 6 years, exhibiting a discrepancy from resistance mutations in PBMC proviral DNA. Moreover, this discrepancy persisted for more than 3 years. Thus, we retrospectively analyzed the gene mutations associated with drug resistance. In addition, we analyzed the sequences of the V3 loop in the envelope (env) gene to identify cell tropism.

The objective of this study was to analyze anti-HIV drug-resistance mutations in plasma HIV-1 RNA and PBMC proviral DNA by genotypic assay in a patient showing mutational discrepancies, in order to clarify the relationship between the test results in different specimens.

Sequential blood was used from a patient infected with HIV-1 through homosexual activity. Anti-HIV therapy was initiated in November 1997, and the anti-HIV drugs administered are shown in Table 1. Informed consent for performance of the study was obtained from the patient.

Plasma was separated by centrifugation from 5 ml of blood from the HIV-1-infected patient, and PBMCs were isolated by the Ficoll method and stored at −80°C. Plasma HIV-1 RNA and PBMC proviral DNA were extracted. When viral isolation was possible, culture supernatant was used for extraction. For amplification of gene fragments of the reverse transcriptase (RT), protease (Pro), and gp120 V3 regions, RT-nested PCR was used for culture supernatant and plasma HIV-1 RNA, and nested PCR was used for PBMC proviral DNA.

PBMCs were isolated by the Ficoll method from 20 ml of blood collected from the HIV-1-infected patient at designated times C (February 2002) and D (February 2003). Using anti-CD8 antibody-conjugated beads (Dynabeads M-450 CD8), $CD8^+$ cells were removed from PBMCs from a healthy subject and the patient, and 5×10^6 PBMCs/5 ml from each were mixed and co-cultured for 3 days in the presence of anti-CD3 antibody to enhance the T-cell response (8). P24 antigen in the supernatant of the passage culture was measured to confirm replication of HIV.

The base sequences were determined by direct sequencing of the PCR products of plasma HIV-1 RNA, PBMC proviral DNA, and cloned colonies. A BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif., USA) was used for the cycle sequence reaction, and sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Amplicons (326 bp) of the RT region and amplicons (165 bp) of the gp120 V3 region were used for phylogenetic tree analysis. Nucleotide and amino acid sequences were aligned using Genetyx Mac software version 10.1.1 (Software Development Co.,

*Corresponding author: Mailing address: Yokohama City Institute of Health, Takigashira 1-2-17, Isogo-ku, Yokohama City, Kanagawa 235-0012, Japan. Tel: +81-45-754-9804, Fax: +81-45-754-2210, E-mail: sh00-usuku@city.yokohama.jp

Tokyo, Japan). Multiple alignments were performed with CLUSTALW (9). The phylogenetic tree was constructed by the neighbor-joining (NJ) method based on Kimura's two-parameter distance matrix with 1,000 bootstrap replicates. The trees were visualized using TREEVIEW (10). The base sequences obtained for the RT and Pro region were analyzed by a beta test program provided by Stanford University Medical Center (available online at http://hivdb6.stanford.edu), and drug resistance-associated mutations were identified.

HIV-1 env sequences corresponding to the gp120 V3 loop were used to predict syncytium-inducing (SI) and non-syncytium-inducing (NSI) genotypes, based on the presence of basic or acidic residues at positions 11 and 25 of the V3 loop (11,12).

Codons related to drug resistance in the Pro and RT regions of plasma HIV-1 RNA and PBMC proviral DNA and viral isolates were analyzed in an AIDS patient undergoing treatment with HAART (Table 1). There were no major drug-resistance mutations in plasma HIV-1 RNA and PBMC proviral DNA before initiation of antiretroviral therapy in September 1997. However, we subsequently observed discrepancies in the genotypes associated with drug resistance, especially after July 1999. At time A (March 2000), four drug resistance-associated mutations, D67N, K70R, Y188L, and T215Y, were detected in the RT region, and two primary mutations, I84V and L90M, were detected in the Pro region in plasma HIV-1 RNA. In contrast, no mutations were detected in PBMC proviral DNA. At time B (April 2001), D67N, K70R, Y188L, and T215Y were detected in the RT region, and M46L, I84V, and L90M were detected in the Pro region in plasma HIV-1 RNA, while only L90L/M in the Pro region was detected as a resistance-associated mutation in PBMC proviral DNA. The ratios of clones containing resistance mutations at times A and B were compared using the cloning method, and these data are summarized in Table 2. At times A and B, D67N, K70R, Y188L, and T215Y were detected in all the plasma HIV-1 RNA clones, but not in the PBMC proviral DNA clones. In contrast, for DNA, 9 of the 13 clones and 8 of the 14 clones contained no resistance mutations at times A and B, respectively. In addition, some DNA clones contained multiple resistance-related mutations: T215Y, D67N + K70R; and D67N + K70R + T215Y. No DNA clones contained the RT-region mutation Y188L, which is related to resistance to non-nucleic acid RT inhibitors, at either time A or time B. Since efavirenz (EFV) therapy was initiated 4 months before time A, and all plasma HIV-1 RNA clones contained Y188L, PBMC proviral DNA clones containing resistance mutations were not actively replicating viral clones, but instead clones accumulated due to resistance mutations that had occurred in the past.

Isolates at times C (February 2002) and D (February 2003) were obtained by a high-sensitivity culture method. In the genotypic assay for times C and D, L210W at time C, and L210W, M41L, and M184V/I at time D in the RT region were added in plasma HIV-1 RNA as drug-resistance gene mutations compared to the results of time B. The genotypic results at times C and D were inconsistent between plasma HIV-1 RNA and PBMC proviral DNA, as were the results at times A and B, and drug-resistance gene mutations in the viral strains obtained by isolation culture were similar to those in PBMC proviral DNA, except for the Pro region at time D (Table 1).

A phylogenetic tree for the RT and gp120 V3 region at time A revealed that plasma HIV-1 RNA clones were descendants of PBMC proviral DNA (Fig. 1): one DNA clone in the gp120 V3 region (Fig. 1B) had the same sequence as that of RNA clones.

Deduced amino acid sequences for the V3 loop were compared between the plasma HIV-1 RNA and PBMC proviral DNA clones at time A (Fig. 1). The basic amino acid lysine (K) at positions 25 of the V3 loop associated with the SI phenotype were found in DNA clones (11,12). All RNA clones were of the NSI genotype, while 7 of the 10 DNA clones were of the SI genotype, with the basic amino acid lysine (K)

Table 1. Resistance mutations in the RT gene and major resistance mutations in the protease gene obtained by direct sequencing[1)]

	Date	Plasma RNA (copies/ml)	Plasma HIV-1 RNA		PBMC proviral DNA		Viral isolate		Antiviral medications		
			RT	Protease	RT	Protease	RT	Protease	NRTIs	NNRTIs	PIs
	Sep-97	1.9×10^5	(–)	(–)	(–)	(–)	NT	NT			
	Aug-98	8.4×10^4	D67N, K70R, T215Y	D30N	T215Y/T	D30N	NT	NT	AZT, ddI		NFV
	Jul-99	NT	D67N, K70R, T215F/Y	(–)	(–)	(–)	NT	NT	d4T, ddI		RTV, SQV
	Oct-99	NT	D67N, K70R, T215Y	L90M	(–)	L90M	NT	NT	d4T, ddI		RTV,SQV
	Dec-99	1.8×10^3	D67N, K70R, T215Y	I84V, L90M	(–)	(–)	NT	NT	d4T, ddI	EFV	APV
A	Mar-00	3.1×10^4	D67N, K70R, Y188L, T215Y	I84V, L90M	(–)	(–)	NT	NT	d4T, ddI	EFV	APV
	Dec-00	4.1×10^4	D67N, K70R, Y188L, T215Y	I84V, L90M	(–)	(–)	NT	NT	d4T, ddI		APV
	Feb-01	1.3×10^4	D67N, K70R, Y188L, T215Y	I84V, L90M	(–)	(–)	NT	NT	d4T, ddI		APV
B	Apr-01	2.0×10^4	D67N, K70R, Y188L, T215Y	M46L, I84V, L90M	(–)	L90L/M	NT	NT	d4T, ddI		APV
	Sep-01	2.8×10^4	D67N, K70R, Y188L, T215Y	M46L, I84V, L90M	(–)	M46M/L, L90M/L	NT	NT	d4T, ddI		APV
C	Feb-02	3.7×10^4	D67N, K70R, Y188L, L210W ,T215Y	M46L, I84V, L90M	(–)	I84V/I, L90M/L	(–)	I84V/I, L90M/L	d4T, ddI		APV
D	Feb-03	5.0×10^3	M41L, D67N, K70R, M184V/I, Y188L, L210W, T215Y	M46I, I84V, L90M	(–)	M46M/L, I84I/V, L90L/M	(–)	(–)	3TC, ddI		LPV/RTV

[1)]: A major mutation was defined based on reference (17).
NT, not tested; RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. (–): No drug-resistant mutations were detected.

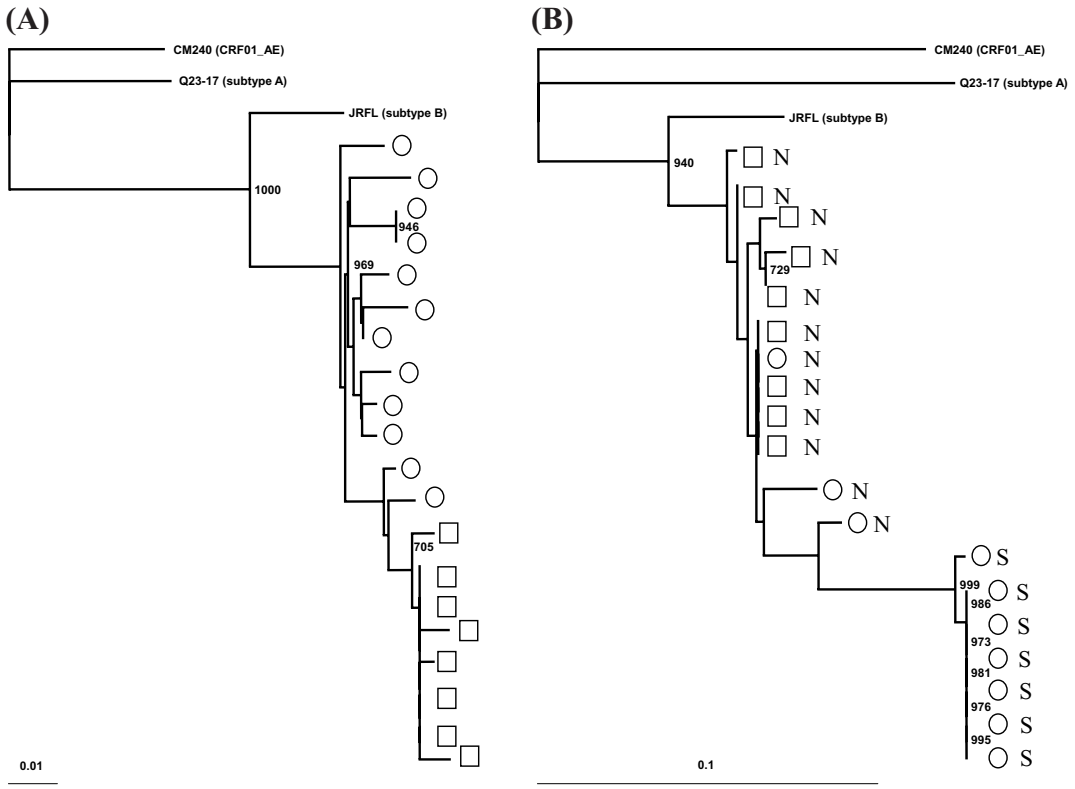

Fig.1. Neighbour-joining phylogenetic tree depicting the relationship of the nucleotide sequences of partial reverse transcriptase (A) and gp120 V3 (B) clones in plasma HIV RNA and PBMC proviral DNA at time A (Mar-2000). Branch lengths are drawn to scale, and bootstrap values greater than 70% are shown at the nodes for 1,000 replicates. S or N indicates the SI or NSI genotype, respectively. The reference sequence CM240 was used as an outgroup. GenBank accession numbers are shown below. CM240 (U54771), Q23-17 (AF004885), JRFL (U63632). PBMC proviral DNA, ○; Plasma RNA, □.

Table 2. Frequencies of drug-resistance mutations of the RT region in plasma HIV-1 RNA and PBMC proviral DNA

Resistant mutations of RT	Time A (Mar-00)		Time B (Apr-01)	
	Plasma RNA	PBMC DNA	Plasma RNA	PBMC DNA
(–)	0	9	0	8
T215Y	0	2	0	1
D67N, K70R	0	1	0	3
D67N, K70R, T215Y	0	1	0	2
D67N, K70R, Y188L, T215Y	11	0	12	0
Total	11	13	12	14

RT, reverse transcriptase. (–): No drug-resistant mutation was detected.

at position 25 in the V3 loop. The other 3 clones were of the NSI genotype.

We identified a unique case of HIV-1 infection that showed discrepancies in drug-resistance mutations between plasma HIV-RNA and PBMC proviral DNA for more than 3 years. Multiple drug-resistant viral clones were observed in all of plasma HIV-RNA clones at time A, and the genotype in the RT region was shown to be notably discordant with that of PBMC proviral DNA by cloning and sequencing (Table 1, 2). Furthermore, deduced amino acid sequences in the V3 loop showed that all the plasma HIV-RNA clones analyzed at time A had NSI genotypes, whereas SI genotypes were predominant in the PBMC proviral DNA clones (Fig. 1). These findings indicate that an actively replicating virus with drug resistance in plasma has distinct sequence properties from those of PBMC proviral DNA, and that an NSI/macrophage-tropic virus can contribute to acquisition of drug resistance. Zhang et al. (13) studied env region sequences prior to and during the course of AZT therapy, and demonstrated that different HIV substrains coexist and evolve independently within an individual. Our results are consistent with this observation.

On the other hand, we cannot rule out the possibility that resistant viruses in plasma that replicate and flow into the circulation in patients under drug therapy are derived not from the PBMC provirus population but from other infected cell populations. The cell population associated with acquisition of drug resistance is unclear, but the present results indicate that HIV-1 infected macrophages may be the cell population that acquires drug resistance under antiretroviral therapy because the NSI/macrophage-tropic genotype is a major population in plasma HIV-RNA.

The genotypes of viral isolates were found to be similar to those of PBMCs in genotypic assays performed at time C and D (Table 1). This finding indicates the presence of drug selection-unloaded infectious viruses in PBMCs. Our results are congruent with those reported by Ruff et al. (14), who observed the dynamic characteristics of replication-competent viruses isolated from resting memory $CD4^+$ T cells that developed HIV-1-drug resistance following HAART, and demonstrated that archival wild-type HIV-1 persists in resting $CD4^+$ T cells free of selective pressure from antiretroviral drugs. Several studies have indicated the existence of long-lived reservoirs, composed primarily of resting memory $CD4^+$ T cells, and recovered viruses do not show mutations associated with resistance to the relevant antiretroviral drugs (8, 14,15). In addition, Wang et al. recently found that IL-7 is a more effective enhancer of the HIV-1 provirus than IL-2, or a

combination of IL-2 with phytohemagglutinin (PHA), in CD8-depleted PBMCs; they showed by phylogenetic analysis that distinct proviral quasi-species were activated by IL-7, compared with those activated by IL-2 and PHA (16). Our culture technique might have activated resting $CD4^+$ T cells or another population activated by a cytokine such as IL-7. Although further studies are needed to clarify which population of proviruses in PBMCs was obtained in our culture technique, the results suggest that the recovered viruses in this study are archival drug-sensitive replicates and have the potential to serve as a stable viral reservoir. It is particularly striking that these reservoirs appear to be in major compartments in PBMCs, and are maintained for a long period.

ACKNOWLEDGMENTS

We thank Dr. Singo Kato, Keio University, for providing valuable assistance with the viral cell culture techniques.

This study was partially supported by a grant for AIDS study groups, sponsored by the Ministry of Health, Labour and Welfare, Japan.

REFERENCES

1. Smith, M. S., Koerber, K. L. and Pagono, J. S. (1993): Zidovudine-resistant human immunodeficiency virus type 1 genomes detected in plasma distinct from viral genomes in peripheral blood mononuclear cells. J. Infect. Dis., 167, 445-448.
2. Kaye, S., Comber, E., Tenant-Flowers, M. and Loveday, C. (1995): The appearance of drug resistance-associated point mutations in HIV type 1 plasma RNA precedes their appearance in proviral DNA. AIDS Res. Human Retroviruses, 11, 1221-1225.
3. Koch, N., Yahi, N., Ariasi, F., Fantini, J. and Tamalet, C. (1999): Comparison of human immunodeficiency virus type 1 (HIV-1) protease mutations in HIV-1 genomes detected in plasma and in peripheral blood mononuclear cells from patients receiving combination drug therapy. J. Clin. Microbiol., 37, 1595-1597.
4. Kroodsma, K. L., Kozal, M. J., Hamed, K. A., Winters, M. A. and Merigan, T. C. (1994): Detection of drug resistance mutations in the human immunodeficiency virus type 1 (HIV-1) pol gene: differences in semen and blood HIV-1 RNA and proviral DNA. J. Infect. Dis., 170, 1292-1295.
5. Devereux, H. L., Burke, A., Lee, C. A. and Johnson, M. A. (2002): In vivo HIV-1 compartmentalisation: drug resistance-associated mutation distribution. J. Med. Virol., 66, 8-12.
6. Paolucci, S., Baldanti, F., Campanini, G., Zavattoni, M., Cattaneo, E., Donssena, L. and Gerna, G. (2001): Analysis of HIV drug-resistant quasi-species in plasma, peripheral blood mononuclear cells and viral isolates from treatment-naïve and HAART patients. J. Med. Virol., 65, 207-217.
7. Sarmati, L., Nicastri, E., Uccela, I., d'Ettorre, G., Parisi, S. G., Palmisano, L., Galluzzo, C., Concia, E., Vullo, V., Vella, S. and Andreoni, M. (2003): Drug-associated resistance mutations in plasma and peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected patients for whom highly active antiretroviral therapy is failing. J. Clin. Microbiol., 41, 1760-1762.
8. Wong, J. K., Hezareh, M., Günthard, H. F., Havlir, D. V., Ignacio, C. C., Spina, C. A. and Richman, D. D. (1997): Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science, 278, 1291-1295.
9. Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22, 4973-4680.
10. Page, R. D. M. (1996): An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci., 12, 53-60.
11. Bhattacharyya, D., Brooks, B. R. and Callahan, L. (1996): Positioning of positively charged residues in the V3 loop correlates with HIV type 1 syncytium-inducing phenotype. AIDS Res. Human Retroviruses, 12, 83-90.
12. Hung, C. S., Vander Heyden, N. and Ratner, L. (1999): Analysis of the critical domain in the V3 loop of human immunodeficiency virus type 1 gp120 involved in CCR5 utilization. J. Virol., 73, 8216-8226.
13. Zhang, Y. M., Dawson, S. C., Landsman, D., Lane, H. C. and Salzman, N. P. (1994): Persistence of four related human immunodeficiency virus subtypes during the course of zidovudine therapy: relationship between virion RNA and proviral DNA. J. Virol., 68, 425-432.
14. Ruff, C. T., Ray, S. C., Kwon, P., Zinn, R., Pendleton, A., Hutton, N., Ashworth, R., Gange, S., Quinn, T. C., Siliciano, R. F. and Persaud, D. (2002): Persistence of wild-type virus and lack of temporal structure in the latent reservoir for human immunodeficiency virus type 1 in pediatric patients with extensive antiretroviral exposure. J. Virol., 76, 9481-9492.
15. Finzi, D., Hermankova, M., Pierson, T., Carruth, L. M., Buck, C., Chaisson, R. E., Quinn, T. C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D. D., Richman, D. D. and Siliciano, R. F. (1997): Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science, 278, 1295-1300.
16. Wang, F. X., Xu, Y., Sullivan, J., Souder, E., Argyris, E. G., Acheampong, E. A., Fisher, J., Sierra, M., Thompson, M. M., Najera, R., Frank, I., Kulkosky, J., Pomerantz, R. J. and Nunnari, G. (2005): IL-7 is a potent and proviral strain-specific inducer of latent HIV-1 cellular reservoir of infected individuals on virally suppressive HAART. J. Clin. Invest., 115, 128-137
17. Johnson, V. A., Brun-Vézinet, F., Clotet, B., Conway, B., D'Aquila, R. T., Demeter, L. M., Kuritzkes, D. R., Pillay, D., Schapiro, J. M., Telenti, A. and Richman, D. D. (2004): International AIDS Society-USA Drug resistance mutations group: Update of the drug resistance mutations in HIV-1. Top. HIV Med., 12, 119-124.