two different antibodies recognizing *Gag*. We were unable to detect a significant increase in the *Env*:*Gag* ratio (unpublished data), suggesting that the sparsomycin's effect on *Gag–Pol*:*Gag* ratio was specific. These data suggested that the translational efficiencies of viral proteins were not equally enhanced by sparsomycin. Altogether, it was strongly suggested that the sparsomycin's replication-boosting effect on HIV-1 was partly due to the enhancement of the –1 frameshift efficiency.

Discussion

In the present study, we have demonstrated that sparsomycin is an enhancer of HIV-1 replication in many human T cell lines at concentrations between 400–500nM. Our preliminary observation suggested that HIV-1 replication was also enhanced in primary peripheral blood monocyte culture (data not shown). Sparsomycin should be able to accelerate the study on the low-fitness HIV-1 such as drug-resistant mutants. As sparsomycin did not alter the IC_{50} of multiple antiretroviral drugs on both wt and drug-resistant HIV-1, its usage should be able to facilitate the phenotypic resistance testing of clinical isolates and as a result, benefit HIV-1-infected patients. Our observation raised an immediate concern as to whether sparsomycin-producing Streptomyces species caused an opportunistic infection in humans, which influenced AIDS progression. However, we did not find any reports suggesting so.

Sparsomycin and puromycin are the only antibiotics that can inhibit protein synthesis in bacterial, archaeal and eukaryotic cells (Ottenheijm *et al.*, 1986; Porse *et al.*, 1999). Sparsomycin has the ability to enhance the –1 frameshift in mammalian cells as well as *S. cervisiae* (Dinman *et al.*, 1997). The proposed molecular mechanism behind this ability was either through a higher affinity of the donor stem for the ribosome and slowing down the rate of the peptidyl transfer reaction, or a change in the steric alignment between donor and acceptor tRNA stems resulting in decreased peptidyl-transfer rates. Conversely, puromycin is not known to enhance the –1 frameshift in mammalian cells. At sub-toxic concentrations, puromycin was unable to enhance the HIV-1 replication (unpublished data). These data, along with the data provided in this paper, implied that the sparsomycin's unique ability to enhance the –1 frameshift might play a role in boosting the HIV-1 replication.

The maintenance of the –1 frameshift efficiency at the optimal range is critical for HIV-1 to replicate (Jacks *et al.*, 1988). Therefore, limiting *Gag–Pol* production should lead to an inhibition of viral replication because *pol* encodes enzymes essential for viral replication (Levin *et al.*, 1993). In contrast, it was also reported that increasing the *Gag–Pol* to *Gag* ratio by twofold resulted in a reduction of viral replication (Hung *et al.*, 1998; Shehu-Xhilaga *et al.*, 2001). Thus, a modest alteration of the –1 frameshift efficiency should markedly affect the replication capacity of HIV-1. Our data indicated that sparsomycin increased the efficiency of –1 frameshift by 1.3-fold, which produces a better replication capacity for HIV-1. As a result, we hypothesize that HIV-1 has a 'suboptimal' –1 frameshift efficiency. In theory, the 1.3-fold difference per one replication cycle becomes approximately 10-fold after 10 rounds of viral replication cycle because the effect accumulates exponentially. The difference should become larger when HIV-1 replicates with the slower kinetics and the replication profile is monitored over a longer time course. In fact, our experimental data were in good agreement with the above estimation. In nature, HIV-1 does not accumulate mutations within the frameshift signal to achieve the higher frameshift efficiencies. This implies that there are multiple and complex regulatory mechanisms that keep the efficiency of the –1 frameshift at suboptimum. Under these conditions, the best efficiency of HIV-1 survival in the host might be achieved. Altogether, one of the possible mechanisms that sparsomycin boosted the HIV-1 replication could be the enhancement of the –1 frameshift efficiency.

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Original Article

Mutations of Conserved Glycine Residues within the Membrane-Spanning Domain of Human Immunodeficiency Virus Type 1 gp41 Can Inhibit Membrane Fusion and Incorporation of Env onto Virions

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SUMMARY: The membrane-spanning domain (MSD) of HIV-1 envelope protein (Env) has an additional glycine residue within a well-conserved putative transmembrane helix-helix interaction motif, GXXXG, and forms a $G^{690}G^{691}XXG^{694}$ sequence (G, glycine; X, any residues; the numbering indicates the position within the Env of an infectious molecular clone, HXB2). Different from vesicular stomatitis virus G (VSV-G), the glycine residues of the GXXXG motif of HIV-1 showed higher tolerance against mutations, and a simultaneous substitution of G690 and G694 with leucine residues only modestly decreased fusion activity and replication capacity of HIV-1. When G691 was further substituted with alanine, phenylalanine or leucine residue while G690 and G694 were substituted with leucine residues, the efficiency of membrane fusion decreased, with the decrease greatest occurring with the leucine substitution, a less severe decrease with phenylalanine, and the least severe decrease with alanine. Substitution with leucine residue also decreased the incorporation of Env onto virions, and the mutant showed the most delayed replication profile. Thus the presence of the extra glycine residue, G691, may increase the tolerance of the other two glycine residues against mutations than VSV-G. The fact that a more severe defect was observed for the leucine residue than the phenylalanine residue suggested that the function of Env depended on the steric nature rather than on the simple volume of the side chain of the amino acid residue at position 691. Based on this result, we propose a hypothetical model of the association among MSDs of gp41, in which G^{691} locates itself near the helix-helix interface.

INTRODUCTION

The envelope protein (Env) of human immunodeficiency virus type 1 (HIV-1) is a trimer of non-covalently associated heterodimers of gp120 and gp41. As with other retroviruses, gp120 (SU) and gp41(TM) play key roles in the determination of host range and membrane fusion, respectively.

For the three subdomains of HIV-1 gp41, the structure-function relationship of the ectodomain during membrane fusion has been elucidated at the molecular level (1,2). After gp120 binds to the receptor/coreceptor, the ectodomain undergoes a conformational change to form a six-helix bundle, a common structure observed for the class I fusion protein (3,4). Information on the structure-function relationships of the membrane-spanning domain (MSD) is rather limited. Although the amino acid sequence of the MSD of HIV-1's gp41 is highly conserved among different clades, the significance of the specific amino acid sequence within MSD has been underestimated, because some heterologous MSDs can substitute functionally for the native MSD of gp41 (5,6). However, truncation of HIV-1 MSD by a glycosylphosphatidylinositol anchor abolished the fusion activity (7). Furthermore, in viruses such as simian immunodeficiency virus and the influenza virus, mutations introduced into the MSD have been shown to impede the late stage of membrane fusion (8-11). These data suggest the importance of the MSD for the function of Env.

A glycine-containing helix-helix interaction motif, a GXXXG motif, has been found in MSDs of many membrane proteins such as glycophorin A (GpA) (12,13) and the hepatitis C virus envelope glycoproteins (14). In the case of HIV-1, it occurs as a $G^{690}G^{691}XXG^{694}$ sequence (G, glycine residue; X, any amino acid residue; the number indicates the position of each glycine residue in the Env of a molecular clone HXB2 [15]) within the MSD of gp41. The glycine residues within the GXXXG motif are critical for the proper fusogenicity of vesicular stomatitis virus G (VSV-G) (16). In a previous study we have shown that the point mutations of the individual glycine residue of the GXXXG motif of gp41 MSD were well tolerated (17). The molecular basis for this high tolerance of gp41 MSD against mutations was not identified.

Here we hypothesize that the GXXXG motif of gp41, like in other transmembrane helices, forms the helix-helix interface of gp41 MSDs. We reevaluated the role of glycine residues within the gp41 MSD by introducing a simultaneous substitution of several glycine residues. We confirmed the high tolerance of gp41 MSD against mutations through the finding that any combinatorial mutations of two glycine residues with leucine residues were well tolerated. By using one of the mutants, the $LG^{691}XXL$ mutant, we evaluated the role

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of the extra glycine residue by substituting it with several different amino acid residues, such as alanine, leucine, and phenylalanine residue. These substitutions negatively affected the function of Env, such as its fusogenicity or virion incorporation. We also found that there was a correlation between the steric characteristics of the side chain of the residue replacing G^{691} and the alteration in the function of Env. Based on these findings, we propose a potential association model of gp41 MSD.

MATERIALS AND METHODS

Construction of plasmids: The substitution mutants were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif., USA) using the subclone containing the 1.2-kb *Nhe*I-*Bam*HI fragment covering the *env* portion of HXB2RU3ΔN as described previously (17). The complementary oligonucleotide pairs used were as follow: 691F, ATGATAGTAG GATTCTTGGT AGGTTTA/ TAAA CCTACC AAGAATCCTA CTATCAT; 690/691-2L, GTACT GCTCT TGGTAGGTTT AAGAATAGTT TTTG/ CAAA AACTAT TCTTAAACCT ACCAAGAGCA GTAC; 690/694-2L, ATTCATAATG ATAGTACTGG GCTTGGTACT TTTAAG/ CTTAAAAGT ACCAAGCCCA GTACTATCAT TATGAAT; 691/694-2L, TTCATAATGA TAGTAGGACT CTTGGTACTT TTAAG/ CTTAAAAGTA CCAAGAGTCC TACTATCATT ATGAA. PCR was performed using PfuTurbo (Stratagene). The three glycine (G691) substitution mutants were created by site-directed mutagenesis using one subclone of the 2L mutants, 690/694-2L, as a PCR template and the following complementary oligonucleotide pairs: 690/694-2L + 691A, ATTCATAATG ATAGTACTGG CCTTGGTACT TTTAAG/ CTTAAAAGTA CCAAGGCCAG TACTATC ATT ATGAAT; 690/694-2L + 691L, CATAATGATA GTA CTGCTCT TGGTACTTTT AAGAAT/ ATTCTTAAAA GTACCAAGAG CAGTACTATC ATTATG; 690/694-2L + 691F, ATTCATAATG ATAGTACTGT TCTTGGTACT TTTAAG/ CTTAAAAGTA CCAAGAACAG TACTATC ATT ATGAAT). Following mutagenesis, the 1.2-kb *Nhe*I-*Bam*HI fragments were sequenced and cloned back into the pSP65HXB2RU3ΔN or pElucEnv (17) plasmid. The entire *Nhe*I-*Bam*HI portion, together with the junction, was verified by sequencing after the fragments were back.

Cells and antibodies: COS7 cells, 293 cells and 293CD4 cells (17) were grown in Dulbecco's modified essential medium (DMEM; Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah, USA) and penicillin-streptomycin (Gibco-BRL, Rockville, Md., USA). Jurkat cells were grown in RPMI 1640 (Sigma) supplemented with 10% FBS and penicillin-streptomycin. Cells were kept under 5% CO_2 in a humidified incubator. Anti-gp120 polyclonal antibody was obtained from Fitzgerald Industries International, Inc. (Concord, Mass., USA). The hybridoma 902 and Chessie 8 were obtained from Bruce Chesebro and George Lewis, respectively, through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (18-20). Serum from a patient infected with HIV-1 was kindly provided by T. H. Lee of Harvard School of Public Health, Boston, Mass., USA.

Protein analysis: The provirus DNA constructs were transfected into COS7 cells by electroporation or lipofection. In electroporation, COS7 cells (4 μg proviral DNA per 1×10^7 cells) were suspended in serum-free DMEM and electroporated at a 250-kV, 950-μF setting using Gene Pulsar II (Bio-Rad, Hercules, Calif., USA). In lipofection, COS7 cells (3×10^6 cells) were transfected with 7 μg proviral DNA by FuGene6 (Roche Molecular Biochemicals, Mannheim, Germany). At 72 h after transfection, transfected COS7 cells were collected by scraping and were centrifuged at 2,000 $\times$ *g* for 10 min (Allegra 6KR system; Beckman Coulter, Fullerton, Calif., USA). The cell pellets were dissolved in radioimmunoprecipitation assay (RIPA) lysis buffer (0.05 M Tris-Cl [pH 7.2] including 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and the clear lysates were centrifuged at 314,00 $\times$ *g* for 45 min at 4°C (Himac CS 120fx system; Hitachi, Tokyo, Japan). The virus was sedimented from pre-cleared supernatants (centrifuged at 2,000 $\times$ *g* for 20 min; Allegra 6KR system, then filtered through 0.45-μm-pore-size filters: Millipore, Bedford, Mass., USA) by centrifuging at 113,000 $\times$ *g* for 1.5 h at 4°C on 3 ml of a 20% sucrose cushion (SW28 rotor; Beckman Coulter). Virus pellets were dissolved in RIPA lysis buffer. Both cell and virus lysates were run on a 7.5-15% gradient in a SDS-polyacrylamide electrophoresis gel (PAGE) system (DRC, Tokyo, Japan), and proteins were blotted onto Immobilon-P (Millipore) by passive transfer, as described previously (21). The immunoblotting procedure was as described previously (17). Enhanced chemiluminescence (Roche Molecular Biochemicals) and a LAS-3000 (Fuji Photo Film, Kanagawa, Japan) were used to detect the bands.

Infection study: For the infection study, the virus seed was prepared by transfecting 1 μg of the proviral DNA into 10^6 of the COS7 cells using FuGene6 (Roche Molecular Biochemicals). Jurkat cells were infected with each virus adjusted by the p24 amount (10 ng per 10^6 cells). The infection was monitored by measuring the amount of p24 in the culture supernatant at specific time points after infection (0, 8, 15, 22 and 25 days). A p24 ELISA was performed using a p24 RETRO-TEK ELISA kit (ZeptoMetrix, Buffalo, N.Y., USA).

Flow cytometry: The level of Env expressed on the cell surface was monitored by fluorescence-activated cell sorting analysis as described previously (17). Briefly, 48 h after the COS7 cells were transfected with each Env expression vector by FuGene6, the cells were stained with the 902 monoclonal antibody for 1 h at 4°C (10 μg/ml in phosphate-buffered saline [PBS] with 2% FBS), incubated with biotin-XX goat anti-mouse immunoglobulin G (Molecular Probes, Eugene, Oreg., USA) for 30 min at 4°C, treated with streptavidin Alexa Fluor 555 (Molecular Probes) for 30 min at 4°C, and finally fixed with 1% paraformaldehyde in PBS. Cells were suspended in PBS with 2% FBS and analyzed with Becton Dickinson FACSCalibur and CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, Calif., USA). A double gate was defined by forward versus side scatter and by the amount of GFP (FL-1). A total of 10,000 events within this gate were collected for analysis. An Env KO that fails to express Env was used as a negative control, as described previously (17).

T7 RNA polymerase (RNApol) transfer assay: The efficiency of fusion pore formation was examined using T7 RNApol transfer assay as described previously (17). Briefly, COS7 cells were transfected with each Env expression vector together with the reporter plasmid pTM3hRL using FuGene6. The reporter plasmid contains the T7 promoter-driven renilla luciferase. At 48 h after transfection, the transfected COS7 cells were cocultured with the 293CD4 cells

that had been transfected with the T7 RNApol expression vector, pCMMP T7RNApoliresGFP (the ratio of cells was 1:1). At 12 h after the coculture, the cells were lysed. Firefly luciferase activities, derived from the Env expression vector, and renilla luciferase activities, activated by the T7 RNApol transferred from 293CD4 cells through the generated fusion pores, were determined using the Dual-Glo luciferase reporter assay system (Promega, Madison, Wis., USA).

RESULTS

Mutagenesis of glycine residues within the GGXXG sequence: In our previous study, mutations to an individual glycine residue within gp41 MSD were well tolerated, and high tolerance of gp41 MSD against mutations was expected (17). Therefore this time we simultaneously mutated several glycine residues within the MSD (Fig. 1). First, we substituted two glycine residues with leucine residues to create three forms of 2L mutants: 690/691-2L, 690/694-2L, and 691/694-2L. In the mutant 690/694-2L, the conserved glycine residues corresponding to the GXXXG motif were substituted. A similar mutation introduced into that of VSV-G resulted in functionally defective VSV-G (16). Next, to address the significance of the additional glycine residue at position 691, we substituted G691 with alanine, phenylalanine, or leucine residue while G690 and G694 were substituted with leucine residues. These substitutions formed the 2L + 691X mutants 690/694-2L + 691A, 690/694-2L + 691F, and 690/694-2L + 691L. Thus the sequence context of 2L + 691X mutants is LXxxL (the small x represents the original sequence of HXB2). As a control, the single substitution of the G691 with phenylalanine (691F) in which the other two glycine residues were left intact was generated (Fig. 1). The single substitution of the G691 to alanine (691A) or leucine residue (691L) was well tolerated and described in our previous study as mentioned above (17).

The replication profile of the MSD mutants: To examine replication capacity, Jurkat cells were infected with the mutant viruses after the p24 amount was adjusted for. Virus replication was monitored by measuring the amount of p24 released into the culture supernatants at 0, 8, 15, 22, and 25 days after infection. A representative result of the Jurkat cell experiment is shown in Figure 2. The single substitution mutant, 691F, was replicated with a slight delay compared with the wild type (WT). Other single substitution mutants, 691A or 691L, were replicated as efficiently as the WT (described in a previous study, data not shown) (17). The 2L mutants showed slightly delayed replication kinetics compared to those of the WT. The replication of the 2L + 691X mutants was less efficient than that of the WT. Substitution with leucine residue (690/694-2L + 691L) produced the slowest replication profile in repeated experiments. These data confirmed the high tolerance of the glycine residues within the GXXXG motif of gp41 MSD against mutation. Although the replication kinetics were generally slower in the H9 cells, a similar replication profile was observed (data not shown).

Analysis of the protein profiles of MSD mutants: The protein profiles of the mutants depicted in Figure 1 were examined by immunoblotting analysis using the serum from an individual infected with HIV-1 and anti-gp120 polyclonal antibody for both cell and virus lysates. Similar protein profiles were observed for all mutants and the WT in cell lysates (Fig. 3A). Almost equivalent amounts of gp160 and gp120 were observed for all constructs, and there was no obvious alteration in the processing. There was no difference found in the processing of Gag and Pol products. In the virus lysates, as in the cell lysates, no differences were found in the profiles of Gag and Pol products (Fig. 3B). However the amounts of gp120 found on 690/694-2L + 691L virions were 50 - 60% those of the WT (51.9% ± 14.4 %, $n = 3$, the amount of the incorporated Env was estimated by determining the ratio of the intensity of the Env to the p24 bands). This result is not due to the shedding of gp120, because the probing of the virus lysates with anti-gp41 monoclonal antibody detected a smaller amount of gp41 in 690/694-2L + 691L than in the WT (Fig. 3C). Therefore the alteration of the replication profiles observed for 690/694-2L + 691L (Fig. 2) may be accounted for by a defect in the incorporation of Env onto the virions. The reason other mutants manifested the slower replication is not evident from the protein profiles.

Fusion activity of mutants evaluated by the efficiency of fusion pore formation: Our previous study as well as others (11,17,22), have shown that mutations of the MSD sometimes negatively affect the fusogenicity of the Env. Therefore, to investigate the reason for the delayed replication observed in Figure 2, we evaluated the fusion efficiency of our mutants using the Env expression vector (Fig. 4A).

```
WT: yikLFIMIVGGLVGLRIVFAVLSIVnrv
691F: yikLFIMIVGFLVGLRIVFAVLSIVnrv
2L mutants
  690/691-2L: yikLFIMIVLLLVGLRIVFAVLSIVnrv
  690/694-2L: yikLFIMIVLGLVLLRIVFAVLSIVnrv
  691/694-2L: yikLFIMIVGLLVLLRIVFAVLSIVnrv
690/694-2L+691 mutants
  690/694-2L+691A: yikLFIMIVLALVLLRIVFAVLSIVnrv
  690/694-2L+691L: yikLFIMIVLLLVLLRIVFAVLSIVnrv
  690/694-2L+691F: yikLFIMIVLFLVLLRIVFAVLSIVnrv
```

Fig. 1. The mutants of gp41 MSD studied. The primary structures of the MSD mutants used in this study are shown using the one-letter abbreviation of amino acid residues. The position numbering is based on that used for HXB2 Env. The portion of the predicted MSD is shown in capital letters. WT corresponds to the wild type HXB2. Mutated residues are underlined.

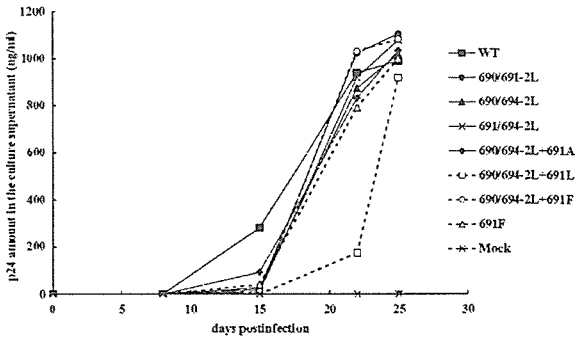

Fig. 2. The replication profile of gp41 MSD mutants in Jurkat cells. The replication of HIV-1 was monitored by measuring the p24 amount in the culture medium at 0, 8, 15, 22 and 25 days. The replication of 691A and 691L was similar to that of WT, as reported previously (17) and not shown here.

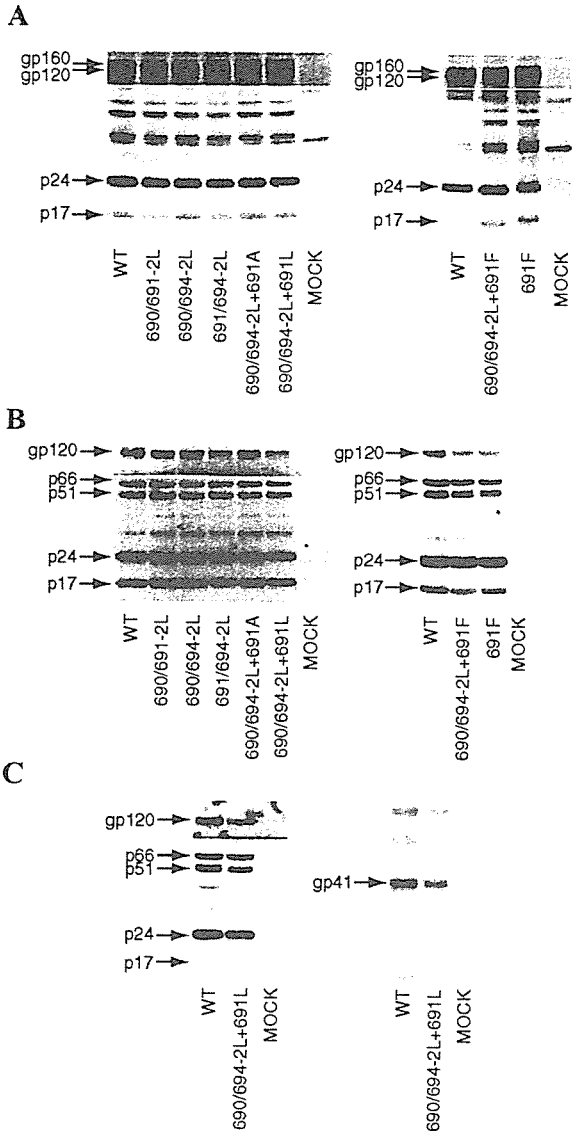

Fig. 3. Protein profiles of cell and virus lysates of the WT and mutants. The protein profiles of mutants were examined by immunoblotting. The cell (A) and virus (B, C) lysates prepared from transfected COS7 cells were used. The Env proteins (gp160, gp120 and gp41) were detected using anti-gp120 polyclonal antibody or anti-gp41 monoclonal antibody, respectively. Gag (p17 and p24) and Pol (p51 and p66) were detected using serum from an individual infected with HIV-1. The names of the mutants are shown.

First, we confirmed that the surface expression level of Env was similar by using the 902 monoclonal antibody in a flow cytometric analysis (Fig. 4B). This confirmation further supported the hypothesis that 690/694-2L + 691L has a defect in the incorporation of Env onto the virions rather than a defect in the intracellular transport of Env. We then analyzed the fusion activity of Env mutants using the T7 RNApol transfer assay. In this assay, the T7 RNApol that is transferred through the fusion pore between the Env- and receptor-expressing cells activates the T7 promoter-driven renilla luciferase. The renilla luciferase activity was normalized for transfection efficiency by the firefly luciferase activity derived from the Env expression vector. The representative data are shown in Figure 4C. Compared with WT, the 2L mutants showed a decrease in fusion efficiency of about 30%. There were no significant differences in fusogenicity among the different 2L mutants, a finding that was consistent with the replication profile shown in Figure 2. Therefore, the delayed replication observed for 2L mutants may be due to a defect in fusion. The delayed replication of 691F may also be due to a similar mechanism, although the observed decrease in the fusogenicity of 691F is not statistically significant (Fig. 4C).

The effect of the mutations of the conserved glycine residues within the GXXXG motif of gp41 MSD was less severe than in the case of VSV-G, in which a simultaneous substitution of two glycine residues corresponding to G690 and G694 resulted in almost complete elimination of fusion activity (16). The presence of an additional glycine residue within the GXXXG motif generates the GG^{691}XXG sequence in gp41 MSD, which led us to evaluate the effect of substituting other amino acid residues for the glycine residue at position 691 in the 690/694-2L context for membrane fusion. Substituting glycine residue with alanine residue did not reduce the fusion efficiency further (for example, compare 690/694-2L with 690/694-2L + 691A in Fig. 4C). However, changing the glycine residue to phenylalanine reduced the fusion efficiency significantly (690/694-2L versus 690/694-2L + 691F in Fig. 4C). The introduction of leucine residue in place of the glycine residue had the most severely negative effect. Thus the presence of a glycine residue at position 691 seems to produce an apparently higher fusion efficiency for gp41 even when the two other glycine residues constituting the GXXXG motif were replaced with leucine residue. Furthermore, because substitution with leucine resulted in less fusogenic gp41 than did substitution with phenylalanine residue, it seemed that the steric nature of the side chain at position 691, not simple bulkiness, affected the outcome.

DISCUSSION

The importance of the GXXXG motif in helix-helix association has been well established through the studies of GpA. The glycine residues within the GXXXG motif play a critical role in helix-helix interaction (13,23). Here we showed that the mutations introduced in the conserved glycine residues within the GXXXG motif of gp41 MSD were well tolerated. This finding is quite different from that of the previous study of VSV-G MSD, where a similar mutation corresponding to the 690/694-2L mutant almost abolished the fusion activity (16). Furthermore, we also observed that the substitution of any two glycine residues within the GGXXG sequence of gp41 MSD only modestly decreased the fusion activity (Fig. 4C). The expression, processing, and transport of Env proteins to the cell surface were preserved (Fig. 3A and Fig. 4B). Consistent with the modest decrease in fusion activity, the replication profiles of these mutant viruses were only slightly retarded when compared to that of the WT virus (Fig. 2). This result confirmed the high tolerance of gp41 MSD that allows the substitution of any two glycine residues within the GGXXG sequence. The results for the 690/694-2L mutant may suggest that the GXXXG motif of gp41 MSD does not play a role as a helix-helix interaction motif or that there is another mechanism that cancels out the introduced mutations.

The presence of the additional glycine residue within the GXXXG motif is a well-conserved feature of gp41's MSD (15), and this feature is absent from VSV-G. The three

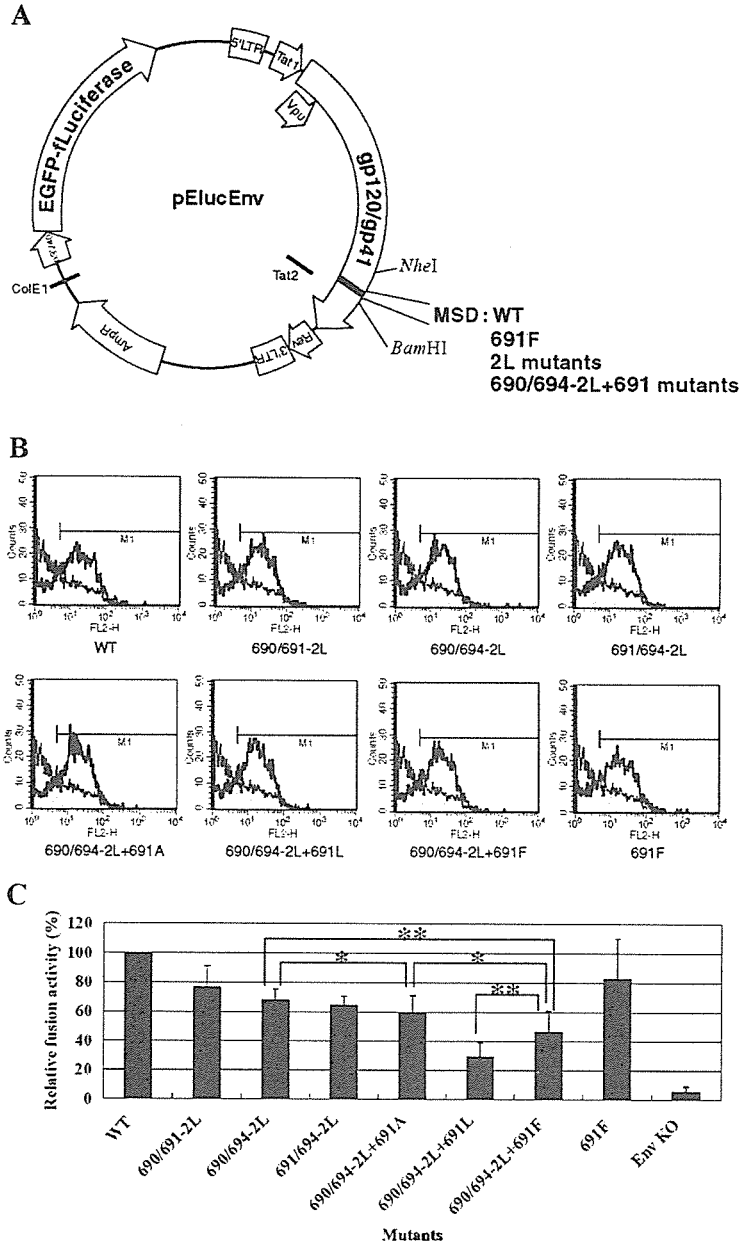

Fig. 4. Evaluation of the cell surface expression and measurement of the fusion activity of mutant Env. (A) The map of the Env expression vector, pElucEnv. pElucEnv supports the expression of HIV-1 *env* (gp120/gp41) and the gene of the EGFP-firefly luciferase (EGFP-fLuciferase) hybrid protein, respectively, from two separate promoters. The *Nhe*I and *Bam*HI sites used for cloning are indicated. The LTR, *tat*, *vpu*, and *rev* of HIV-1 are shown. AmpR, β-lactamase gene; SV40, SV40 late promoter; ColE1, ColE1 replication origin. (B) Cell-surface expression of Env. FACS analysis of Env expressed on the surface of COS7 cells transfected with each pElucEnv construct was accomplished as described in the Materials and Methods section. The signal for each Env is shown as a gray line. The filled area depicts the signal obtained for the control vector, Env KO. (C) Fusion activity of the mutants evaluated by T7 RNApol transfer assay. The cell-cell fusion assay between the Env expressing cells (containing T7 promoter-driven plasmid) and CD4+ cells (bearing the T7 RNApol expression plasmid) was used to evaluate the fusion efficiency of mutant Envs. A representative result of four independent experiments is shown (*statistically not significant, **statistically significant difference: $P < 0.05$ by Student's t test). The results shown are means ± s.d. ($n = 4$).

glycine residues will cluster within the gp41 MSD helix (Fig. 5A, prepared by the program Insight II; Accelrys, San Diego, Calif., USA). Having a hydrogen atom as its side chain, the clustering of three glycine residues may give the region more flexibility to accommodate mutations. To test whether the high tolerance of gp41MSD against mutations is attributable to the presence of the extra glycine residue within the gp41 GXXXG motif, we further mutated the extra glycine residue (G691) while the other two glycine residues were mutated to leucine residues under the $LG^{691}XXL$ context. This also allows us to obtain information on a potential helix-helix interface among gp41 MSDs. We would expect the mutation

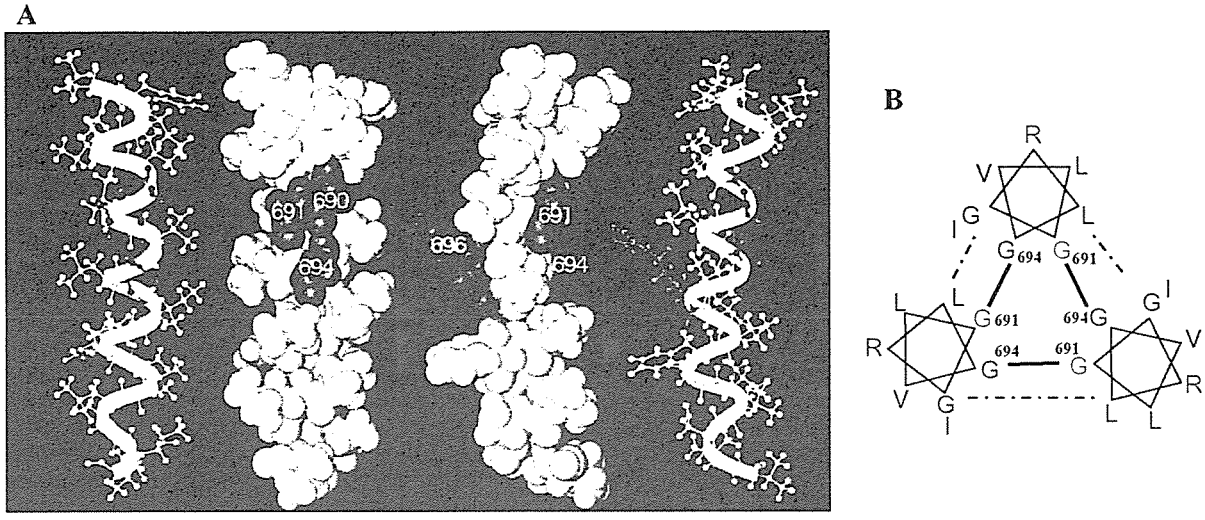

Fig. 5. A structural model of MSD and a hypothetical arrangement of the MSD helices. (A) The MSD region (position 684-705 of HXB2 Env, the amino acid sequence: LFIMIVGGLVGLRIVFAVLSIV) of the WT HXB2 was modeled in an a-helix conformation. Molecular dynamics simulations were carried out in the lipid-like environment, using the Generalized Born method with a relative permittivity of 4.0 (25, 26). The most representative structure seen in the simulation was extracted by a principal component analysis and is shown graphically. Three glycine residues (shown in red) colocalize each other and generate a hollow surface on one aspect of the helix. Two different views (ball-and-stick and CPK) of the same helix are shown. (B) A hypothetical arrangement of the MSD helices. The glycine residue at positions 691 and arginine residue at position 696 are shown in red and blue, respectively. The potential interactions between amino acid residues are shown by the bold and dotted lines, respectively.

to have no significant effect if G691 was facing outward to the lipid environment and was not involved in the helix-helix interaction. We took a genetic approach and we created the mutants 690/694-2L + 691A, 690/694-2L + 691F, and 690/694-2L + 691L. None of these mutations changed the processing or surface expression of Env (Figs. 3A and 4B). This suggests that these mutations did not induce drastic conformational changes that could be detected by a quality control mechanism of endoplasmic reticulum. Indeed, our molecular dynamics analyses of these mutants failed to detect severe deformation of the structure of the individual helix (data not shown).

When the fusion activity of the 690/694-2L + 691X mutants was evaluated, there was no statistically significant difference between glycine and alanine residue (Fig. 4C). This finding is consistent with the observation that alanine residue can functionally substitute glycine residue in transmembrane helices (23). The substitution of glycine residue with a bulky residue, namely phenylalanine residue (690/694-2L + 691F), resulted in a significant reduction in fusion activity. The fusion efficiency of 690/694-2L + 691F was about 50% that of WT. Substitution with leucine residue (690/694-2L + 691L) had an even greater negative effect on fusogenicity, and the fusion efficiency of 690/694-2L + 691L was about 30% that of WT (Fig. 4C). The replication efficiency somewhat reflected these changes in fusion activity, although we could not attribute the defect of 690/694-2L + 691L to the defect in fusion alone because 690/694-2L + 691L had an additional defect in Env incorporation (Figs. 3B and C). These results showed that the presence of glycine residue at position 691 seemed to reduce the negative effects of the leucine residue substitution of glycine residue at positions 690 and 694.

It is noteworthy that we have observed the phenotypic changes of gp41 according to the nature of the substituted amino acid residue at the position 691 (Figs. 2-4). Interestingly, the negative effect of mutations was not dependent on the mere volume of the side chain (F > L), but rather on the steric nature of the side chain. The introduction of another branched amino acid residue, isoleucine residue, at position 691 also resulted in decreased fusion activity (data not shown). This may suggest that the mutation may affect the interhelix association of MSDs, and the changes in association among MSDs may affect the function of gp41. Indeed our preliminary analysis of the measurement of helix-helix interaction by means of TOXCAT (12) analysis indicated that 690/694-2L + 691L had a slightly stronger association than WT (E. Matthews and D. M. Engelman, unpublished data). Based on these data, we present our hypothetical model of the association of the three MSDs in which the GXXXG motif is facing inward as shown in Figure 5B. Under this configuration, the G691 locates itself near the helical interface. Although this model is consistent with our observation, it places the highly conserved arginine residue downstream of the GXXXG motif toward the lipid environment. This may not be a thermodynamically favorable arrangement despite the recent finding that suggested that arginine residue can be accommodated into a lipid bilayer more easily than previously expected (24). Therefore we cannot rule out alternative arrangements of the gp41 MSDs at present. The observed high tolerance of glycines constituting this hypothetical interface may also suggest that the potential interaction among gp41 MSDs may be a rather weak one. To prove our model, the physical structure of the gp41 MSDs in lipid environments must be determined.

Here we have shown that the efficiency of the Env-mediated fusion pore formation and the incorporation of Env onto virions were affected by alterations within the MSD of gp41. This confirms that the specific primary structure of MSD is important for its proper function. Our findings also suggest that a subtle change in the structure within MSD can affect the conformations of other subdomains of gp41. Conversely, they may suggest that the conformational changes

in other subdomains may affect the structure of the MSD. This may have implications for the mechanism of disassembly and for that of uncoating events, and may also suggest that the MSD of gp41 may become another target for therapeutic intervention against HIV-1 infection.

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A simple competitive RT-PCR assay for quantitation of HIV-1 subtype B and non-B RNA in plasma

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Abstract

An easy, inexpensive competitive RT-PCR assay for HIV-1 RNA quantitation was constructed. A 138-bp sequence in the HIV-1 *gag* p24 region was selected as the target and co-amplified with competitor RNA containing an internal 44-bp deletion. Quantitation of serial dilutions of control RNA samples prepared from the LAI isolate demonstrated a good linearity ($R^2 = 0.991$) within the range between 10 and 250 copies/sample. The detection limit of the assay was determined to be 3.8 copies/sample by Probit analysis and corresponded to 110 copies/ml in plasma. The intra-assay CV value was 9.1%, and the inter-assay value was 25.9%. Both were comparable to those obtained with commercially available HIV-1 RNA quantitation kits. The correlation efficient for the results obtained in 47 plasma samples from HIV-1-infected individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16) with the competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5 was 0.956 for subtype B and 0.947 for subtype non-B. The assay devised is a good alternative for monitoring antiretroviral therapy in resource-poor countries.
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Keywords: HIV-1; Monitoring; Competitive RT-PCR; Viral load

1. Introduction

The pandemic of human immunodeficiency virus type 1 (HIV-1) infection has been the greatest threat to human health worldwide since its emergence two decades ago, and today about 40 million people are living with HIV/AIDS (Simon et al., 2006). As has been the pattern with respect to many other infectious diseases, the regions of the world experiencing the most significant spread of HIV-1 infection are poor. Ninety five percent of individuals infected with HIV-1 are living in developing countries with few resources, such as the countries of sub-Saharan Africa and Southeast Asia. According to the World Health Organization (WHO), nearly 6 million HIV/AIDS patients are in urgent need of antiretroviral therapy (ART), yet only 5% have access to the necessary drugs, and the remaining 95% are hopelessly waiting to die (WHO, 2004). To address this problem, WHO has launched the so-called "3 by 5" initiative (Jong-wook, 2003), which sought to treat 3 million people by the end of the year 2005, a goal made more likely by agreements enabling the use of generic antiretroviral drugs. The project appears to be operating effectively, and more patients than ever are being treated with antiretrovirals. Ironically, the introduction of antiretrovirals to countries with few resources has raised another issue: how best to monitor patients' response to treatment. In most developing countries where the "3 by 5" initiative has been implemented, it has been difficult to routinely monitor patients' CD4-positive T-cell counts or, what is worse, their plasma viral RNA concentrations (Russell, 2004). The plasma viral load is an important surrogate marker that is used to make prognoses, evaluate response to treatment, and estimate the probability of emergence of drug resistance (Kalish et al., 1999; Kempf et al., 1998; Leriche-Guerin et al., 1997; McDermott et al., 2005; Piliero, 2003). Three types of viral load quantitation kits are available commercially: the Cobas Amplicor HIV-1 Monitor test (Roche Diagnostics, Branchburg, NJ) (DiDomenico et al., 1996), the Quantiplex HIV-1 RNA Assay (Bayer, Mannheim,

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Germany) (Collins et al., 1997; Pachl et al., 1995), and the NucliSens EasyQ HIV-1 assay (bioMérieux, Boxtel, The Netherlands) (Kievits et al., 1991; de Baar et al., 1999; Yao et al., 2005). All three commercial kits have high sensitivity and reproducibility, and have been strictly validated. One significant drawback, however, is their high price. The cost per sample between 50 and 80 US dollars is too expensive to use for routine monitoring in resource-poor countries.

Competitive reverse transcription (RT)-PCR was the first method to quantitate HIV-1 RNA in plasma and used to show that viral load in plasma can be used as an important surrogate marker to assess the disease progression and the efficacy of ART (Piatak et al., 1993). Competitive RT-PCR involves co-amplification of a competitive RNA template that uses the same primers as for the target cDNA but that can be distinguished from the target after amplification by introducing a deletion, insertion, or a new restriction enzyme site. In conventional competitive RT-PCR, the amplified products of the target sequence and competitor are quantitated on agarose gel electrophoretograms, which is easy and inexpensive to perform, but has never been developed as a commercial assay. Several in-house competitive RT-PCR methods have been reported (Ernest et al., 2001; Johanson et al., 2001), but they have not been fully evaluated for quantitation of HIV-1 subtype non-B RNA in plasma samples. This paper describe a simplified, less expensive protocol for plasma viral load quantitation that is also applicable to HIV-1 subtype non-B.

2. Materials and methods

2.1. Subjects

Blood samples were obtained from 47 HIV-1 seropositive individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16). The subtype was determined by phylogenetic analysis of sequences in RT and PR regions in *pol* and the *env* V3 region (Kato et al., 2003). The patients provided written informed consent to the study, and the study was approved by the local ethics committee.

2.2. Preparation of competitor RNA and control HIV-1 RNA

A template DNA consisting of an HIV-1 *gag* sequence (positions 1372–1509) with a 44-bp deletion (1418–1461) (Fig. 1) and a T7 promoter (TAATACGACTCACTATAGGGAGA) at the 5′ end was constructed from HIV-1 DNA clone NL4-3 by the recombinant PCR technique (Higuchi, 1990). Competitor RNA was synthesized from the template DNA with an *in vitro* transcription system and T7 RNA polymerase (Promega, Madison, WI). QIAamp UltraSens Virus Kit (QIAGEN, Hilden, Germany) was used to extract HIV-1 LAI RNA from the culture supernatant of peripheral blood mononuclear cells infected with the LAI isolate, and the extracted LAI RNA was stored at −20 °C until used as an external control for HIV-1 quantitation. The exact HIV-1 RNA concentration of the LAI RNA solution was determined by spectrophotometry and Poisson distribution analysis of the positive scores of nested RT-PCR at the endpoint dilution (Kato et al., 2006).

Fig. 1. Construction of the competitive RT-PCR assay: target genome and primer design. An HIV-1 *gag* p24 region was selected as the target for the competitive RT-PCR. Both the target and the competitor RNA molecules were co-amplified with a primer mixture of GAR, GAF1, and GAF2. The amplicons of the target and the competitor were distinguished by a 44-bp deletion. The primer sequences of GAR, GAF1, and GAF2 are shown. The nucleotide numbers are based on the HIV-1 NL4-3 sequence (GenBank accession no. M19921).

2.3. Competitive RT-PCR assay for viral RNA quantitation

Primer binding sites were selected in the most conserved sequences in the p24 region of the *gag* gene among all subtypes and CRFs of HIV-1 (Fig. 1) according to the HIV sequence database (Los Alamos database— http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). Two oligonucleotides that differ in nucleotide at positions 1374 and 1380, GAF1 and GAF2, were used together as upstream primers to minimize mismatches with HIV-1 sequences reported in the database (Fig. 1). The size of amplicons of the target RNA and competitor RNA was expected to be 138 and 94 bp, respectively.

RNA was extracted from 140 μl of patient plasma with a QIAamp Viral RNA kit (QIAGEN) with elution in 80 μl. A 20 μl volume of 1, 1/10, and 1/100 dilutions of the eluate were used for subsequent competitive RT-PCR. Three different copy numbers of LAI RNA (10, 50, and 250) were assayed in duplicates as external controls.

RT-PCR was carried out in a 50 μl solution containing primers (0.2 μM GAR, 0.1 μM GAF1, and 0.1 μM GAF2), competitor RNA equivalent to 50 copies, and 1 × SuperScript III RT/Platinum *Taq* Mix (Invitrogen, Carlsbad, CA) with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal profile of RT-PCR was: 10 min at 50 °C for RT; 2 min at 94 °C for initial denaturation; 5 s at 94 °C, 10 s at 48 °C, and 15 s at 72 °C for 5 cycles; 5 s at 94 °C, 10 s at 62 °C, and 15 s at 72 °C for 35 cycles; and 1 min at 72 °C for extended elongation. Introduction of the elongation step at 48 °C was intended to minimize the effect of mismatches between primers and naturally occurring HIV-1 variants.

The plasma samples were also assayed with COBAS Amplicor HIV-1 Monitor Test v1.5 (Roche Diagnostics).

2.4. Electrophoresis and evaluation

A 20 μl sample of PCR products was electrophoresed in a 2% agarose gel at 160 V for 40 min. The gel image was photographed with a Polaroid camera under UV illumination and

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scanned with a CanoScan FB-636U scanner (Canon, Tokyo, Japan). The band intensity of targets and competitors was evaluated with the NIH Image program (http://rsb.info.nih.gov/nih-image/Default.html). A calibration curve was obtained from the ratios between the bands of the external controls and competitors with MS Excel software (Microsoft, Seattle, WA). The copy numbers of the samples were determined by fitting their ratios to the calibration curve.

3. Results

3.1. Competitive RT-PCR system

Five different copies (10, 25, 50, 125, and 250) of control HIV-1 LAI RNA were amplified together with a fixed dose of competitor RNA, and the ratios between the band intensity of target RNA and the band intensity of competitor RNA were plotted against the input copy numbers of target RNA (Fig. 2A). Good linearity was obtained, with a high correlation coefficient of 0.995.

A photograph of a typical electrophoretogram of competitive RT-PCR for RNA samples from plasma of HIV-1-infected individuals is shown in Fig. 2B. DNA fragments of two different sizes were observed: a 138-bp fragment amplified from target HIV-1 RNA and a 94-bp fragment amplified from competitor RNA. In practical runs, a calibration curve was obtained from co-amplification of each of three different numbers of copies (250, 50, and 10) of LAI RNA with competitor RNA. Fig. 2B shows that ratios of target and competitor RNA bands for patients 1 and 2 were calculated as 2.66 and 4.10, respectively, and applying them to a calibration curve revealed HIV-1 RNA copy numbers per milliliter of 4500 and 6600, respectively.

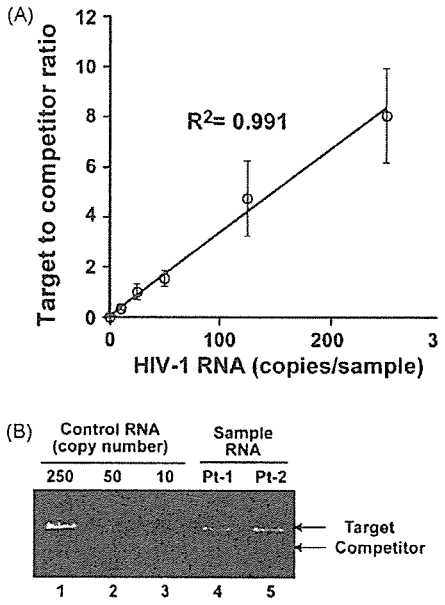

Fig. 2. Linearity and a typical agarose electrophoretogram of the results of competitive RT-PCR. (A) Linear relationship between target/competitor band ratios and initial HIV-1 RNA copy numbers. Each measurement was performed in five replicates. Means and standard deviations are indicated by circles and bars, respectively. (B) Lanes 1–3 are control LAI RNA at levels of 250, 50, and 10 copies/sample, respectively. Lanes 4 and 5 are two clinical samples (Pt-1 and Pt-2). Target/competitor band ratios of the clinical samples (2.66 and 4.10) were translated into copy numbers (160 and 240 copies per sample) using a calibration curve obtained from lanes 1–3.

Table 1
Detection limit of competitive RT-PCR by limiting dilution assay

HIV-1 RNA (copies/sample)	Target amplification	
	+	–
20	10	0
10	10	0
5	7	3
2.5	2	8

Control LAI RNA samples of two-fold serial dilutions were assayed in 10 replicates, and the detection limit was calculated by Probit analysis.

3.2. Detection limit and reproducibility of the competitive RT-PCR

The lower detection limit of the assay was assessed by testing multiple replicates of dilutions of the HIV-1 LAI RNA (subtype B) that had been quantitated by using the Poisson distribution analysis. Dilutions equal to 560, 280, 140, and 70 copies/ml were tested in 10 replicates at each dilution (Table 1). A Probit analysis showed that the 50% detection limit was 110 copies/ml. Since samples of HIV-1 subtypes A, B, C, and F and CRF01_AE exhibited equivalent efficiency of amplification and detection as described below (Fig. 3), the detection limit for subtype non-B viruses was concluded to be at the same level. The dynamic range of 110–700,000 copies/ml was obtained by testing three serial dilutions (1, 1/10, and 1/100) of the RNA sample.

The reproducibility of the competitive RT-PCR assay was assessed by assaying five seronegative plasma samples spiked

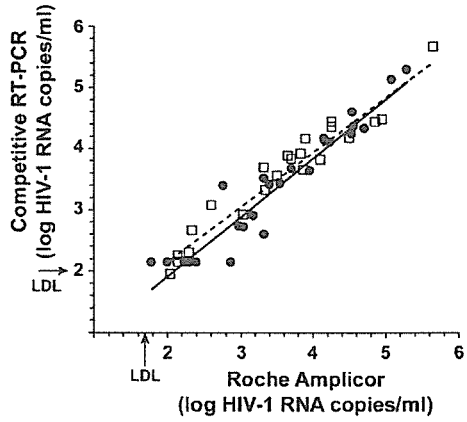

Fig. 3. Comparison between the results of competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5. Plasma HIV-1 RNA levels of 47 clinical specimens containing subtype B (solid circle) and non-B (open square) are plotted. The lower detection limit (LDL) by competitive RT-PCR and Amplicor was 110 and 50 copies/ml, respectively. The correlation coefficient was 0.956 for subtype B and 0.947 for non-B. Simple regression lines for subtype B (dotted line) and non-B (solid line) are shown.

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Table 2
Reproducibility of competitive RT-PCR

Test[a]	Sample[b] (copies)					
	A	B	C	D	E	Mean ± SD
1	48.5[b]	39.4	37.2	36.8	30.9	37.2 ± 6.4
2	47.6	35.8	37.9	34.8	27.4	35.8 ± 7.3
3	41.5	47.8	40.2	36.2	33.2	40.2 ± 5.6

[a] Viral RNA extraction and competitive RT-PCR reaction were performed in triplicate in three independent experiments.
[b] Fifty copies of LAI RNA were added to the plasma from a healthy human subject in each sample tube.

with 50 copies of HIV-1 LAI RNA in triplicate (Table 2). The inter-assay and intra-assay CV values were 25.9% and 9.1%, respectively, and comparable to those obtained with commercially available quantitation kits (16.2–87.5%) (Lin et al., 1998; Muyldermans et al., 2000).

3.3. Quantitation of clinical samples

The HIV-1 RNA concentration of 47 seropositive plasma samples of subtypes A, B, C, and F and CRF01_AE was analyzed with both Cobas Amplicor HIV-1 Monitor test v1.5 and the competitive RT-PCR assay (Fig. 3). The results showed good agreement between the two assays: the correlation coefficient for subtype B and subtype non-B was 0.956 and 0.947, respectively; and the ratio between the two measurements was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Although the number of samples in this study was limited, these data suggest that the competitive RT-PCR allows quantitation of both subtype non-B and subtype B of the viral load.

4. Discussion

In order to establish a quantitation method for HIV-1 RNA in plasma that could be used as a routine clinical test in resource-poor countries, an attempt was made to develop an alternative to the commercial kits. The assay described above is based on a competitive RT-PCR technique and is characterized by several improvements over similar methods reported previously. First, it enabled quantitation of HIV-1 RNA in 47 plasma samples containing different HIV-1 subtypes (A, B, AE, C, F) belonging to group M. The viral loads measured with the present assay were consistent with those determined with Cobas Amplicor HIV-1 Monitor, and the ratio was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Two competitive RT-PCR assays have been previously reported to detect or quantitate non-subtype B viruses (Emery et al., 2000; Ernest et al., 2001), but since both assays tested primary isolates containing extremely high titers instead of viruses in patients' plasma, the inhibitory effect of plasma components on RT or PCR was not examined. The target of PCR in the present study was selected as the most conserved sequence in the *gag* gene among all subtypes and CRFs of HIV-1 group M (HIV Sequence Database, Los Alamos). Furthermore, to mitigate the influence of sequence variations among subtypes on viral RNA quantitation, a mixture of two upstream primers was employed that differ at two nucleotide positions to minimize mismatches between primers and HIV-1 RNA variants, and 5 cycles with a low annealing temperature of 48 °C were introduced during the early phase of PCR.

The present assay is simple and easy to perform, and the entire procedure, from RNA extraction to analysis of electrophoresis images, takes only 4 h. The use of hot-start DNA polymerase (Platinum *Taq*) enables preparation of PCR samples at room temperature and combination of RT and PCR into one step. Because of the shortness of the amplicon (138 bp), brief reaction times were adequate for RT (10 min) and DNA elongation (15 s each) during PCR. High sensitivity for HIV-1 RNA detection was also achieved. A Probit analysis showed a 50% detection limit of 110 copies of HIV-1 RNA per ml in plasma, which is comparable with the results reported in previous studies (Piatak et al., 1993; Venturi et al., 2000), although a smaller sample volume (140 μl) was used than in those studies (2.8 and 1.8 ml, respectively).

Several commercial HIV-1 quantitation assays, such as Amplicor (Roche Diagnostics), Nuclisens (bioMérieu), and Quantiplex (Bayer), are being used as routine tests to monitor patients being treated with antiretroviral drugs in developed countries. However, they are expensive and require the use of special instruments that are often unaffordable in developing countries. The present method costs only about 10 dollars per sample and does not require any special instruments except a microcentrifuge, gel electrophoresis system, and ultraviolet transilluminator, and the simplicity of the method means that no special training program is needed for clinical technologists. These features should be advantageous in regard to adoption of the assay in resource-poor countries.

One common problem of competitive RT-PCR is the narrow dynamic range. In the current assay, 1-, 1/10-, and 1/100-fold dilutions of plasma RNA samples were subjected to RT-PCR separately to cover a quantitation range from 110 to 700,000 copies/ml. Because an adequate dilution ratio cannot be predicted for each plasma sample, a fixed amount of competitor RNA (50 copies) is added to a reaction mixture rather than a plasma sample. Thus, this protocol does not correct measurements of viral loads in terms of RNA extraction efficiency, although the recovery of RNA by QIAamp Viral RNA kits (QIAGEN) was found to be almost constant at 77% (Table 2).

In conclusion, the competitive RT-PCR assay described in this study is a good candidate for an HIV-1 viral load assay for use in monitoring ART in resource-poor settings.

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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

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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.,

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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	1.8×10^5	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	5.0×10^5	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.

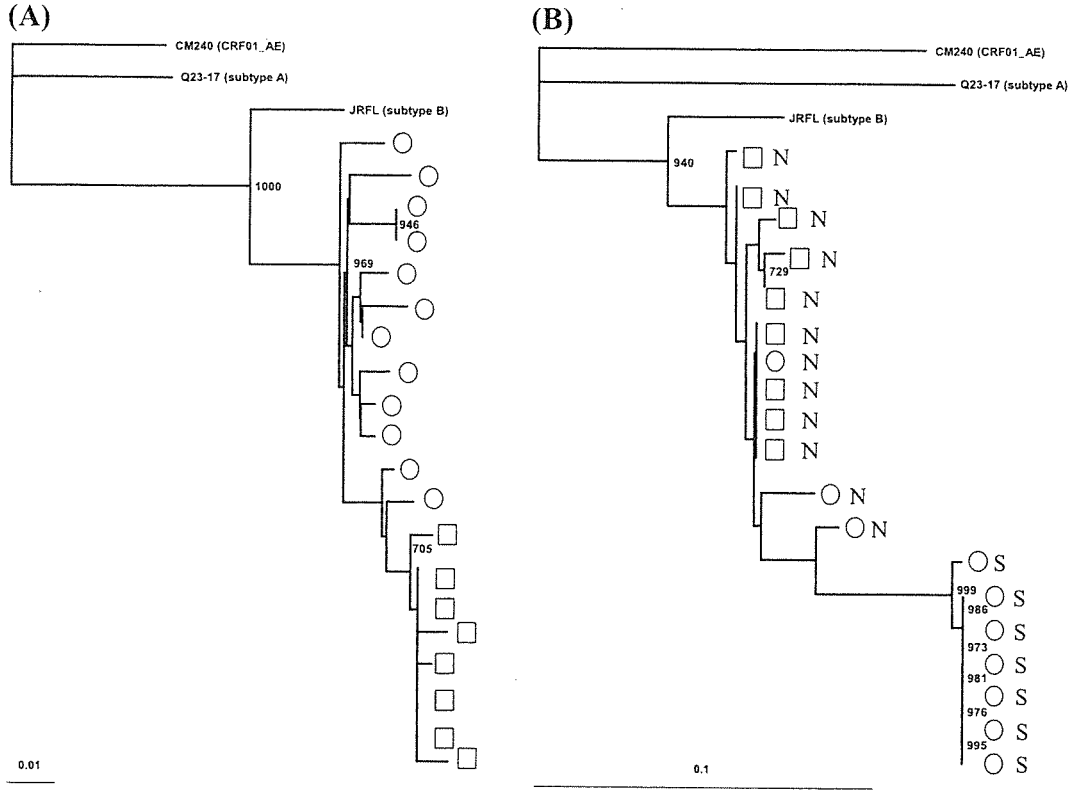

Fig.1. Neighbour-joining phylogenetic tree depicting the relationship of the nucleotide sequences of partial reverse transcriptase (A) and gp120 V3 (B) clones in plasma HIV RNA and PBMC proviral DNA at time A (Mar-2000). Branch lengths are drawn to scale, and bootstrap values greater than 70% are shown 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 PBMC's 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.

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長野県におけるHIV陽性者の診断契機と免疫不全進行度

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Diagnostic Clues and Development of Immunodeficiency in Patients with HIV Infection in Nagano Prefecture

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Nagano ranks third in Japan in terms of the percentage of HIV-positive people in its population.

We analysed the number of CD4+ T cells, the ratio of AIDS in HIV-positive people, diagnostic clues and the past histories in 125 patients who visited the AIDS base hospitals in Nagano from January 2001 to August 2005.

58.4% of patients had developed to AIDS when they were diagnosed as HIV positive. CD4+ T cells were below 200/μl in 64.8% and severe immunosupression (CD4+ T cells below 50/μl) was observed in 40.8%. The median number of CD4+ T cells at diagnosis was 70.5/μl. Frequent clues to the diagnosis of HIV infection were found to be pneumocystis pneumonia, oral candidasis and body weight loss. Herpes zoster infection, sexually transmitted infections (STIs), and refractory eruptions were observed in past histories.

In Nagano Prefecture, many of the patients were diagnosed as HIV positive when they had already developed to AIDS. Past histories such as herpes zoster infection, STIs, and refractory eruptions seemed to be clues for making an early diagnosis by general clinicians, which will be essential to improve the prognosis and prevent the spread of the disease. *Shinshu Med J 54 : 183—187, 2006*

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Key words: HIV, AIDS, Nagano Prefecture, early diagnosis, STI
HIV，エイズ，長野県，早期診断，性感染症

I はじめに

長野県は人口あたりの累積のエイズ患者数・HIV感染者数とも，東京都，茨城県に次いで全国で3番目に多い地域である。2004年における都道府県別の新規のHIV陽性者数（発症とキャリアの合計）では長野県は7番目であるが，人口あたりで比較すると，東京に次いで第2位である。さらに，エイズ発症者に限ると，人口あたりで東京をしのいで全国第1位となっている[1)]。これは，進行してエイズを発症してから初めてHIV感染症と診断される例が極めて多いことを表している。今回我々は長野県内のHIV感染症・エイズの実状について調査・解析を行い，HIV感染判明時の進行度を明らかにし，HIV感染者の既往歴から早期診断の手がかりをつかむため検討を行ったので報告する。

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II 対象および方法

2001年1月から2005年8月までの4年8カ月間に長野県内のエイズ治療拠点病院（長野赤十字病院，厚生連佐久総合病院，信州大学医学部附属病院，国立病院機構松本病院，長野県立須坂病院，国立病院機構長野