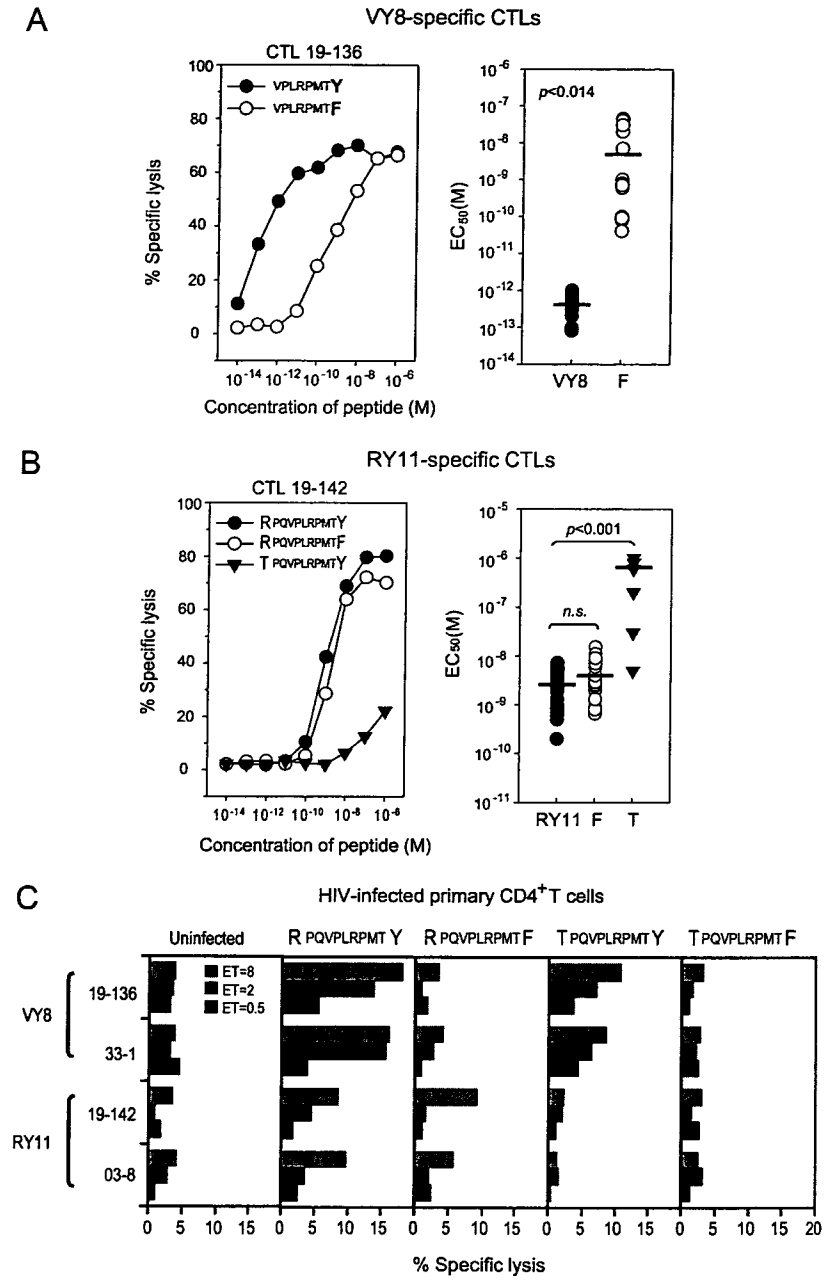

FIGURE 3. CTL responses to variant Ags. *A* and *B*, VY8 and RY11-specific CTL clones (same clones as in Fig. 2, *A* and *B*) were tested for their ability to respond to variant peptides by using C1R-B3501 cells pulsed with various concentrations of the wt or variant peptides (ET = 2). Representative peptide-titration data obtained for CTL 19-136 and 19-142 are shown (each *left panel*). EC_{50} values thus obtained for an additional 9 clones (total 10 clones) are also shown (each *right panel*). Horizontal bars indicate means, and statistic analysis was performed by using the paired *t* test. Cytotoxic activity in the absence of the peptide was always <3%. *C*, The VY8- and RY11-specific CTL clones were analyzed for their cytolytic activity toward target cells at ET = 0.5, 2, and 8 as indicated. The target cells were primary $CD4^+$ T cells that had been isolated from an HIV-negative donor (HLA-$B3501^+$), activated by PHA, and infected with wt or various variant viruses. The frequency of HIV-infected cells among target cells as determined by intracellular p24 Ag expression was 31.5, 33.2, 34.5, and 29.8% for wt, RF, TY, and TF variants, respectively. An additional experiment conducted by using a different blood donor (HLA-$B3501^+$) showed similar results.

combination of both mutations selectively diminishes the HLA-I down-regulation activity by Nef.

Effects of Nef mutations on cytolytic activity of CTL clones with other specificity

To test whether the observed differences in HLA-I down-regulation affect the susceptibility of HIV-infected cells to recognition by CTLs, we assessed the cytolytic activity of CTL clones with specificity to HIV-1 gene products other than Nef and other restriction toward primary CD4 T cells infected with wt and Nef variant viruses.

Freshly isolated CD4 T cells from an HIV-negative donor (HLA-$B35^+$ and HLA-$A24^+$) were infected with various HIV-1 as above and mixed with CTL clones specific for Pol and Env epitopes presented by HLA-B*3501 as well as with a clone specific for another Nef epitope presented by HLA-A*2402 (designate as B35-Pol, B35-Env, and A24-Nef, respectively). Although the amino acid sequences in the epitope regions of B35-Pol, B35-Env, and A24-Nef were the same among the wt and variant viruses tested, CTL-mediated killing activity appeared to be different among target cells infected with these viruses (Fig. 4*C*). Both B35-Pol and B35-Env CTLs showed most potent cytotoxic activity toward target cells infected with the ΔNef variant, whereas the same CTLs showed weak cytolytic activity toward wt virus-infected cells (Fig. 4*C*). Interestingly, CTLs markedly killed cells infected with the TF double mutant virus, whereas they weakly killed cells infected with either T75 or F85 single mutant virus (Fig. 4*C*). Moreover, in A24-Nef CTL-mediated cytotoxic activity, we also observed that the TF double mutant virus-infected cells were more potently killed than cells infected with wt or single mutant viruses (Fig. 4*C*). These data suggest that the diminished HLA-I down-regulation (i.e., increased level of cell surface HLA-I) in CD4 T cells infected with the TF double mutant virus resulted in increased susceptibility to killing by CTLs, leading to a possible selective disadvantage for the variant virus in vivo.

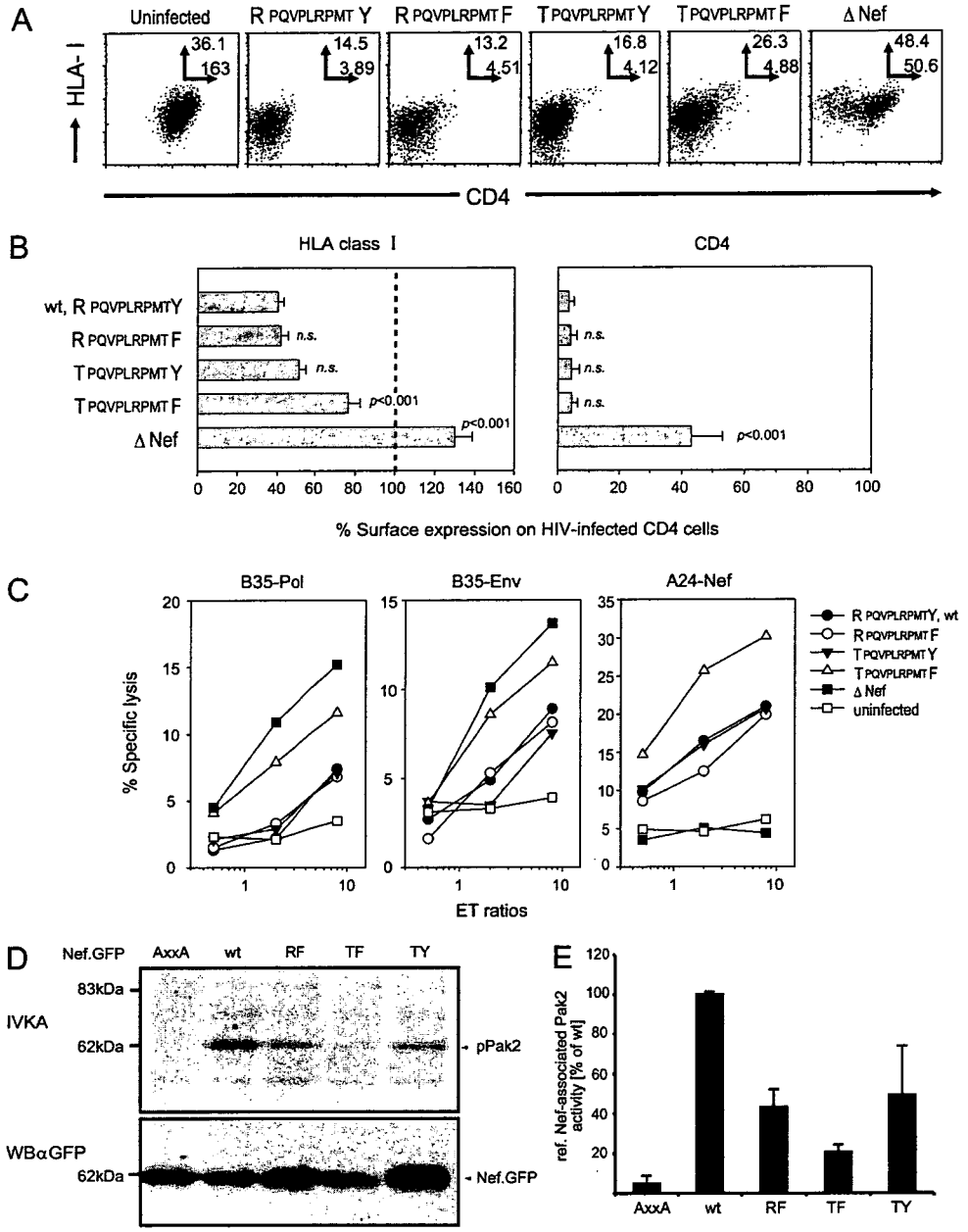

FIGURE 4. Functional consequences of CTL escape Nef mutations. *A*, Freshly isolated primary $CD4^+$ cells from an HIV-negative donor (HLA-$B35^+$) were activated by PHA for 3 days and then infected with wt or various variants for 5 days. The cells were stained with anti-HLA-Bw6 mAb (clone: SFR8-B6) and anti-CD4 mAb, and 7-AAD followed by intracellular staining for p24 Ag. In flow cytometric analysis, cells negative for 7-AAD and positive for p24 Ag were gated and analyzed for their fluorescence intensity for HLA-Bw6 and CD4. The frequency of infected cells was 29.6, 34.3, 30.5, 31.9, and 26.2% for HIV-1 wt, RF, TY, TF, and ΔNef variants, respectively. The mean fluorescence intensities (MFI) for HLA-Bw6 and CD4 are shown in the *right upper corner* of the dot plots. *B*, The same experiment as above was done by using three additional HIV-negative donors. The Ab specific for HLA-I allotypes used was either SFR8-B6 or A11,1M as appropriate for each donor. The MFI level of HLA-I and CD4 on uninfected cells was set to 100% and indicated by the dotted vertical line in the graph. Statistical analysis was performed by ANOVA with multiple comparisons vs wt. *n.s.*, not significant. *C*, Primary $CD4^+$ cells infected with wt or various variant HIV-1s as in Fig. 3 (the donor carries both HLA-A*2402 and HLA-B*3501) were used as target cells for cytolysis by CTL clones specific for HLA-B3501-restricted Pol ($Pol_{273-282}$: VPLDKDFRKY), Env (Env_{77-85}: DPNPQEVVL), or HLA-A2402-restricted Nef epitope ($Nef_{138-147}$: RYPLTFGWCF). An additional experiment using a different blood donor (positive for both HLA-A*2402 and HLA-B*3501) showed similar results. *D*, Nef-associated Pak2 activity. Jurkat cells were electroporated with plasmid DNAs encoding the indicated Nef-GFP fusion proteins. Total cell lysates were immunoprecipitated with anti-GFP Ab, and the resultant immunoprecipitates were analyzed by IVKA for Pak2 autophosphorylation (pPak) (*upper panel*). The same IVKA reactions were directly separated by SDS-PAGE and analyzed for immunoisolated Nef-GFP levels by Western blotting with anti-GFP Ab (*lower panel*). *E*, Quantification of Nef-Pak2 association. The indicated values represent the Nef-associated Pak2 activity after the levels of pPak2 had been normalized to the amounts of immunoisolated Nef-GFP. Values presented are the mean of at least three independent experiments with the indicated SEM expressed relative to the wt control that was arbitrarily set to 100%.

Effects of Nef mutations on the association of Nef with the cellular kinase Pak2

Given this reduced ability to down-modulate cell surface HLA-I, we also wanted to assess whether other Nef activities that depend on the interaction of the PxxP motif with SH3 domain-containing ligands are affected by the CTL escape mutations. To this end, we analyzed the association of Nef with cellular Pak2 kinase activity. This interaction is conserved among a variety of lentiviruses (39),

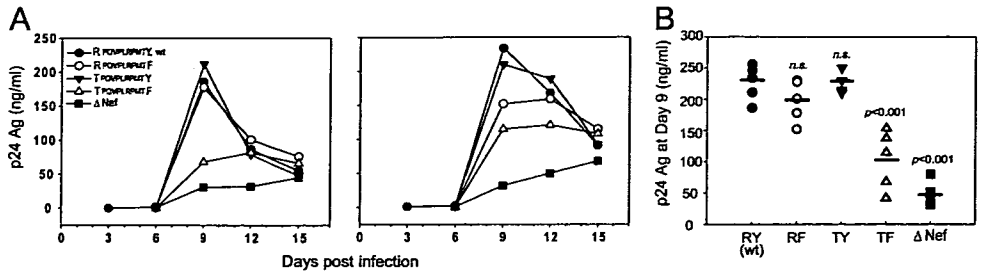

FIGURE 5. Effects of Nef mutations on viral replication in PBMC. *A*, Freshly isolated PBMC samples from two HIV-negative donors were first infected with wt or various variant HIV-1s and 3 days later cells were activated by PHA. For monitoring viral replication, culture supernatants were collected every 3 days and replaced with fresh medium containing rIL-2. *B*, The same experiment as above was done by using three additional HIV-negative donors. The level of p24 Ag obtained at day 9 postinfection was plotted and statistically analyzed based on ANOVA with multiple comparisons vs the wt. *n.s.*, not significant. Horizontal bars indicate means of data obtained for the five different PBMC donors.

strictly depends on the integrity of the PxxP motif and has multiple functional consequences that may optimize virus production (10, 40, 41). Expectedly (34, 39), wt Nef from HIV-1 SF2 (wt) showed robust association with phosphorylated Pak2 (pPak2) while the AxxA mutant (both Pro^{76} and ^{80} to Ala) did not show substantial association with pPak2 (Fig. 4*D*). Pak2 association was substantially reduced but not abrogated for the F85 (RF) and T75 (TY) single variants. According to phosphorimager quantification of the Nef-associated Pak2 signal and normalization to the levels of Nef present in the IVKA (Fig. 4*E*), Pak2 association was more than two-fold reduced for these two Nef variants relative to wt. The TF double mutant displayed an even stronger reduction to ~20% Pak2 association relative to wt Nef (Fig. 4, *D* and *E*). These data suggest that the T75 and F85 mutations in the PxxP region of Nef affect its ability to interact with SH3 domain-containing ligands.

Effects of Nef mutations on viral replication in PBMC

Nef significantly enhances virus replication in primary CD4 T cells, particularly if these cells are exposed to HIV-1 before activation with mitogens (42, 43). Because amino acid substitutions from prolines to alanines in the PxxP region have been shown to decrease this activity (25, 26, 32), we asked whether the T75 and F85 mutations would have similar effects.

Freshly isolated PBMC from two HIV-negative donors were first exposed to wt or various variant viruses for 3 days and subsequently activated by PHA. In both donors' PBMC, the wt and T75 variant viruses showed comparable replication kinetics, whereas the replication of the ΔNef virus was substantially delayed (Fig. 5*A*). The replication of the F85 variant virus was comparable to that of the wt virus in PBMC of a donor and was partially impaired with PBMC of another one (Fig. 5*A*). In contrast, the double TF variant virus showed delayed replication kinetics in PBMC from both donors (Fig. 5*A*).

To account for this donor variability, results from a total of five donors are summarized in Fig. 5*B*. As all PBMC samples showed a peak on day 9 after infection with the wt virus, the amounts of p24 Ag at day 9 after infection with the variant viruses were measured and statistically analyzed by multiple comparisons vs the wt (Fig. 5*B*). The ΔNef virus showed reproducibly the weakest replicative capacity under this assay condition, in good agreement with previous reports (26, 32). In addition, the TF double variant virus showed diminished capacity for viral replication compared with the wt; whereas each type of single variant virus did not show much difference in replication capacity (Fig. 5*B*). These data demonstrate that, even in the absence of HIV-specific CTL responses, the combination of T75 and F85 mutations is disadvantageous for Nef's ability to enhance virus replication.

Discussion

It is thought that the *nef* gene has higher levels of mutational plasticity in response to selective pressures compared with genes exhibiting structural or functional constraints (e.g., Gag, protease, reverse transcriptase, or integrase), because it exhibits considerable sequence diversity in vivo. In fact, some CTL escape variants of Nef, such as those with the mutations located in the CTL epitopes restricted by HLA-B*57 and HLA-A*24, have been suggested to have minimum fitness cost on the virus. This is because, in such a region, reversions are not often observed after transmission of the virus to new hosts who are negative for that particular *HLA-I* allele and the mutations are readily fixed in the population in the meantime (44, 45). In contrast, we show in the present study that the naturally arising mutations in the well-conserved PxxP region of HIV-1 Nef are selected under active CTL-mediated selective force at work and these mutations alone or in combination can modulate the pathogenic function by HIV-1 Nef including HLA-I down-regulation, enhancement of viral replication, and association with an activated cellular kinase, strongly suggesting that these mutations can impose functional constraints on the Nef activity and viral replication in vivo. Considering that various Nef activities substantially vary during the course of an infection at different stages of disease progression (4) and that there are substantial numbers of HLA-I-associated sequence variations in Nef (46–48), immunosurveillance by the Nef-specific CTLs plays additional roles in modulating the pathogenic potential of HIV-1 through selection of CTL-escape mutations in Nef particularly those in a well-conserved functional region.

It is obvious that HLA-B35-restricted CTL responses were shifted in patients during the early to chronic phase of an HIV-1 infection in our study, as the Nef VY8 epitope was dominantly recognized by CTLs relatively early in the infection, whereas the N-terminal extended RY11 epitope was recognized by CTLs in the chronic phase. This observation is in line with previous reports showing that CTL epitope specificity is different during the course of an HIV infection (23, 49, 50). Particularly, an immunodominant response directed against the HIV Gag p17-derived, HLA-A0201-restricted SL9 epitope (SLYNTVATL) was not detected early in an infection (50). Although the mechanisms underlying this phenomenon are not yet known, one possible explanation is that the responses detected in the early stage of an infection could have "mutated away," opening the field for a second wave of CTL specificities taking over in their place. The CTLs induced by a second or third waves of CTL specificities may have decreased antiviral effectiveness as predicted in the mathematical antigenic oscillation

model proposed by Nowak et al. (51). Our data support this scenario that the highly active VY8-specific CTLs elicited early in an infection were rendered ineffective apparently due to the acquisition of the F85 Nef mutation by the virus and that subsequently the cross-reactive RY11-specific CTLs, yet having moderate antiviral activity, became dominant. It is interest to note that the T75 variant, which had been selected by RY11-specific CTLs during the chronic phase, can induce de novo variant-specific CTLs with less effective Ag-specific proliferative capacity, further reducing antiviral activity of CTLs in vivo (29).

HLA-B*35 has been documented to be associated with rapid disease progression to AIDS (52). However, a further detailed study showed that individuals having HLA-B*35 allelic variants, including B3502/3503/3504, progress more rapidly to AIDS than do those with HLA-B*3501 (53). All the HLA-B35$^+$ subjects in this study were considered to carry HLA-B*3501, as the HLA-B*3501 is highly prevalent in the HLA-B35$^+$ Japanese population, though we have not yet done the genotypic analysis of *HLA-B* loci of all of the subjects. Further studies are needed to clarify whether CTL responses toward the PxxP region of Nef may be associated with the difference in the disease progression among HIV-infected patients having different HLA-B35 allelic variants.

Although the TF double mutation provided the best CTL escape of the Nef variants tested here, this variant was barely selected in HLA-B35$^+$ patients. This suggested that important functional constrains imposed by these combinatorial mutations precluded selection of these variants. The present study revealed at least two independent possible reasons for such a counterselection. First, the T75 and F85 double mutation in HIV-1 Nef significantly reduced the down-regulation activity of HLA-I and resulted in increased recognition by Pol- and Env-specific CTLs. Because down-regulation of MHC-I by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo (14), these results suggest that the sustained HLA-I down-regulation activity by HIV-1 Nef is required for efficient viral replication in vivo. This observation is in line with a previous report demonstrating that Nef mutations selected by Nef-specific CTLs in vitro, although most mutations disrupted *nef* reading frames in their study, leads to progeny virions that are increased in their susceptibility to CTLs with specificities for proteins other than Nef (54). However, the mutations in that report are different from representative naturally arising variations (54) as the *nef* reading frame is highly maintained intact in vivo (55) and large deletions or frame shifts are rarely observed. In contrast, the current study focused on the naturally arising mutations that are selected under Nef-specific CTL responses in vivo.

Second, the double mutation also affected PxxP-dependent activities of Nef in the absence of HIV-specific CTLs and significantly impaired Nef's ability to boost HIV-1 replication in primary human T lymphocytes. Because the individual mutations caused no significant impairment to HIV replication in the experimental system used, these results also help to explain why the double mutant is counterselected in HIV-infected patients. On the molecular level, Nef's effects on viral replication are likely mediated by a number of yet to be fully defined protein interactions. Among others, its association with Pak2 activity has also been implicated in the Nef-mediated enhancement of virus infectivity and replication (40, 56, 57). In this scenario, our results suggest that the reduction of Nef-Pak2 below a certain threshold activity may contribute to the reduction of Nef's ability to boost HIV spread. More importantly, the reduction of Pak2 association indicates that CTL escape Nef variants are impaired in their interaction with SH3 domains, which is expected to have select functional consequences in various cellular environments.

Together, these results demonstrate that CTL escape has severe consequences on the functionality of the PxxP motif in Nef, both for its role in immunoevasion and intrinsic replicative potential of the virus. Thus, a vaccine regimen that can elicit CTL responses targeting the regions involved in HLA-I down-regulation activity by Nef could be a potent candidate for future vaccine design.

Acknowledgments

We thank Y. Idegami and T. Akahoshi for their technical help. We also thank Drs. A. Kawana-Tachikawa, Zabrina Brumme, and Chanson Brumme for helpful discussion.

Disclosures

The authors have no financial conflict of interest.

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Different immunodominance of HIV-1-specific CTL epitopes among three subtypes of HLA-A*26 associated with slow progression to AIDS

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Received 1 November 2007
Available online 21 November 2007

Abstract

It is speculated that HLA-A*26-restricted HIV-1-specific CTLs can control HIV-1, since HLA-A*26 is associated with a slow progression to AIDS. In three major HLA-A*26 subtypes, HLA-A*2601-restricted, and HLA-A*2603-restricted HIV-1 epitopes have been identified, but HLA-A*2602-restricted ones have not. We here identified HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and compared the immunodominance of the epitopes among the three subtypes. Out of 110 HIV-1 peptides carrying HLA-A*26 anchor residues, only the Gag169-177 peptide, which had been previously identified as an HLA-A*2601- and HLA-A*2603-restricted immunodominant epitope, induced Gag169-177-specific $CD8^+$ T cells from only two of six HLA-A*2602^+ HIV-1-infected individuals. No difference in affinity of this epitope peptide was found among these three HLA-A*26 subtypes, indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected HLA-A*2602^+ individuals. These findings indicate different immunodominance of Gag169-177 epitope among 3 HLA-A*26 subtypes.

Keywords: HLA-A*26; HLA-A*2602; HIV-1; Epitope; CTL

Human immunodeficiency virus type-1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) responses play an important role in the control of HIV-1 infections [1–5]. However, it is thought that HIV-1 can escape from the host immune system, since it fails to completely eradiate HIV-1 from infected individuals. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific $CD8^+$ T cells [6–11]. A mutation within the viral epitopes recognized by CTLs is one of these mechanisms [6,7]. Identification and characterization of HIV-1 CTL epitopes are therefore necessary for studies on the immunopathogenesis of AIDS. In addition, since HIV-1-specific CTLs are expected to suppress HIV-1 replication *in vivo*, characterization of these epitopes is also necessary for studies aimed at developing HIV-1 vaccines and immunotherapy to induce HIV-1-specific CTLs, either of which might be expected to prevent HIV-1 infection and the progression to AIDS.

HLA-A*26 is one of the alleles associated with a slow progression to AIDS [12]. Therefore, identification and characterization of HIV-1-specific epitopes presented by this allele are necessary for studies on the immunopathogenesis of AIDS and vaccine development. Three HLA-A*26 subtypes, i.e., HLA-A*2601, HLA-A*2602, and HLA-A*2603, are found at a gene frequency of 7.7%, 2.3%, and 1.5%, respectively, in the Japanese population [13]. We previously identified four HLA-A*2601- and two HLA-A*2603-restricted HIV-1 epitopes by using reverse immunogenetics [14,15]. Both HLA-A*2601 and -A*2603 presented one immunodominant epitope, Gag169-177

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doi:10.1016/j.bbrc.2007.11.031

(EVIPMFSAL), which overlaps with the HLA-B*57-restricted immunodominant epitope KF11 (KAFSPEVIPMF) [16]. Gag169-177-specific CTLs have been speculated to control HIV-1 replication.

In the present study, we sought to identify HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and to compare them with HLA-A*2601- and HLA-A*2603-restricted ones [14,15].

Materials and methods

Cells. C1R and TAP-defective cells of mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2601, -A*2602 or -A*2603 (C1R-A*2601, C1R-A*2602 or C1R-A*2603, respectively) were generated by transfecting the C1R cells with the HLA-A*2601, -A*2602 or -A*2603 gene, respectively [17]. RMA-S transfectants expressing HLA-A*2602 (RMA-S-A*2602) were previously generated [18]. C1R-A*2601, -A*2602 and -A*2603 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A* 2602, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Synthetic peptides. Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2602-binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

HLA-stabilization assay. Binding of HIV-1-derived peptides to HLA-A*2602 was measured as previously described by using RMA-S-A*2602 cells [18]. RMA-S-A*2602 cells were cultured for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [19] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, CA, USA). HLA-A*2602-binding peptides were defined as those which at a concentration of 10^{-3} M caused a >25% increase in MFI compared with the MFI of control RMA-S-A*2602 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value.

Patients. Blood samples were collected with informed consent from six HIV-1 clade B-infected patients with HLA-A*2602 (KO-003, KI-021, KI-030, KI-082, KI-382, and KI-478), 11 those with HLA-A*2601, and eight those with HLA-A*2603 at the AIDS Medical Center, National Hospital Organization, Osaka National Hospital or the AIDS Clinical Center, International Medical Center of Japan. Clinical stage of all patients tested was chronic one. Significant difference of CD4 count was not found among three subtype groups (HLA-A*2601:458±257, HLA-A*2602:564±299, HLA-A*2603:314±109). This study was approved by the ethical committees of Kumamoto University, International Medical Center of Japan, and Osaka National Hospital. Informed consent was obtained from all subjects, according to the Declaration of Helsinki.

Intracellular cytokine staining (ICC assay). After C1R-A*2601, C1R-A*2602 or C1R-A*2603 cells had been incubated for 60 min with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These C1R-A*26 cells and cultured PBMCs were incubated at 37 °C for 6 h at an effector-to-stimulator ratio of 1:4 after the addition of Brefeldin A (10 μg/ml). Next, the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized at 4 °C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing 2% paraformaldehyde, and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was determined by using the FACSCalibur.

*ICC assay using C1R-A*2602 cells infected with recombinant HIV-1 vaccinia.* C1R-A*2602 cells were infected for 1 h at 37 °C with 10 plaque-forming units (per target cell) of recombinant vaccinia virus expressing HIV-1 SF2 Gag protein or of WT vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells at 37 °C for 6 h after the addition of Brefeldin A (10 μg/ml). The ability of the effector cells to produce IFN-γ was tested at an E:S ratio of 1:4. The cells were then stained with anti-CD8 mAb and anti-IFN-γ mAb.

Results and discussion

HLA-A*2602-binding peptides have two anchor residues, Val, Phe, Ile, Leu or Thr at position 2 and Tyr, Phe, Met or Leu at the C-terminus [17]. A previous study demonstrated that acidic amino acids (Asp and Glu) and a broad range of amino acids with the exception of positively charge amino acids function as an anchor at position 1 and the C-terminus, respectively [18]. Therefore, to identify HLA-A*2602-binding HIV-1 peptides, 8-mer to 11-mer sequences containing the anchor residues Asp or Glu at position 1, Val, Thr, Ile, Leu or Phe at position 2, and any amino acids except positively charged ones at the C-terminus were selected from the sequence of Gag, Pol, Nef, and Env proteins in the HIV-1 SF2 strain; and then 110 peptides matching these sequences were synthesized. The binding affinity of these synthesized peptides for the HLA-A*2602 molecule was tested by using the HLA-stabilization assay [18,20]. Representative results are shown in Fig. 1. Thirty-two out of these 110 peptides bound to HLA-A*2602 (Table 1). The frequency of HLA-A*2602-binding HIV-1 peptides was similar to that of HLA-A*2601- or HLA-A*2603-binding HIV-1 peptides previously identified by using the same 110 peptides [14,15].

PBMCs from three HLA-A^*2602^+ HIV-1-infected individuals (KO-003, KI-030 and KI-082) were stimulated

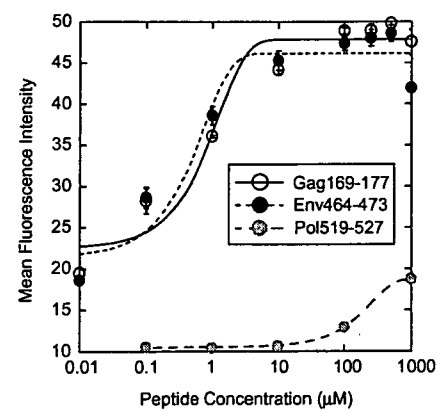

Fig. 1. Binding to HLA-A*2602 of HIV-1 peptides carrying HLA-A*2602 motif. Binding of the peptides carrying A*2602 anchors was measured by a stabilization assay using RMA-S-A*2602 cells. Representative results showing high-affinity peptides (Gag169-177 and Env464-473) and a very low-affinity peptide (Pol519-527) are given in this figure.

Table 1
HLA-A*2602-binding peptides

Sequence	Position	BL50[a]
EVFRPGGGDM	Env464-473	3.98×10^{-7}
EVIPMFSAL	Gag169-177	5.84×10^{-7}
ELKKIIGQV	Pol872-880	9.88×10^{-6}
EVNIVTDSQY	Pol647-656	1.21×10^{-5}
EVVIRSDNF	Env272-280	4.1×10^{-5}
ELNKRTQDF	Pol234-242	1.02×10^{-4}
EIKGEIKNCSF	Env147-157	1.62×10^{-4}
DIVIYQYMDDL	Pol332-342	1.94×10^{-4}
DTTNQKTEL	Pol626-634	2.78×10^{-4}
EIVASCDKCQL	Pol750-760	3.50×10^{-4}
ETVPVKLKPGM	Pol161-171	4.26×10^{-4}
ETWEAWWMEYW	Pol551-561	6.28×10^{-4}
ETWEAWWMEY	Pol551-560	$>1 \times 10^{-3}$
EICGHKAIGTV	Pol121-131	$>1 \times 10^{-3}$
EVVLGNVTENF	Env82-92	$>1 \times 10^{-3}$
DLRSLCLFSY	Env758-767	$>1 \times 10^{-3}$
DLWIYHTQGYF	Nef115-125	$>1 \times 10^{-3}$
EVIPLTEEA	Pol446-454	$>1 \times 10^{-3}$
ETPGIRYQY	Pol293-301	$>1 \times 10^{-3}$
EVYYDPSKDLV	Pol471-481	$>1 \times 10^{-3}$
EVYYDPSKDL	Pol471-480	$>1 \times 10^{-3}$
ETKLGKAGYV	Pol604-613	$>1 \times 10^{-3}$
EVHNVWATHAC	Env63-73	$>1 \times 10^{-3}$
EVQLGIPHPA	Pol244-253	$>1 \times 10^{-3}$
ELYPLTSLRS	Gag484-493	$>1 \times 10^{-3}$
DLNTMLNTV	Gag185-193	$>1 \times 10^{-3}$
DVKQLTEAV	Pol519-527	$>1 \times 10^{-3}$
ELYPLTSLRSL	Gag484-494	$>1 \times 10^{-3}$
DIQKLVGKL	Pol411-419	$>1 \times 10^{-3}$
DIAGTTSTL	Gag235-245	$>1 \times 10^{-3}$
ELRQHLLRW	Pol359-367	$>1 \times 10^{-3}$
DTKEALEKI	Gag96-104	$>1 \times 10^{-3}$

[a] The half maximal binding level was calculated as the peptide concentration yielding the half-maximal MFI.

in vitro for 14 days with a cocktail of HLA-A*2602-binding peptides containing 5–7 peptides (cocktail 1: Env464-473, Gag169-177, Pol647-656, Pol872-880, and Pol551-560; cocktail 2: Env272-280, Pol121-131, Env82-92, Pol551-561, Env758-767, Pol234-242, and Nef115-125; cocktail 3: Pol446-454, Pol293-301, Pol471-481, Pol471-480, Pol604-613, Env147-157, and Env63-73; cocktail 4: Pol244-253, Gag484-493, Gag185-193, Pol626-634, Pol519-527, Gag484-494, and Pol411-419; and cocktail 5: Gag235-243, Pol359-367, Pol161-171, Gag96-104, Pol750-760, and Pol332-342). IFN-γ production by each bulk culture in response to C1R-A*2602 prepulsed with the corresponding peptide cocktail was assessed by intracellular IFN-γ staining. All five cocktails failed to induce specific $CD8^+$ T cells among the cells in bulk culture obtained from patients KI-030 and KI-082. On the other hand, only cocktail 1 induced specific $CD8^+$ T cells among the cells in bulk culture obtained from patient KO-003 (Fig. 2A). To determine which peptides in the cocktail induced the specific $CD8^+$ T cells, we re-stimulated the cells of this bulk culture with C1R-A*2602 cells prepulsed with each single peptide in this cocktail to detect the specific $CD8^+$ T cells. Only the Gag196-177 peptide induced $CD8^+$ T cells producing IFN-γ (Fig. 2B).

To clarify whether Gag169-177 was a naturally occurring peptide, we investigated the ability of these peptide-specific $CD8^+$ T cells to produce IFN-γ after having stimulated them with C1R-A*2602 cells infected with recombinant HIV-1 vaccinia virus (r-HIV vaccinia). IFN-γ-producing cells were induced in the Gag169-177-specific $CD8^+$ T cell culture after stimulation with r-HIV vaccinia-infected C1R-A*2602 cells, whereas they were not detected in that stimulated with WT vaccinia-infected C1R-A*2602 cells or r-HIV vaccinia-infected C1R cells (Fig. 2C). These results indicate that Gag169-177 is indeed a naturally occurring HIV-1 epitope peptide presented by HLA-A*2602.

Gag169-177-specific $CD8^+$ T cells were induced from only 1 of the 3 HLA-A*2602^+ HIV-1-infected individuals; whereas two HLA-A*2601 epitopes, Pol647-656 and Env464-473, which were also HLA-A*2602-binding peptides, failed to induce specific T cells in these individuals. To address lower frequency of these peptide-specific $CD8^+$ T cells, we investigated the induction of the specific $CD8^+$ T cells by stimulating PBMC from three additional donors carrying HLA-A*2602 with these three peptides. Only Gag169-177 peptide induced the specific $CD8^+$ T cells in one donor. Thus, Gag169-177-specific $CD8^+$ T cells were induced in 2 of 6 HLA-A*2602^+ HIV-1-infected individuals whereas they were induced in 8 of 11 HLA-A*2601^+ and 7 of 8 HLA-A*2603^+ HIV-1-infected ones (Fig. 2D). These results indicate that Gag169-177 is a subdominant epitope in the HLA-A*2602^+ donors.

Gag169-177-specific $CD8^+$ T cells were previously detected in five of seven HLA-A*2601^+ donors and in all four HLA-A*2603^+ ones [14,15], suggesting that Gag169-177 is a dominant epitope in HIV-1-infected individuals carrying either of these HLA-A*26 alleles. Additional experiments in the present study confirmed the immunodominance of this epitope in HLA-A*2601^+ and HLA-A*2603^+ donors (Fig. 2D). In contrast, they were detected in only two of six HLA-A*2602^+ donors, indicating Gag169-177 to be a subdominant epitope in HLA-A*2602^+ individuals. Interestingly, they were elicited in only a long-term non-progressor and a controller having low viral load. HLA-A*1101-restricted Gag349-, Nef73-, and/or Nef84-specific $CD8^+$ T cells were induces in two of four HLA-A*1101^+/A*2602^+ donors who did not have Gag169-177-specific $CD8^+$ T cells (data not shown), supporting that these HLA-A*2602^+ donors maintain HIV-1-specific cellular immunity. The affinity of Gag169-177 for HLA-A*2602 was similar to that for HLA-A*2601 and -A*2603 (Table 2), indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected individuals carrying HLA-A*2602. Pol604-612 and Env63-72 are HLA-A*2601 and HLA-A*2603 epitopes, respectively [14,15]. These epitope peptides failed to bind to HLA-A*2602 (data not shown). Since only one amino acid, at residue 116, differs between HLA-A*2602 and the other two subtypes (Asp

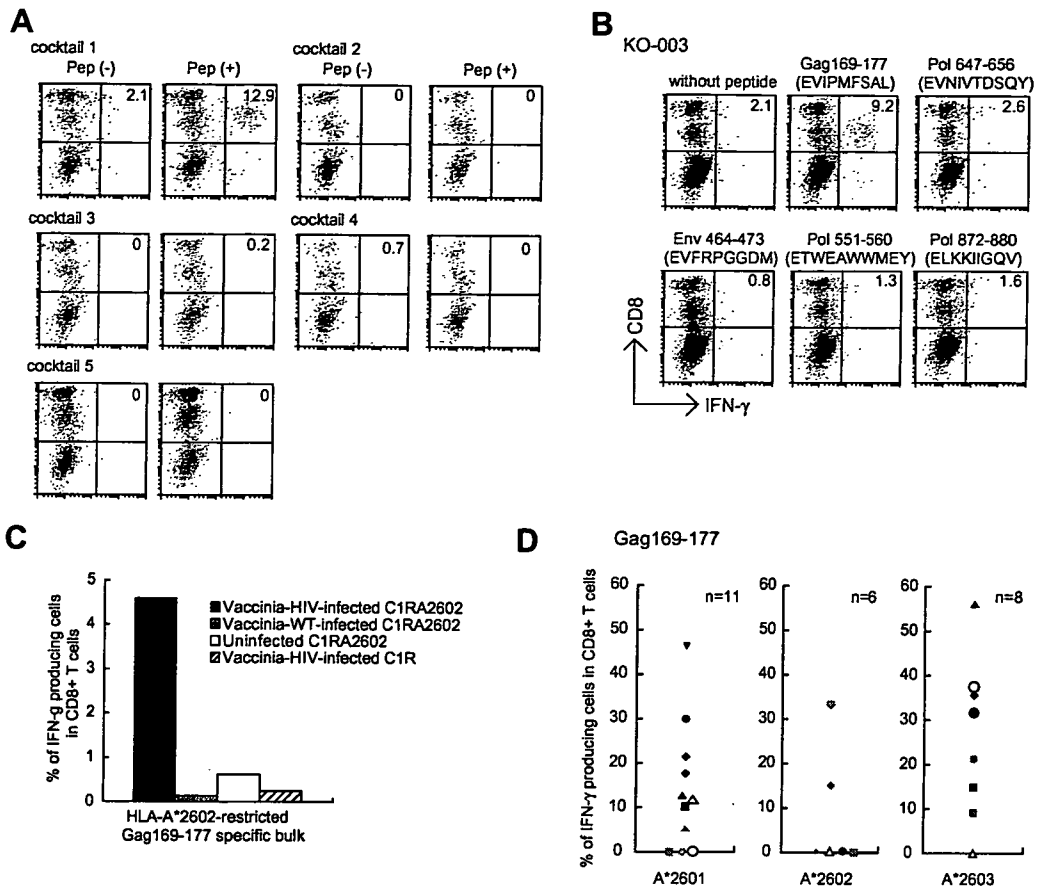

Fig. 2. Identification and recognition of Gag169-177-specific $CD8^+$ T cells. (A) PBMCs from HIV-1-infected individuals with HLA-A*2602 (KO-003) were cultured for 10–14 days after they had been stimulated with the indicated cocktails of HLA-A*2602-binding peptides. The cultured cells were then tested for IFN-γ production by $CD8^+$ T cells after stimulation with C1R-A*2602 cells prepulsed with the peptide cocktails. (B) Induction of Gag169-177-specific $CD8^+$ T cells. PBMCs from KO-003 were stimulated with peptide cocktail 1 and cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2602 cells prepulsed with each single peptide included in cocktail 1. The population of IFN-γ-producing $CD8^+$ T cells was determined by using flow cytometry. The percentage of IFN-γ-producing $CD8^+$ T cells is presented at the right of the upper right-hand quadrant. (C) Presentation of Gag169-177 by HLA-A*2602 on r-HIV-1 vaccinia-infected cells. Bulk cultures containing Gag169-177-specific $CD8^+$ T cells were examined for IFN-γ production after they had been stimulated with C1R-A*2602 cells infected with wild-type vaccinia (Vaccinia-WT) or with C1R-A*2602 cells or with C1R cells infected with r-HIV-1Gag-vaccinia (Vaccinia-HIV-1), or uninfected C1R-A*2602 cells (Uninfected). The percentage of IFN-γ-producing $CD8^+$ T cells was measured by using flow cytometry. (D) Percentage of Gag169-177-specific $CD8^+$ T cells in HIV-1-infected individuals having three HLA-A*26 subtypes. The percentage of IFN-γ-producing cells among $CD8^+$ T cells from each individual was plotted in the graph. The percentage of IFN-γ-producing cells in the cultures was measured by using flow cytometry after they had been stimulated with the corresponding C1R-A*26 cells prepulsed with Gag169-177 peptide.

Table 2
Comparison of binding affinity of HLA-A*26 epitope peptides among three HLA-A*26 subtypes and induction of the peptide-specific $CD8^+$ T cells

	Binding affinity (BL50)			Comparison of binding affinity		Frequency[a]		
	A*2601	A*2602	A*2603	A*2602/A*2601	A*2602/A*2603	A*2601	A*2602	A*2603
Gag169-177	7.5×10^{-7}	5.8×10^{-7}	2.1×10^{-6}	0.77	0.28	8/11	2/6	7/8
Env63-72	1.1×10^{-4}	No binding	7.6×10^{-7}	—	—	0/11	NT	3/8
Pol604-612	6.5×10^{-5}	No binding	No binding	—	—	10/11	NT	NT
Pol647-656	6.3×10^{-5}	1.2×10^{-5}	6.6×10^{-4}	0.19	0.02	1/11	0/6	0/8
Env464-473	1.5×10^{-6}	4.0×10^{-7}	3.7×10^{-5}	0.27	0.01	1/11	0/6	0/8

NT, not tested.
[a] The number of individuals in whom peptide specific $CD8^+$ T cells were induced/the number of tested individuals.

for HLA-A*2601 and -A*2603, but Asn for HLA-A*2602), this substitution in the floor of the peptide binding groove is thought to affect the binding of these peptides.

In summary, Gag169-177 was not an HIV-1 immunodominant epitope in HIV-1-infected individuals carrying HLA-A*2602, whereas it was one in those carrying HLA-

A*2601 or -A*2603. These findings imply the possibility that HLA-A*2602 is not an allele associated with a slow progression to AIDS. However, it still remains unknown that Gag169-177-specific CTLs can control HIV-1. A further study using a cohort of a large number of subjects will clarify the association of these HLA-A*26 subtypes or Gag169-177-specific CTLs with the progression of AIDS.

Acknowledgments

The authors thank Dr. S. Ferrone for the gift of mAb TP25.99, Dr. H. Gatanaka for proving clinical data, and Sachiko Sakai for her secretarial assistance. This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labour, and Welfare of the government of Japan, and by a grant from the Japan Health Science Foundation.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 24, Number 1, 2008

DOI: 10.1089/aid.2007.0129

Trend of Drug-Resistant HIV Type 1 Emergence among Therapy-Naive Patients in Nagoya, Japan: An 8-Year Surveillance from 1999 to 2006

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ABSTRACT

We studied the emergence of drug-resistant human immunodeficiency virus type 1 (HIV-1) with major amino acid mutations in 402 therapy-naive patients at Nagoya Medical Center, Japan, between 1999 and 2006. The mean prevalence of drug-resistant HIV-1 was 6.7% (range, 2.3–10.0%; $n = 27$). HIV-1 variants with protease inhibitor (PI)-resistant mutations alone were most frequently found (3.5%, $n = 14$), followed by those with nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutations alone (1.7%, $n = 7$). Variants with nucleoside reverse transcriptase inhibitor (NRTI)-resistant mutations alone were sporadically found (1.0%, $n = 4$). A variant possessing both NRTI- and PI-resistant mutations was detected in one patient (0.2%) and a variant possessing both NNRTI- and PI-resistant mutations was identified in another patient (0.2%). In addition, another 17 variants (4.2%, $n = 17$) with only 215-revertant mutations (T215C/D/G/L/S) that can easily reconvert to the nucleoside analogue-associated mutation of T215Y/F were found. The 402 viruses were phylogenetically analyzed, revealing three independent clusters comprising PI-resistant variants with the M46I or L90M mutation, NNRTI-resistant variants with the K103N mutation, and 215-revertant variants. The PI-resistant and 215-revertant strains have been spreading since 2000, and the NNRTI-resistant strain has started spreading since 2003. The nature of the epidemic and information for successfully blocking the spread of drug-resistant HIV-1 were clarified in this study.

INTRODUCTION

COMBINATION THERAPY with three or more antiretroviral drugs (highly active antiretroviral therapy, HAART) can strongly suppress the replication of human immunodeficiency virus type 1 (HIV-1) and maintain the amount of HIV-1 RNA in plasma (viral load) under detectable levels in many cases.[1–5] However, HIV-1 variants with decreased susceptibility to antiretroviral drugs are sometimes found under conditions in which the drug concentration is insufficient to suppress viral replication following poor adherence to treatment regimens.[4–9] Such variants might become an origin for HIV-1 transmission, resulting in the finding of drug-resistant HIV-1 in therapy-naive individuals. This represents a serious problem in therapy, as such variants hinder antiretroviral therapy from the first trial.[10–16] Determining whether therapy-naive patients are infected by drug-resistant HIV-1 before starting HAART is thus important. The present study studied emergence trends for drug-resistant HIV-1 with major mutations among therapy-naive patients in the Nagoya Medical Center, Japan, between 1999 and 2006. We also studied the emergence of HIV-1 with 215-revertant amino acid mutations in the reverse transcriptase (RT), as 215-revertant variants can easily change to nucleoside RT inhibitor (NRTI)-resistant variants.[17–20] The final aim of the study was to understand the epidemiological nature of drug-resistant variants and obtain information to successfully block their spread.

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MATERIALS AND METHODS

Patients

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

Genotypic drug-resistance testing for HIV-1

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

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

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

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

Phylogenetic analysis

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

Measurement of viral load and CD4 cell count

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

Statistics

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

RESULTS

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

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

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

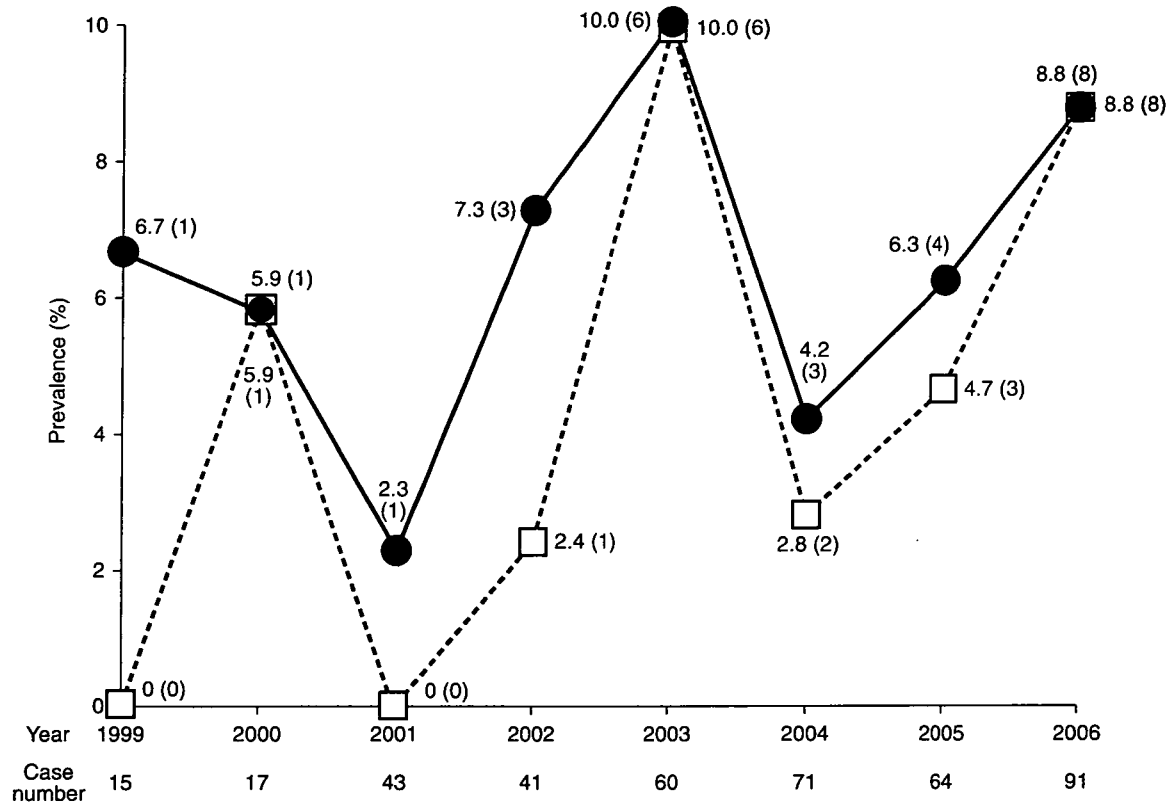

FIG. 1. Changes in prevalence of drug-resistant HIV-1 with major amino acid mutations (●—●) and 215-revertant variants (□—□) in therapy-naive patients. Genotypic drug-resistance testing was performed for 402 therapy-naive patients from 1999 to 2006. Detected numbers are shown in parentheses.

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

Characteristics of drug-resistant HIV-1

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

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

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

Phylogenetic analysis

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

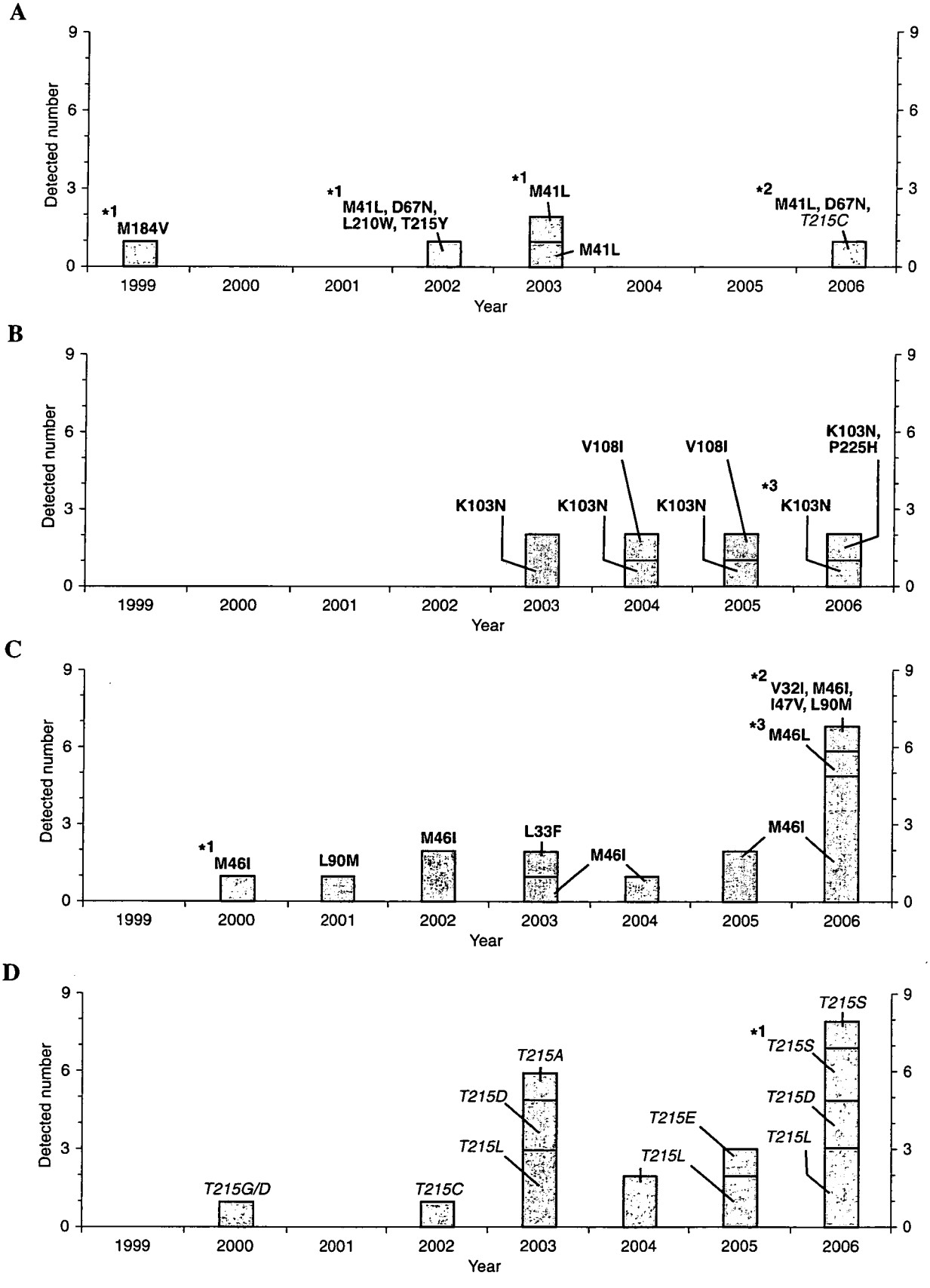
A
Detected number
*1 M184V
*1 M41L, D67N, L210W, T215Y
*1 M41L
M41L
*2 M41L, D67N, T215C
1999 2000 2001 2002 2003 2004 2005 2006
Year
B
Detected number
K103N
K103N
V108I
K103N
V108I
*3 K103N
K103N, P225H
1999 2000 2001 2002 2003 2004 2005 2006
Year
C
Detected number
*1 M46I
L90M
M46I
L33F
M46I
M46I
*3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

TABLE 2. CHARACTERISTICS OF DRUG-RESISTANT HIV-1

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

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

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

Statistical analysis

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

DISCUSSION

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

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

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

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

SEQUENCE DATA

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

ACKNOWLEDGMENTS

Seiichiro Fujisaki and Saeko Fujisaki are research residents of the Japanese Foundation for AIDS Prevention. This study was partly supported by a Health Science Research Grant for Research on HIV/AIDS from the Ministry of Health, Labour and Welfare of Japan (No. H16-AIDS-002 to T.K.).

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

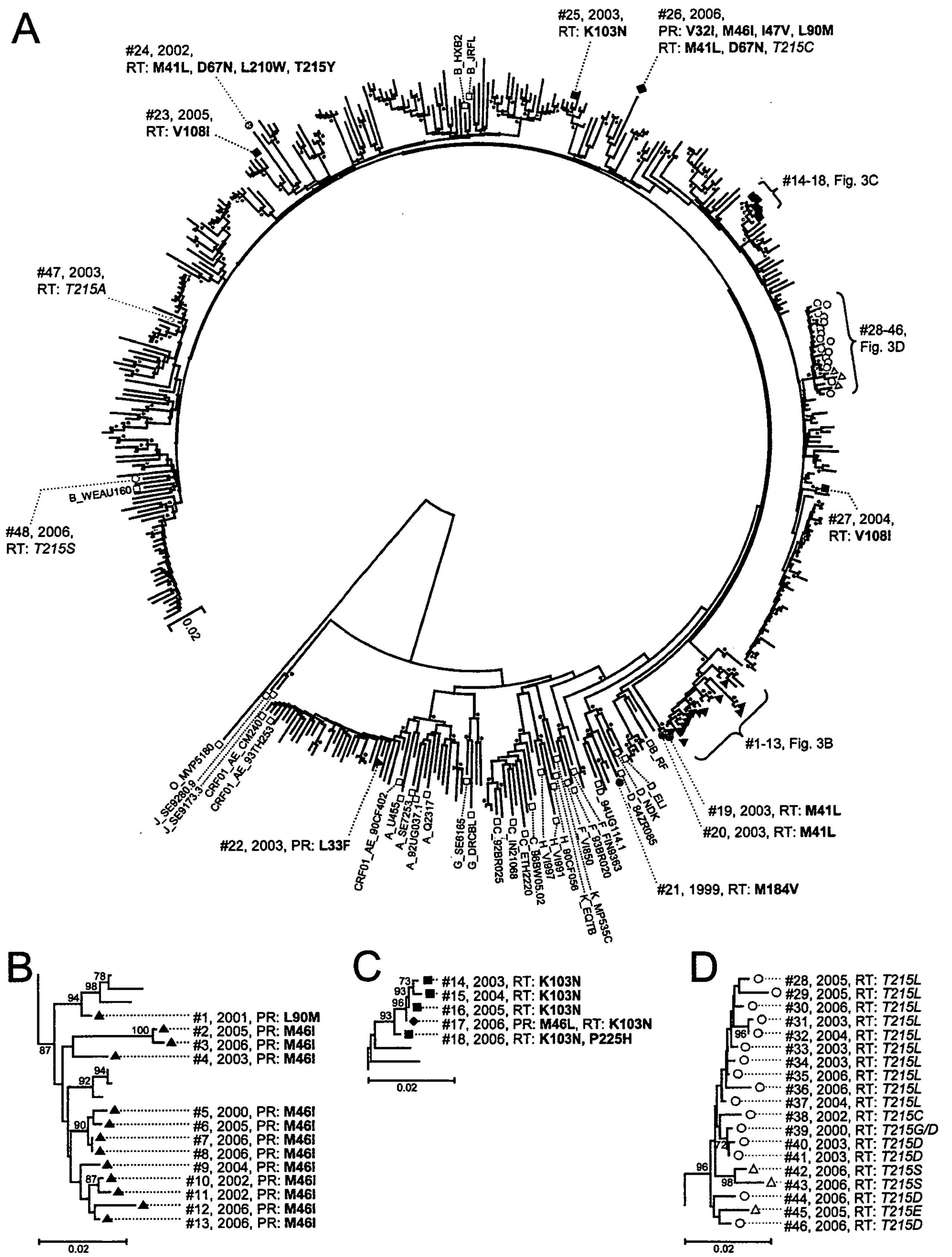
A
#24, 2002,
RT: M41L, D67N, L210W, T215Y
#23, 2005,
RT: V108I
B_HXB2
B_JRFL
#25, 2003,
RT: K103N
#26, 2006,
PR: V32I, M46I, I47V, L90M
RT: M41L, D67N, T215C
#14-18, Fig. 3C
#47, 2003,
RT: T215A
#28-46,
Fig. 3D
B_WEAU160
#48, 2006,
RT: T215S
#27, 2004,
RT: V108I
0.02
#1-13, Fig. 3B
#19, 2003, RT: M41L
#20, 2003, RT: M41L
#21, 1999, RT: M184V
#22, 2003, PR: L33F
O_MVP5180
J_SE9280.9
J_SE9173.3
CRF01_AE_CM240
CRF01_AE_93TH253
CRF01_AE_90CF402
A_U455
A_SE7253
A_92UG037.1
A_Q2317
G_SE6165
G_DRCBL
C_92BR025
C_IN21068
C_ETH2220
C_96BW05.02
H_VI997
H_VI991
H_90CF056
F_VI850
F_93BR020
F_FIN9363
K_EQTB
K_MP535C
D_94UG114.1
D_84ZR085
D_NDK
D_ELI
DB_RF
B
#1, 2001, PR: L90M
#2, 2005, PR: M46I
#3, 2006, PR: M46I
#4, 2003, PR: M46I
#5, 2000, PR: M46I
#6, 2005, PR: M46I
#7, 2006, PR: M46I
#8, 2006, PR: M46I
#9, 2004, PR: M46I
#10, 2002, PR: M46I
#11, 2002, PR: M46I
#12, 2006, PR: M46I
#13, 2006, PR: M46I
0.02
C
#14, 2003, RT: K103N
#15, 2004, RT: K103N
#16, 2005, RT: K103N
#17, 2006, PR: M46L, RT: K103N
#18, 2006, RT: K103N, P225H
0.02
D
#28, 2005, RT: T215L
#29, 2005, RT: T215L
#30, 2006, RT: T215L
#31, 2003, RT: T215L
#32, 2004, RT: T215L
#33, 2003, RT: T215L
#34, 2003, RT: T215L
#35, 2006, RT: T215L
#36, 2006, RT: T215L
#37, 2004, RT: T215L
#38, 2002, RT: T215C
#39, 2000, RT: T215G/D
#40, 2003, RT: T215D
#41, 2003, RT: T215D
#42, 2006, RT: T215S
#43, 2006, RT: T215S
#44, 2006, RT: T215D
#45, 2005, RT: T215E
#46, 2006, RT: T215D
0.02

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

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Jpn. J. Infect. Dis., 60, 113-117, 2007

Original Article

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

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(Received November 27, 2006. Accepted January 23, 2007)

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

INTRODUCTION

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

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MATERIALS AND METHODS

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

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

V35T, T39A, K43E, E122K, I135T, R172K, D177E, Q207H, R211K, L214F, and K238S in RT.

HIV-1 RNA was extracted from the plasma using the QIAamp Viral RNA kit (QIAGEN, Valencia, Calif., USA). DNA fragments sized 1.3 kb containing *gag* (codons #412-500), *protease* (codons #1-99), and *reverse transcriptase* (codons #1-260) were amplified by RT-PCR using SuperScript One-Step RT-PCR (Invitrogen, Carlsbad, Calif., USA) and the primer pair K1 (5′-AAG GGC TGT TGG AAA TGT GG-3′) and U13 (5′-CCC ACT CAG GAA TCC AGG T-3′), followed by a second-round PCR using LA Taq (Takara, Shiga, Japan) with the primer pair INF-ApaI (5′-TGC TGG GCC CCT AGG AAA AAG G-3′) and INF-NheI (5′-TCT GGC TAG CCC AAT TCA ATT TTC CCA C-3′). The amplified fragments were sequenced and the presence of the target mutations was confirmed. The amplicon was then digested with *Apa*I (Takara) and *Nhe*I (New England Biolabs, Ipswich, Mass., USA), and the *Apa*I-*Nhe*I fragment was inserted into the corresponding site of a pSUM9 HXB2 expression vector (kindly provided by Dr. H. Mitsuya, National Cancer Institute, Bethesda, Md., USA) (6). Each clone was transfected into MT-2 cells (approximately 40% confluence in 1 ml of RPMI 1640) with Lipofectamine (Invitrogen). The cells were cultured with RPMI 1640 containing 10% FBS under a 5% CO_2 atmosphere at 37°C. After 4 days, 0.2 ml of the culture supernatant was collected and transferred to 5×10^4 MT-4 cells in 4.8 ml of culture medium. Three days after infection, the culture supernatants were harvested and stored at −80°C until use.

Preparation and shipping of HIV-1 RNA samples: HIV-1 virions were precipitated by ultracentrifugation at 23,000 $\times g$ for 1 h at 4°C and washed twice with PBS(−). The pellets were suspended in 100 μl of PBS(−) and incubated with 3 U of deoxyribonuclease (RT Grade) (Nippon Gene, Tokyo, Japan) at 37°C for 15 min. Virions were precipitated again by ultracentrifugation at 23,000 $\times g$ for 1 h at 4°C and washed twice with PBS(−) to remove the deoxyribonuclease. RNA was extracted using the QIAamp Viral RNA kit. The number of HIV copies was determined by real-time PCR according to the method reported by Nagai et al. (7), and aliquots containing 2×10^4 copies of HIV-1 RNA in 2 ml of PBS(−) were stored at −80°C. The samples were packed on dry ice and shipped to the participating laboratories.

Data collection and evaluation: Each laboratory was requested to provide a detailed protocol for genotypic testing, including the primer sequences and the enzymes used for reverse transcription and PCR conditions. Laboratories were asked to submit electropherograms and a list of drug-resistance mutations determined according to International AIDS Society-USA panel criteria, version March/April 2005 (8).

The rate of successful detection of mutations was expressed as follows: %DR (or NDR) = detected number of drug-resistance mutations (non-drug-resistance mutations)/total number of drug-resistance mutations (non-drug-resistance mutations). Reported mutations absent in the test samples (ghost mutations) were noted as "E1" errors, erroneous categorization as an "E2" error, and errors in preparing the reports as "E3" errors.

RESULTS

Variations in protocols for genotypic drug-resistance testing in 15 laboratories: As shown in Figure 1, the enrolled laboratories used different protocols with respect to primers, sizes of amplified fragments, and the number of amplified fragments. Eight laboratories (A, B, D, E, F, I, J, and K) used the latest NIID protocol with minor modifications, and three laboratories (G, H, and N) used the 1996 version of the NIID protocol with or without modifications. Four laboratories (C, L, M, and O) used their own protocols.

Regarding the reverse transcription and amplification procedures, 12 laboratories used one-step RT-PCR, and the other three laboratories used two-step RT-PCR. In the reverse-transcription process, AMV RT was used in two laboratories, and M-MLV RT was used in one laboratory. As regards the DNA polymerase used for the first and second PCR, most laboratories used Taq polymerase but other few laboratories used KOD polymerase.

For sequencing, Big Dye Terminator V1.1 (Applied Biosystems, Foster City, Calif., USA), Big Dye Terminator V3.1 (Applied Biosystems), CEQ Dye Terminator Cycle Sequencing with a Quick Start kit (Beckman Coulter, Fullerton, Calif., USA), and Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, Ohio, USA) with IRDye™ 800 v2 Terminator Mixes (LI-COR, Lincoln, Nebr., USA) were used in eight, five, one, and one of the laboratories, respectively. For the purification of labeled products, CENTRI SEP Spin Columns (Applied Biosystems), Sephadex G-50 (GE Healthcare Bio-Sciences, Piscataway, N.J., USA), and DyeEx 2.0 Spin kit (QIAGEN) were used in five, three, and one of the laboratories, respectively. The other six laboratories used the ethanol precipitation method.

For electrophoresis, 14 laboratories used a capillary-type auto-sequencer and the remaining laboratory used a plate-type auto-sequencer. Ten laboratories used the ABI PRISM 310 (Applied Biosystems) auto-sequencer and five laboratories used other sequencers, i.e., the CEQ 8000 (Beckman Coulter), LI-COR 4200 IR2 System (LI-COR), ABI PRISM 3100 (Applied Biosystems), ABI PRISM 3100 Avant (Applied Biosystems), and ABI PRISM 3730S (Applied Biosystems) auto-sequencers.

Results of case 1 testing: As shown in Table 1, in the PR mutation analyses, all laboratories except G detected 100% of the drug-resistance mutations. On the other hand, when detecting the non-drug-resistance mutations, the amino acid mutations reported by laboratories G and H were totally different from those reported by the other laboratories. Laboratory G missed one drug-resistance mutation, A71T, and two non-drug-resistance mutations, E35D and R41K. Laboratory H reported all of the three drug-resistance mutations correctly, but not all of the five non-drug-resistance mutations. Laboratories G and H appear to have amplified the wrong samples, suggesting that contamination may have occurred. Checking their protocols, we noticed that laboratories G and H used DRPRO3 primer in their second round of PCR (Figure 1). As the case 1 sample had an insertion mutation in the DRPRO3 annealing region, a mismatch with this primer in the region caused these errors.

Laboratories E and F reported ghost mutations D29N and C95W, respectively. In the case of laboratory E, the error appeared to be due to the high background noise in the electropherogram. In the case of laboratory F, the data were analyzed only by automatic base sequence analysis, and no manual editing was performed to eliminate the error.

Two laboratories (A and B) made errors in their final reports. We confirmed the raw data from these two laboratories, and found that their electropherograms, nucleotide