killing. Furthermore, by using a biochemical approach, we found that the peptide intrinsic cooperative thermodynamics of pMHC could be an important factor to support efficient antiviral cytotoxic activity of CTLs.

Materials and Methods

Generation of CTL clones and analysis of TCR-encoding genes

CTL clones were established as previously described (6, 15) by using PBMC samples taken from *HLA-B*3501*$^+$ individuals (Pt-01, Pt-03, Pt-19, and Pt-33) in the chronic phase of an HIV-1 infection. Briefly, a bulk CTL culture, which had been established by stimulation of PBMC with a synthetic peptide for 1–2 wk, was further seeded at a density of 0.8 or 5 cells/well with a cloning mixture (irradiated allogeneic PBMC and C1R-B3501 cells pulsed with 1 μM peptide in RPMI 1640 with 10% FCS and 100 U/ml recombinant IL-2). Two weeks later, cells showing substantial Ag-specific cytolytic activity were maintained in the medium with peptide stimulation weekly. CTL clone 139 generated from PBMC of Pt-19 was designated as CTL 19-139, and other clones were similarly designated. TCR-encoding genes of CTL clones were obtained by using a SMART PCR cDNA synthesis kit (BD Clontech) and analyzed by the ImMunoGeneTics database (http://imgt.cines.fr) as previously described (27, 28). The study was conducted in accordance with the human experimentation guidelines of Kumamoto University.

Reconstruction of TCRs on TCR-deficient T cells

The cDNAs encoding full-length TCRα and TCRβ of interest were separately cloned into a retrovirus vector pMX provided by T. Kitamura (Tokyo University, Tokyo, Japan) and delivered into a TCR-deficient mouse T cell hybridoma cell line TG40 provided by T. Saito (RIKEN Institute, Saitama, Japan) as previously described (27, 28). The human CD8α gene was similarly delivered into the cells as needed (28). Finally the cells showing bright staining with PE-conjugated anti-mouse CD3ε mAb (2C11; BD Pharmingen) were cloned by a limiting dilution method for further functional assays described below.

HLA-B35 tetramer binding assays

The HLA-B*3501 tetramers in complex with the VY8 or RY11 peptides were prepared as previously described (28). The CTL clones were stained with PE- and allophycocyanin-labeled HLA-B35 tetramers at 37°C for 15 min followed by anti-CD8-PerCP (BD Biosciences) and anti-CD3-FITC (DakoCytomation) at 4°C for 15 min. For the kinetic analysis of HLA-B35 tetramer dissociation, CTL clones were stained with PE-conjugated tetramer (0.2 μM) for 30 min at 4°C. Then the cells were rapidly washed twice and suspended at 4°C in a buffer (2% BSA in PBS) supplemented with the monomeric type of unconjugated peptide-HLA complex (2 μM) for blocking. A portion of the reaction volume was then removed periodically (0.5, 1, 2, 4, and 8 h), and the cells were subsequently stained with anti-CD8 and anti-CD3 mAbs at 4°C. For the flow cytometric analysis, the CD3$^+$CD8$^+$ cells were gated and then analyzed for the tetramer binding by flow cytometry with FACSCalibur (BD Biosciences).

Cytotoxicity assays

Primary CD4$^+$ cells were purified from PBMC taken freshly from HIV-negative donors expressing *HLA-B*3501* by using a magnetic cell separation system (Miltenyi Biotec) and stimulated with PHA (3 μg/ml; Sigma-Aldrich) for 4 days. After having been labeled with ^{51}Cr, the activated CD4$^+$ cells were pulsed with various concentrations of a synthetic peptide for 1 h at 37°C, washed once with culture medium, and then mixed with CTL clones (4000 cells/well) for 4 h at 37°C. For virus-infected target cells, the activated CD4$^+$ cells (4000 cells/well), which had been infected with recombinant HIV-1 or vaccinia virus carrying the *nef* gene of strain SF2 (10), were mixed with CTL clones at various E:T ratios for 6 h at 37°C after having been labeled with ^{51}Cr. It should be noted that 30 ± 5% of the cells were p24 Ag-positive, as revealed by intracellular flow cytometric analysis of HIV-infected CD4$^+$ cells.

IL-2 assays for T cell activation

TCR recognition of cognate Ags was measured in terms of IL-2 secretion by TG40 cells transduced with TCR and CD8α as described earlier (27, 28). Unless otherwise specified, C1R-B3501 cells (10^4 cells/well), TCR-transduced TG40 cells (2×10^4 cells/well), and peptides were mixed and incubated for 48 h at 37°C. The resultant culture supernatant was then collected, and the amount of IL-2 was determined by analyzing the proliferative activity of CTLL-2, an IL-2 indicator cell line. The EC_{50} value of the peptide was calculated as the concentration of peptide that exhibited a half-maximal activation of TCR-transduced TG40 cells with CD3ε mAb-mediated activation of the cells defined as maximal.

pMHC decay assay

For the kinetic analysis of the peptide dissociation from pMHC, C1R-B3501 cells were first incubated with 100 μM peptide at 37°C for 1 h. Then the cells were rapidly washed twice and suspended at 37°C in culture medium. A portion of the peptide-loaded target cells was then removed periodically (10, 20, 30, 60, 120, 240, 360, 720 min), washed once with culture medium, and subsequently mixed with TCR-transduced TG40 cells. The amount of IL-2 produced by the TG40 cells was then determined as described.

Differential scanning calorimetry (DSC) and circular dichroism (CD) analyses

The extracellular domain of HLA-B*3501 H chain (aa residues 1–276) and β_2-microglobulin (β_2m) were produced in *Escherichia coli* as insoluble inclusion bodies. These proteins were dissolved in a buffer containing urea and then refolded in the presence of synthetic VY8 or RY11 peptide as previously described (28). In this construct, there was no biotinylated tag sequence at the C terminus of the H chain. Refolded proteins were purified by size-exclusion and anion-exchange chromatography analysis, pooled, dialyzed against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.5)), and used both for DSC and CD measurements. The resultant protein solutions were in the concentration range from 0.3 to 0.7 mg/ml, as determined by UV absorption at 280 nm; and the molecular masses of the protein complexes were calculated from the amino acid composition.

For DSC measurements, excessive heat capacity curves were monitored by an ultrasensitive scanning microcalorimeter (VP-DSC; MicroCal) at a heating rate of 1 K/min with a sample cell volume of ~0.5 ml. The experimental data were baseline-corrected and subjected to deconvolution by using the software package ORIGIN for DSC (MicroCal), based on the assumption that the macromolecule is composed of a number of domains, each of which is involved independently in a "two-state" transition between folded and unfolded states. Each transition is characterized by two parameters, T_m and ΔH_m, in which T_m is the thermal midpoint of a transition and ΔH_m is the calorimetric heat change calculated from the area under the transition peak.

For CD measurements, changes in the ellipticity (as θ) with heating from 4° to 90°C were monitored at 222 nm and other wavelengths by a JASCO J-725 spectropolarimeter with a sample cell volume of ~0.4 ml in a quartz cell with an optical path length of 2 mm. The T_m value in the CD analysis was calculated by using the standard analysis software provided by the manufacturer (JASCO).

Results

Antiviral cytotoxic activity of CTLs specific for VY8 or RY11

We previously reported that in HIV-infected patients with *HLA-B35*, Nef protein elicited the most dominant CD8 T cell responses (6), with a short epitope (VY8, VPLRPMTY) being dominant early and a subsequent shift to an N-terminal extended long epitope (RY11, RPQVPLRPMTY) in the chronic phase (10). However, VY8 is entirely contained within RY11 and may therefore be the minimum epitope for CTLs. To clarify this issue, we generated CTL clones by stimulating PBMC of four HIV-infected individuals with either VY8 or RY11 peptide and then analyzed their Ag fine specificity by cytotoxic assays. Three CTL clones (01-127, 19-139, and 33-1) generated with VY8 stimulation showed a potent cytotoxic activity toward primary CD4$^+$ cells pulsed with VY8 and an activity of markedly lesser strength toward those pulsed with RY11 peptide (Fig. 1*A*), confirming their optimal epitope to be VY8. In contrast, the other three CTL clones (01-113, 01-231, and 03-8) generated with RY11 stimulation showed a potent cytotoxic activity toward primary CD4$^+$ cells pulsed with RY11 and no activity toward those pulsed with VY8 (Fig. 1*A*), confirming their optimal epitope to be RY11. Ag fine specificity of the CTL clones was also confirmed in terms of the HLA-B35 tetramer binding (see below). These data indicate that

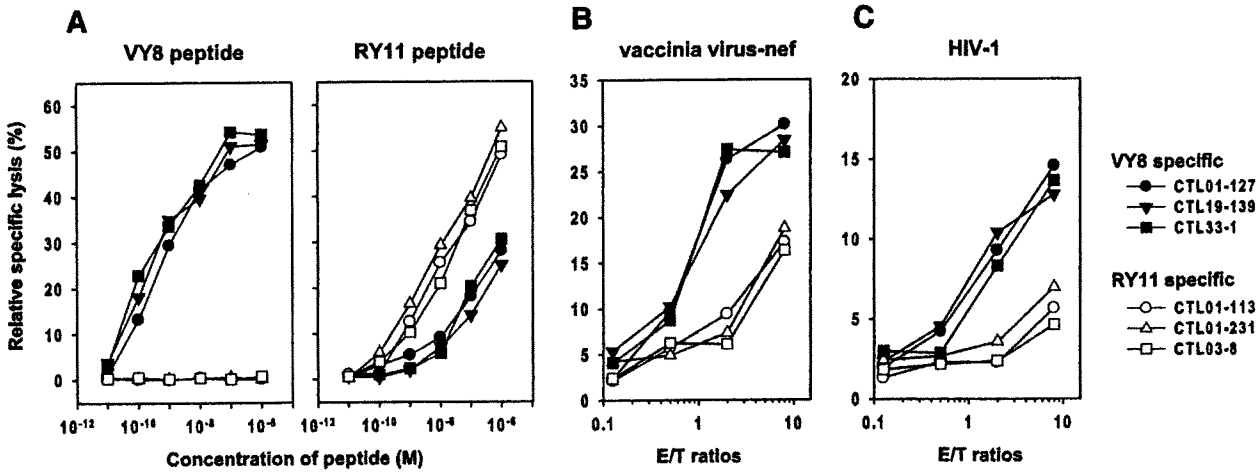

FIGURE 1. Cytotoxic activity of CTL clones. Primary $CD4^+$ cells isolated from an HIV-negative donor were pulsed with various concentrations of VY8 or RY11 peptide (*A*), infected with recombinant vaccinia virus expressing Nef_{SF2} (*B*), or infected with HIV-1 (*C*) and then mixed with the indicated CTL clones. To obtain relative specific lysis values, the cytotoxic activity toward the same target cells not pulsed with peptide, infected with vaccinia virus alone (i.e., lacking *nef* expression) or infected with HIV-1 Δ*nef* variant was determined in parallel and was deducted as a background value. Data presented are the mean of duplicate assays, and an additional set of experiments using another PBMC donor showed similar results.

VY8 and RY11 are optimal epitopes for HLA-B35 and are recognized by a different set of CTL clones.

We next asked whether CTL antiviral cytotoxic activity is different between specificities. The CTL clones showed a significant difference in functional avidity toward their cognate Ags between the specificities ($p = 0.023$, two-tailed t test), with EC_{50} values being $2.9 \pm 0.85 \times 10^{-10}$ and $1.3 \pm 0.37 \times 10^{-8}$ M for VY8 and RY11, respectively (Fig. 1*A*). Next, the same cells were infected with vaccinia virus or HIV-1 expressing Nef_{SF2} and analyzed for their susceptibility to killing by the CTL clones. The VY8-specific CTLs showed more potent cytotoxic activity toward virus-infected $CD4^+$ cells than the RY11-specific ones regardless of the viruses used (Fig. 1, *B* and *C*).

Kinetics of interactions between CTLs and HLA tetramers

We next examined TCR-pMHC interactions by analyzing the binding specificity and activity of CTL clones toward HLA-B35 tetramers. CTL 19-139 and 01-231 were exclusively stained by their cognate HLA-B35 tetramers, whereas an Env-specific CTL clone was not stained by any of the HLA-B35 tetramers examined (Fig. 2*A*), confirming the specificity of the CTL clones as well as the integrity of our peptide-HLA class I complex preparations. Also, titration of the HLA-B35 tetramers showed comparable binding activity of the CTL clones toward the cognate HLA-B35 tetramers (Fig. 2*B*). We then examined the kinetics of the dissociation of cognate HLA-B35 tetramers from CTL clones. There was no substantial difference between CTL 19-139 and CTL 01-231 in dwell time of interaction with the cognate HLA-B35 tetramers (Fig. 2*C*), suggesting comparable kinetic interactions between VY8 and RY11-specific TCRs and their cognate pMHC. However, these results appeared to be inconsistent with the data showing the favorable functional avidity of VY8-specific CTLs (as described).

Functional reconstruction of TCRs on TCR-deficient T cells

It has been shown that TCR reconstruction on TCR-deficient T cells is advantageous to investigate how the TCR-pMHC interaction influences T cell activation (27–30) because primary T cells can increase or decrease their sensitivity/avidity for an epitope through changes in their inhibitory receptor expression and membrane organization as well as via a redistribution of signaling molecules in

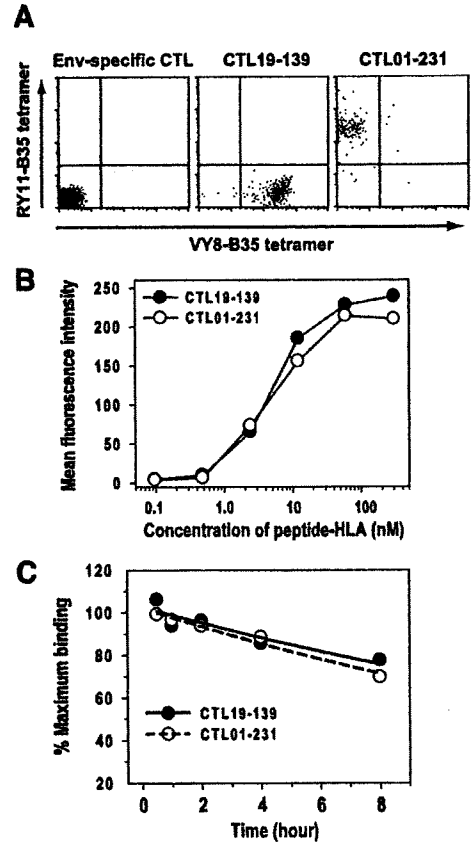

FIGURE 2. HLA tetramer analysis of CTL clones. *A*, CTL clones specific for an Env peptide, VY8 (CTL 19-139) or RY11 (CTL 01-231), were stained with HLA-B35 tetramers in complex with VY8 or RY11 that had been labeled with PE or allophycocyanin, respectively. In the flow cytometric analysis, a live $CD8^+$ subset was gated and analyzed for binding with HLA-B35 tetramers. *B*, CTL 19-139 and CTL 01-231 were separately stained with various concentrations of PE-conjugated HLA-B35 tetramers in complex with their cognate peptides and analyzed by flow cytometry. An independent experiment gave similar results. *C*, Kinetic analysis of dissociation of HLA-B35 tetramers from CTL 19-139 and CTL 01-231 that had been stained with their cognate HLA-B35 tetramers. An independent experiment gave similar results.

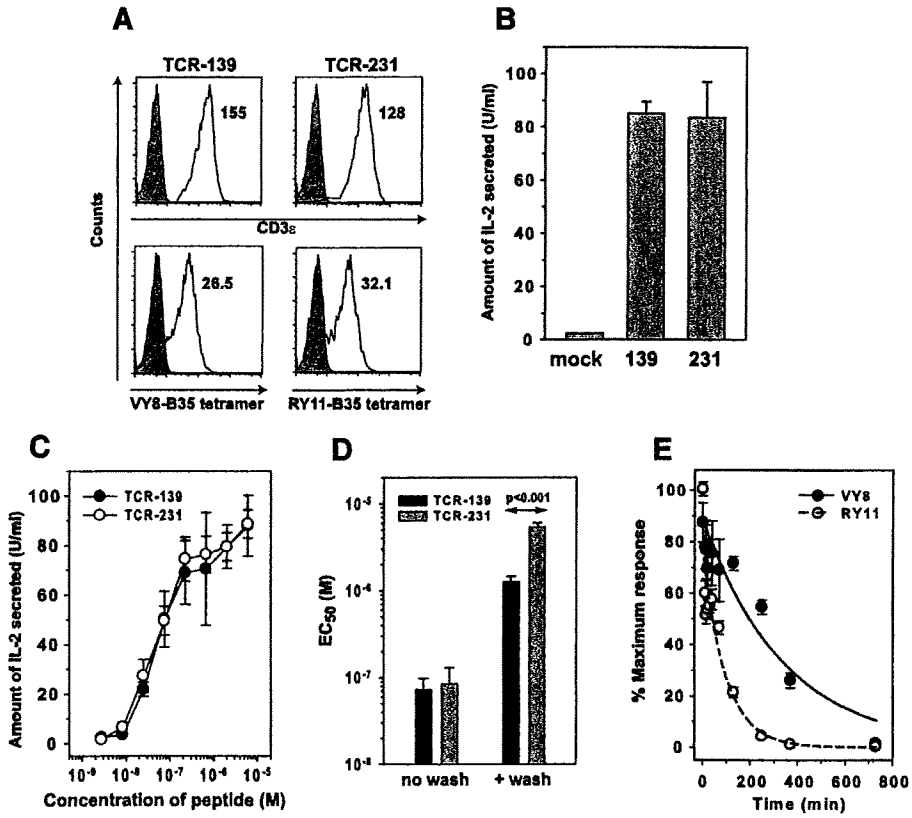

FIGURE 3. TCR-pMHC interactions on TCR-transduced TG40 cells. *A*, TG40 cells alone (shaded histogram) or those expressing TCR-139 or TCR-231 (open histogram) were stained with anti-CD3ε mAb and their cognate HLA-B35 tetramers and then analyzed by flow cytometry. The mean fluorescence intensity is indicated in each histogram. *B*, IL-2 secretion of TG40 cells transduced with mock, TCR-139 or TCR-231 in response to stimulation with CD3ε mAb. Data are the mean ± SD of quadruplicate assays. *C*, IL-2 secretion by TG40 cells transduced with TCR-139 or TCR-231 in response to various concentrations of VY8 or RY11, respectively. TG40 cells, C1R-B3501 cells, and the peptide were coincubated for the duration of the assay. The amount of IL-2 obtained for the mock-transduced TG40 cells was always <5.0. Data are the mean ± SD of quadruplicate assays. *D*, Functional avidity of TG40-139 and TG40-231 cells were dependent of assay conditions. C1R-B3501 cells, the peptide, and TG40 cells were coincubated for the duration of the assay (no wash). C1R-B3501 cells and the peptide were incubated, washed, and subsequently mixed with the TG40-139 or TG40-231 cells (with wash). The EC_{50} values (mean ± SD) were obtained from quadruplicate assays. Statistical analysis was performed using the two-tailed *t* test. *E*, Kinetic analysis of the peptide dissociation from pMHC. C1R-B3501 cells were pulsed with the VY8 or RY11 peptide (100 μM) and washed. A portion of the resultant peptide-loaded cells was taken at each indicated time point and then mixed with TG40-139 or TG40-231 cells for the IL-2 secretion assay. Results are mean ± SD of triplicate assays expressed relative to the maximum response that was arbitrarily set to 100%. The lines shown are based on a single exponential decay.

some circumstances (31–35). To further clarify how the TCR-pMHC interacts, we cloned TCR-encoding genes of CTL 19-139 (VY8 specific) and CTL 01-231 (RY11 specific), and functionally reconstructed their TCRs (designated TCR-139 and TCR-231, respectively) on TCR-deficient T cell line TG40 (27, 28). The resultant TG40-139 and TG40-231 cells showed CD3 expression and HLA-B35 tetramer binding activity at comparable levels (Fig. 3*A*), in good agreement with the observations obtained for the parental CTLs (Fig. 2, *B* and *C*). After further transduction of the cells with human CD8α, both cells showed IL-2 secretion at a comparable level in response to anti-CD3 mAb stimulation (Fig. 3*B*), confirming the integrity of the TCR-mediated signaling machinery in these cells. Then, functional avidity of TG40-139 and TG40-231 cells toward the cognate Ags was determined by coincubation of target cells and peptides. Virtually no difference was observed in their functional avidities (Fig. 3*C*), suggesting comparable TCR-pMHC interactions between the specificities. It should be noted that the peptides were always present for the duration of the assay (see below).

Effect of peptide-off rate on functional avidity of T cells

During a number of attempts to clarify the reasons for the variable observations among assays, we noticed that the avidity of TCR-transduced cells was much decreased when the peptide-loaded target cells were washed before coincubation with the TCR-transduced cells (Fig. 3*D*). Under this washing-off condition, TG40-139 cells showed significantly more potent functional avidity than TG40-231 cells (Fig. 3*D*). We then analyzed the rate of peptide-off from pMHC by using the TCR-transduced cells. The target cells, which had been loaded with a peptide followed by washed-off, were taken and subsequently mixed with TG40 cells expressing the cognate TCR. The extent of the TG40 response should be proportional to the actual pMHC dose retained on the target cell surface. The data showed that the decay of the VY8/HLA-B35 complex was much slower than that of the RY11/HLA-B35 one, as the half-life values of pMHC were $3.3 \pm 0.83 \times 10^2$ and $1.0 \pm 0.03 \times 10^2$ min for VY8 and RY11, respectively (Fig. 3*E*).

Effects of antigenic variations on pMHC decay

To look for variant peptides that could affect pMHC decay and the susceptibility to stimulation of T cells, we examined a series of alanine substitutions in both peptides for their activity to sensitize TCR-transduced T cells under the no-wash condition. VY8 with an alanine substitution at position 5 (designated VY8-5A) showed

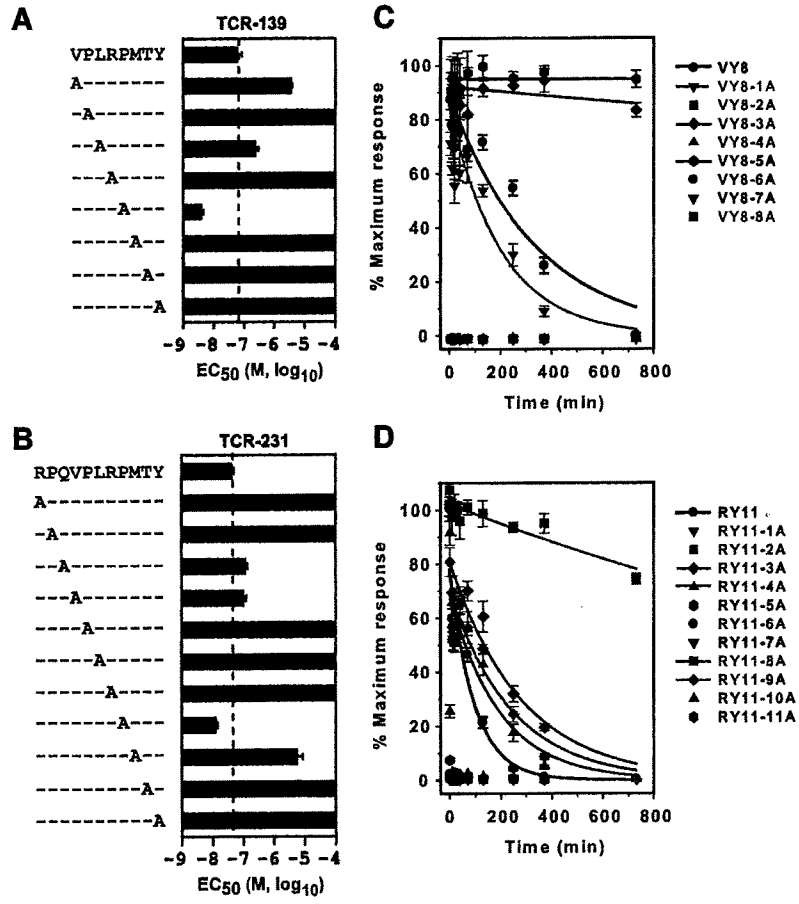

FIGURE 4. Effects of antigenic variations on pMHC decay. *A* and *B*, Alanine variants of VY8 and RY11 were examined by conducting T cell sensitization assays for TCR-139 (*A*) and TCR-231 (*B*), respectively. Amounts of IL-2 secreted by TG40 cells were determined under the no-wash condition. The maximum concentration of the peptides tested was 100 μM. The EC_{50} values at mean ± SD were obtained by performing triplicate assays. An independent experiment gave similar results. *C* and *D*, Dissociation of the wild-type and the alanine variants for VY8 (*C*) and RY11 (*D*) from pMHC was analyzed as in Fig. 3*E*. Data are mean ± SD of triplicate assays. An independent experiment gave similar results. The lines are based on a single exponential decay and given only for the peptides showing positive responses.

more potent reactivity with TCR-139 than did VY8, whereas VY8-1A and VY8-3A had moderate reactivity (Fig. 4*A*). However, TG40-139 cells did not respond to the other five VY8 variants up to a 100-μM concentration (Fig. 4*A*). In TCR-231, RY11-8A showed the most profound response, whereas RY11-3A and RY11-4A had reactivity comparable to that of RY11 (Fig. 4*B*). However, RY11-9A showed weak reactivity; and the other seven RY11 variants had no reactivity (Fig. 4*B*). We also tested various amino acid variations (57 variant peptides in total) for their reactivity toward TCR-139 and TCR-231 (data not shown), but VY8-5A and RY11-8A showed the most pronounced effects on IL-2 production by TCR-transduced T cells.

Next, we examined the decay of a series of alanine variant peptides from HLA-B35. The pMHC decay of VY8-5A and VY8-3A was substantially delayed with a half-life of $>4 \times 10^3$ min, whereas the half-life of VY8-1A was slightly more rapid with one of $2.0 \pm 0.80 \times 10^2$ min (Fig. 4*C*). None of the other VY8 variants showed any reactivity (Fig. 4*C*), consistent with the data obtained under the no-wash condition (Fig. 4*A*). Among the RY11 variants, the decay of RY11-8A was substantially delayed with a half-life of $2.7 \pm 0.44 \times 10^3$ min, whereas that of RY11-3A, RY11-4A, and RY11-9A was slightly delayed with half lives of $2.5 \pm 0.44 \times 10^2$, $2.0 \pm 0.40 \times 10^2$, and $2.2 \pm 0.40 \times 10^2$ min, respectively (Fig. 4*D*). None of the other RY11 variants showed any reactivity (Fig. 4*D*), consistent with the data from the no-wash condition (Fig. 4*B*). Taken together, VY8-5A and RY11-8A provided the most profound effects on pMHC decay and T cell stimulation among the wild-type and variant peptides examined.

Enhancement of susceptibility of Nef variant-expressing cells to CTL killing

To ask whether the variant Ags can improve the susceptibility to killing by CTLs, we took advantage of the fact that the alanine substitution at Pro^{82} of Nef_{SF2} resulted in the generation of both

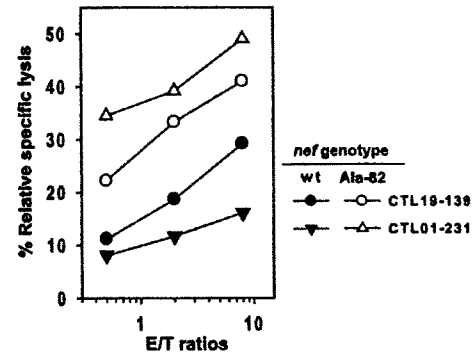

FIGURE 5. Effect of an amino acid substitution on CTL killing of Nef-expressing cells. Primary $CD4^+$ cells isolated from an HIV-negative donor (*HLA-B*3501*$^+$) were transfected with a gene encoding GFP alone, wild-type, or the Ala^{82} mutant of Nef_{SF2}-GFP fusion protein, and then mixed with CTL 19-139 or CTL 01-231 at the indicated E:T ratios. The transfection efficiency was 60 ± 5% as determined by GFP expression. Cytotoxic activity toward cells expressing GFP alone was always <10%. Data are the means of duplicate assays, An independent experiment using another PBMC donor and another set of CTL clones gave similar results.

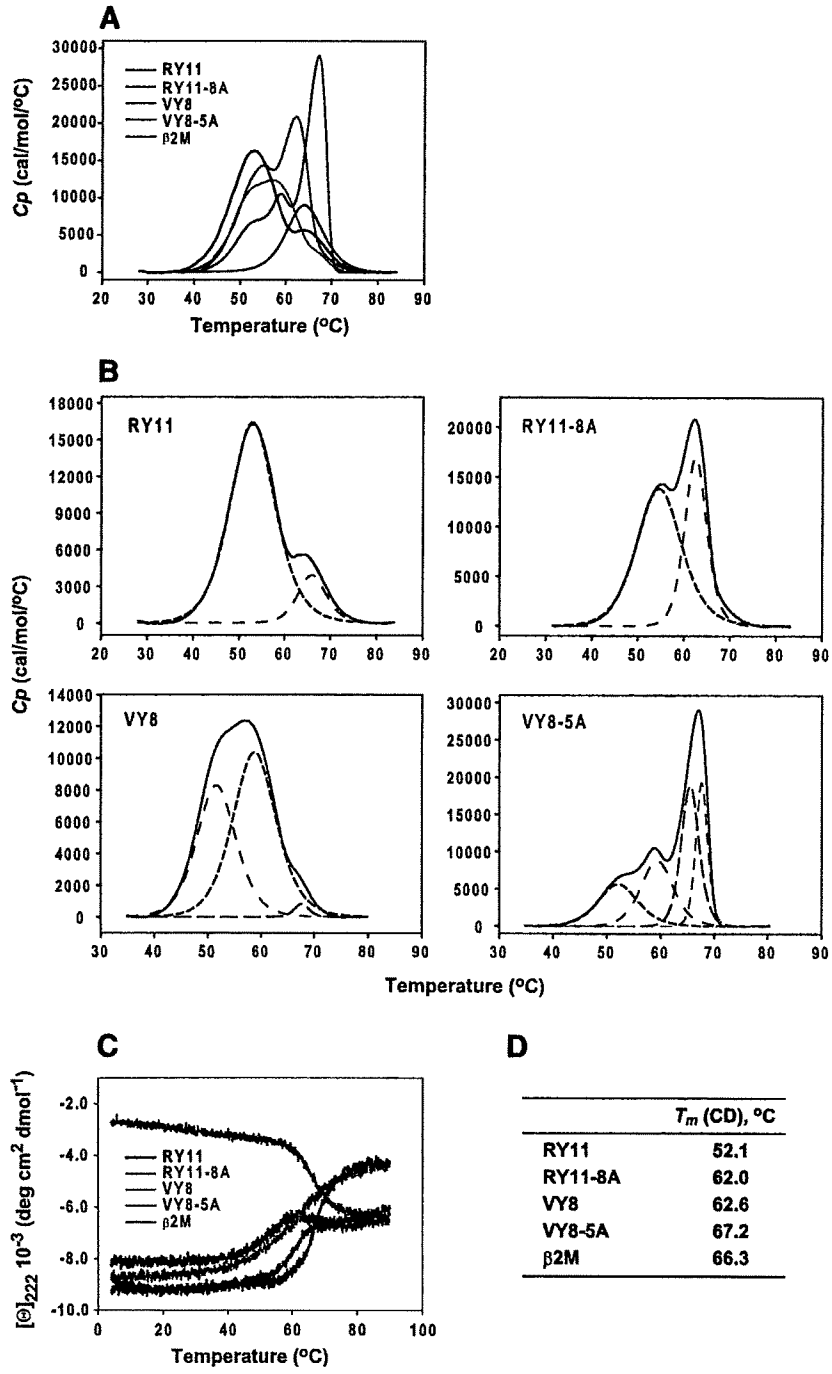

FIGURE 6. Thermostability analysis of peptide-HLA-B35 complexes. *A* and *B*, DSC analysis of pMHC complexes. *A*, The excessive heat capacity of β_2m alone and that of HLA-B35 in complex with indicated peptides was measured by DSC analysis. *B*, Deconvolution of the experimental curves (solid line histogram) of HLA-B35 in complex with the indicated peptides. Deconvolution results in two-state transitions (broken line histogram). T_m and ΔH_m values obtained are given in Table I. *C* and *D*, CD analysis of pMHC complexes. The ellipticity (as θ) at 222 nm of β_2m alone and that of HLA-B35 in complex with the indicated peptides was measured by CD analysis (*C*). The CD melting temperatures, T_m (CD), are also shown (*D*).

VY8-5A and RY11-8A variant Ags. Primary $CD4^+$ cells were transfected with the wild-type or the Ala^{82} variant *nef* genes. The cells with the Ala^{82} variant showed substantially increased susceptibility to killing by both CTL 19-139 and CTL 01-231 (Fig. 5), suggesting that the variant antigenic peptides with slower pMHC decay rendered HIV-infected cells more susceptible to CTL-mediated viral containment.

Thermostability analysis of pMHC

To characterize the biochemical differences between VY8/HLA-B35 and RY11/HLA-B35 complexes, we analyzed the thermostability of free β_2m and that of these heterotrimers (composed of β_2m, H chain, and peptide) by DSC. The heat capacity curve of β_2m, a protein composed of a stable domain containing exclusively β strands, showed a single two-state transition at T_m of 64.18°C (Fig. 6*A* and Table I), in good agreement with a previous report (36). In contrast, the heat capacity curve of RY11/HLA-B35 was characterized by two partly overlapping peaks with the melting temperature of the first peak (T_m^1) substantially below that of β_2m (Fig. 6, *A* and *B* and Table I). The other single transition at the high temperature peak appeared to result from the melting of β_2m, as the T_m^2 value of RY11/HLA-B35 was comparable to that of free β_2m (Table I). These results suggest that the melting of RY11/HLA-B35 started with unfolding of the H chain and concomitant

Table I. *DSC measurements of pMHC complexes*

Sample	Transition Temperature, T_m (°C)				Calorimetric Enthalpy, ΔH_m (kcal/mol)				
	T_m^1	T_m^2	T_m^3	T_m^4	ΔH_m^1	ΔH_m^2	ΔH_m^3	ΔH_m^4	ΔH_{tot}
VY8/HLA35	51.66 ± 0.06	58.83 ± 0.055	67.72 ± 0.087	N/A	80.72	112.7	2.9	N/A	196.32
VY8-5A/HLA35	52.22 ± 0.36	59.42 ± 0.13	65.57 ± 0.3	67.71 ± 0.10	55.44	70.52	84.63	55.6	266.19
RY11/HLA35	52.07 ± 0.019	66.10 ± 0.068	N/A	N/A	215.1	32.16	N/A	N/A	247.26
RY11-8A/HLA35	54.68 ± 0.065	62.56 ± 0.013	N/A	N/A	178.8	108.6	N/A	N/A	287.40
β_2m	64.18 ± 0.0031	N/A	N/A	N/A	96.9	N/A	N/A	N/A	96.9

N/A, Not applicable.

release of folded β_2m, which subsequently melted at the higher temperature.

In contrast, the heat capacity curve of VY8/HLA-B35 appeared to be quite different from that of the RY11 counterpart, as the deconvolution of the VY8/HLA-B35 experimental heat capacity profile showed three peaks, which heavily overlapped each other and were less well separated (Fig. 6, *A* and *C* and Table I). The transition of the third peak, which was separated by a shoulder at the high temperature side of the second peak (Fig. 6*C*), could be correlated with the melting of β_2m, as both melting temperatures were comparable, although interestingly, the ΔH_m^3 value of VY8/HLA-B35 was much lower than that of free β_2m (Table I). The melting of the H chain of VY8/HLA-B35 could not be annotated on a single transition, either T_m^1 or T_m^2. Rather, the results suggested that the melting of the entire VY8/HLA-B35 complex occurred simultaneously and cooperatively with the H chain and β_2m.

To examine the contribution of peptides on the thermostability profile of pMHC, we also analyzed RY11-8A and VY8-5A in complex with HLA-B35 by DSC. Both single mutations showed substantial effects on the heat capacity profiles of overall pMHC complexes and increased total enthalpy values compared with those of their respective wild-type counterparts (Fig. 6, *A*, *D*, and *E* and Table I). Notably, transitions at high temperature peaks in the variant peptide complexes, corresponding to T_m^2 for RY11-8A/HLA-B35 and T_m^3 or T_m^4 for VY8-5A/HLA-B35, appeared to rely on a contribution from β_2m, although these enthalpy costs were considerably larger than the enthalpy change of free β_2m (Table I), suggesting a substantial contribution from the H chain to these transitions.

To further examine the contribution of peptides on the thermostability profile of pMHC, we obtained CD profiles of these pMHC heterotrimers to see the thermally induced changes in their secondary structures. We observed substantial differences in CD profiles between β_2m alone and all pMHC heterotrimers examined (Fig. 6*C*). β_2m alone melted with T_m in the CD analysis of 66.3°C (Fig. 6*D*), in good agreement with the T_m^1 value in the DSC analysis (Table I) as well as with a previous report (36). In contrast, all pMHC heterotrimers had much larger negative molar ellipticity at 222 nm at a low temperature range than β_2m alone (Fig. 6*C*), most likely reflecting the presence of α helices in their H chain subunits. In addition, each pMHC complex showed a different reduction in their negative molar ellipticity with increasing temperatures (Fig. 6*C*), highlighting the contribution of peptides on the thermostability of the secondary structure in their H chain subunits. Specifically, RY11/HLA-B35 melted with a T_m (CD) of 52.1°C (Fig. 6*D*), a value consistent with the T_m^1 in the DSC analysis, confirming the observation made by DSC that melting of RY11/HLA-B35 started with unfolding of the H chain subunit. In addition, VY8/HLA-B35 melted at a much higher temperature (Fig. 6*D*) than RY11/HLA-B35, confirming the potent thermostability of the VY8/HLA-B35 complex as observed in the DSC analysis. Finally, the HLA-B35 complexes with the variant peptides melted at higher temperatures than their wild-type counterparts (Fig. 6, *C* and *D*), confirming again the contribution of variant peptides on the profound thermostability in these pMHC complexes as observed in DSC analysis.

Discussion

In the present study, using TCR-reconstructed cells we clearly showed that the difference in antiviral cytotoxic activity between mature CTLs specific for two different but closely related antigenic Nef peptides (VY8 and RY11) was not caused by the difference in functional avidity of TCR-pMHC interactions. Rather, our data demonstrated that the antiviral activity of these effector CTLs was much influenced by peptide intrinsic factors including peptide-off rate and cooperative thermodynamics of the cognate pMHC. The data obtained by introduction of a mutation in the Nef protein that resulted in the alteration of both epitopes to VY8-5A and RY11-8A further confirmed the association between these peptide intrinsic factors and the susceptibility of Nef-expressing cells to killing by the cognate CTLs. Our results are partly in line with those of previous studies demonstrating that the peptide-off rate of pMHC is an important factor for generating immunodominance hierarchy in class I (34, 37–39) and class II (40, 41) MHC-restricted T cell responses, i.e., the slower the peptide-off rate, the greater the abundance of a given pMHC on the surface of APCs, which leads to the generation of immunodominant T cell responses (26). However, immunodominant peptides are not always those with the highest density presented at the target cell surface (23, 24), and immunodominant CTLs do not always play a dominant role in containment of HIV replication (25). The results shown here extend these previous findings that interdependent and cooperative thermostability profiles of pMHC induced by antigenic peptides can be associated with efficient recognition by CTLs for killing virus-infected target cells.

The DSC and CD measurements showed significantly different thermostability profiles among HLA-B35 in complex with VY8, RY11, and their variant peptides. In comparison of the thermostability of HLA-B35 complexes between wild-type peptides and their variants, we found significant effects of the mutations on thermal stabilization of the entire pMHC, as the total enthalpy values required for unfolding of HLA-B35 in complex with the variant peptides were substantially increased compared with those for their respective wild-type counterpart. This thermal stabilization by the variant peptide was corroborated by the DSC and CD analyses and is most likely associated with slow dissociation of these peptides from pMHC, as observed in the cell-based assays. In contrast, the calorimetric unfolding enthalpy of RY11/HLA-B35 obtained by deconvolution of the experimental heat capacity curve was higher than that of its VY8 counterpart, although the cell-based assays showed rapid dissociation of RY11 from pMHC. In this regard, it is possible that RY11 and HLA-B35 may bind with multiple different conformations; because relatively long

peptides can be accommodated on the peptide binding groove of HLA class I molecules with their central region bulged (42–45) or either end extended away (46). This result is less likely in this study, however, because TCR-231-transduced cells responded to target cells pulsed with RY11 but not to those with truncated peptides such as VY8 and RM9 (RPQVPLRPM, data not shown). Also, the HLA-B35 tetramers prepared with RY11 showed binding exclusively with TCR-231 but not with other TCRs including VY8-specific TCR-139. It is also conceivable that the peptide-off rate from the membrane-bound and glycosylated form of pMHC (i.e., present on the cell surface) is not correlated with the thermostability of the soluble form of pMHC (i.e., using bacterially produced extracellular domain). Alternatively, peptides that are endogenously loaded onto the empty MHC class I with the assistance of a specialized multimeric unit called the peptide-loading complex (47, 48) in the endoplasmic reticulum could have conformational characteristics different from those of molecules refolded in vitro. More importantly, the RY11/HLA-B35 complex showed two relatively simple two-state transitions in thermal unfolding, in which a high-temperature transition corresponds to free β_2m. Such an unfolding pattern has been reported for various self-peptides in association with HLA-B27 (36, 49). In contrast, the VY8/HLA-B35 complex and two other variant complexes showed significantly interdependent and cooperative unfolding processes among heterotrimer subunits and structural domains, suggesting the critical contribution of β_2m in maintaining antigenicity of the peptide in association with the H chain. However, whether such a conformational characteristic in a given pMHC can be directly attributable to efficient docking by cognate TCRs, to preferential loading of peptides in an intracellular peptide selection process, or to both events needs to be examined by further intense experiments.

In HIV-infected cells, antigenic peptides are generated through the endogenous MHC class I Ag processing and presentation pathway for CTL recognition. Peptides generated in the cytosol mainly by the proteasome are translocated into the endoplasmic reticulum by mediation of the TAP, and then loaded onto the empty MHC class I (47, 50). It has been reported that sequence specificities by TAP can influence the efficiency of epitope presentation by cell surface MHC class I molecules (47, 50, 51). It is therefore conceivable that the increased susceptibility of Nef-expressing cells to CTL recognition by the Ala^{82} mutation observed in our study might be attributable to the enhancement of the TAP efficiency, in addition to peptide intrinsic factors including peptide-off rate and thermodynamics of the cognate pMHC. However, this scenario is less likely because it is well known that the amino acid substitutions in the middle of epitopes, such as VY8-5A and RY11-8A in our study, have only a limited effect on TAP efficiency (52–54). Considering that a number of human viruses including HIV-1 can somehow abrogate the TAP function (50, 55), how the TAP efficiency influences the susceptibility of cells infected with various variant viruses to CTL killing is an important question to be addressed in future studies.

Both VY8 and RY11 share anchor residues, proline at position 2 and tyrosine at the C terminus, which are optimal for binding with HLA-B*3501 (56, 57); and both Ags are dominantly recognized in *HLA-B*3501*$^+$ individuals with an HIV-1 infection (6, 10, 58). Even in such a case, the improved thermostability of pMHC by an amino acid substitution within the epitopes, even other than a primary anchor residue, can substantially enhance the susceptibility to recognition by CTLs for killing target cells, suggesting that the altered peptide ligand strategy is capable of enhancing CTL-mediated immune responses against HIV-1 infection similar to that used for anti-cancer vaccines targeting self-Ags (34, 39). Our data thus highlight the importance of incorporating thermostability data in the process of rational optimization of Ags that support profound antiviral activity by HIV-specific CTLs.

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Disclosures

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Selection of escape mutation by Pol154-162-specific cytotoxic T cells among chronically HIV-1-infected HLA-B*5401-positive individuals

Masao Hashimoto [a], Mitsutaka Kitano [a], Kazutaka Honda [a], Hirokazu Koizumi [a], Sachi Dohki [a], Shinichi Oka [b,c], Masafumi Takiguchi [a,*]

[a] Division of Viral Immunology, Centers for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[b] Division of Infectious Disease, Centers for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[c] AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

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ABSTRACT

Most escape mutations have been identified on cytotoxic T lymphocyte (CTL) epitopes presented by Caucasian or African human leukocyte antigen (HLA) class I alleles, whereas a limited number of studies have identified the escape mutations on epitopes presented by Asian alleles. HLA-B54 is a common HLA allele in Asian countries. We recently identified five HLA-B*5401-restricted HIV-1-specific CTL epitopes. We here investigated escape mutations in these CTL epitopes in Japanese HIV-1-infected individuals. The frequency of substitution from Glu (E) to Asp (D) at position 7 (FV9-7D) in the Pol 154-162 (FV9) epitope was significantly higher in HLA-B*5401$^+$ HIV-infected individuals than in HLA-B*5401$^-$ individuals, whereas substitutions that were significantly higher in HLA-B*5401$^+$ individuals than in HLA-B*5401$^-$ individuals were not found in the other four epitopes. FV9-specific CTLs showed reduced killing activity against target cells pulsed with the FV9-7D mutant peptide and failed to kill those infected with the FV9-7D mutant virus, strongly suggesting that FV9-7D is an escape mutant. Furthermore, longitudinal sequence analysis of the FV9 epitope in two HLA-B*5401$^+$ individuals revealed that the sequence had changed from the wild type to the FV9-7D during the clinical course. Taken together, these results indicate that the FV9-7D escape mutant had been selected by FV9-specific CTLs among chronically HIV-1-infected HLA-B*5401$^+$ individuals.

1. Introduction

Cytotoxic T lymphocytes (CTLs) play an important role in controlling HIV-1 replication during acute and chronic phases of an HIV-1 infection [1,2]. However, HIV-1 escapes from the host immune system by various mechanisms, including mutations of immunodominant CTL epitopes [3–7]. The escape of HV-1 from CTLs has been proposed to be a major obstacle for HIV-1 vaccine development [8–10].

A number of studies have demonstrated that CTL-mediated immune pressure selects escape mutants during acute and chronic phases of HIV-1 infections [3–5,11]. Several escape mechanisms have been proposed when mutations occur within CTL epitopes: a substitution of an amino acid abrogates peptide binding to HLA molecules, reduces the recognition of T-cell receptor, and/or interferes with efficient antigen processing [8]. Any of these mechanisms results in impaired CTL activities against target cells infected with HIV-1 mutants, which contribute to the selection of HIV-1 escape mutants. The appearance of escape mutants can lead to the loss of immune control and eventually accelerate the disease progression [2,3,6,12].

Many studies have focused on HIV-1 escape mutants associated with Caucasian or African human leukocyte antigen (HLA) alleles [6,8,13–18]. In contrast, a paucity of data is available on HIV-1 escape mutants selected in Asian populations [7]. HLA-B54 is a common HLA allele in Asia, including Japan. HLA-B*5401, which is the only genotype of HLA-B54 in the Japanese population, is reported in approximately 13% of Japanese people [19]. Previously, we identified five HLA-B*5401-restricted HIV-1-specific CTL epitopes [20]: Pol154-162 (FPISPIETV, FV9), Pol303-312 (LPQGWKGSPA, LA10), Pol792-800 (HVASGYIEA, HA9), Nef125-133 (FPDWQNYTP, FP9), and Nef150-160 (VPVEPEKVEEA, VA11). However, it remains unknown whether escape mutants among these epitopes are selected by HIV-1-specific CTLs in the Japanese population.

The objective of the present study is to determine whether some escape mutants are selected among those five HLA-B*5401-restricted HIV-1-specific CTL epitopes. Here, we first analyzed the sequences of those five HLA-B*5401-restricted HIV-1-specific CTL epitopes in chronically HIV-1-infected Japanese individuals. When the frequency of some amino acid substitutions was significantly higher in HLA-B*5401$^+$ than in HLA-B*5401$^-$ individuals, we ex-

* Corresponding author.
E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

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amined whether such substitutions indeed contributed to a failure of recognition by CTLs. In addition, we performed longitudinal analyses of sequences of those epitopes to confirm that those substitutions had actually been selected in chronically HIV-1-infected HLA-B*5401$^+$ individuals.

2. Subjects and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the International Medical Center of Japan and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. For sequence analyses, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The patients' HLA types were determined by standard sequence-based genotyping.

2.2. Sequence of autologous virus

Viral RNA was extracted from samples of plasma from HIV-1-infected individuals using a QIAamp MinElute virus spin kit (Qiagen GmbH, Germany), and cDNA was synthesized from RNA with SuperScript RNase H-reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). Proviral DNA was extracted from PBMCs of HIV-1-infected individuals using a QIAamp DNA blood mini kit (Qiagen). Corresponding Pol or Nef regions were amplified by nested PCR using Taq DNA polymerase (Promega, Madison, WI). The PCR products were then purified with agarose gel and sequenced directly. DNA sequencing was performed using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

2.3. Cells

C1R cells expressing HLA-B*5401 (C1R-B*5401) were previously generated [20] and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.2 mg/ml neomycin. 721.221-CD4-B*5401 cells were generated by transfecting the CD4 and HLA-B*5401 genes into 721.221 cells and maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B. MT2 and H9 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.1 mg/ml kanamycin. MAGIC-5 cells (CCR-transduced HeLa-CD4/LTR-β-gal cells) were cultured and used as described previously [21].

2.4. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium containing 10% FCS and 200 U/ml human recombinant interleukin-2), 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R-B*5401 pulsed with 1 μM concentration of the appropriate HIV-1-derived peptides. Wells positive for the growth after about 2 weeks were examined for CTL activity by the standard ^{51}Cr-release assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.5. HIV-1 clones

An HIV-1 mutant was generated by introducing the FV9-7D mutation into NL432 (NL432-FV9-7D) using site-directed mutagenesis (Invitrogen) based on overlap extension.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity was measured by the standard ^{51}Cr-release assay, as previously described [7]. Target cells (2×10^5) were incubated for 1 hour with 100 mCi $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% newborn calf serum. Labeled target cells (2×10^3/well) were added to 96-well round-bottom microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After 1 hour of incubation, effector cells were added and the mixtures were then incubated for 4 hours at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells.

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4$^-$B*5401 cells were exposed to NL432 or NL432-FV9-7D for several days. The cells were used as target cells for CTL assays when approximately 60% of cells were infected, which was confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ^{51}Cr as described above. Labeled target cells were added along with effector cells into round-bottom microtiter plates (Nunc), and the mixtures were incubated for 6 hours at 37°C.

2.8. Replication kinetics assay

MT-2 cells (1×10^5) were exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 2 hours, washed twice with phosphate-buffered saline (PBS), and

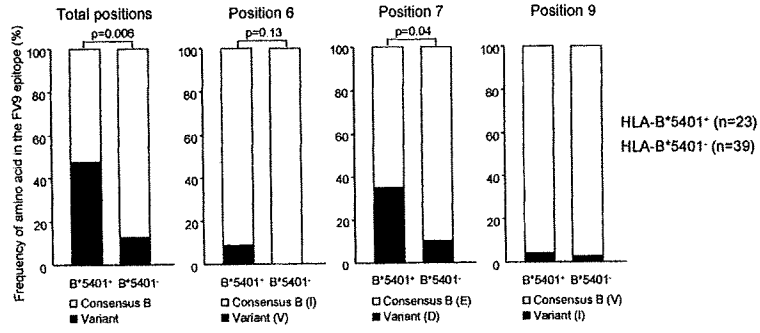

Fig. 1. Frequency of mutations in the FV9 epitope among HLA-B*5401$^+$ and HLA-B*5401$^-$ HIV-1-infected individuals. The consensus sequence of this epitope in clade B is FPISPIETV. The frequency of mutations in the total sequence and at a given position of the epitope is illustrated for both HLA-B*5401$^+$ and HLA-B*5401$^-$ HIV-1-infected individuals. *p* values were determined using Fisher's exact test.

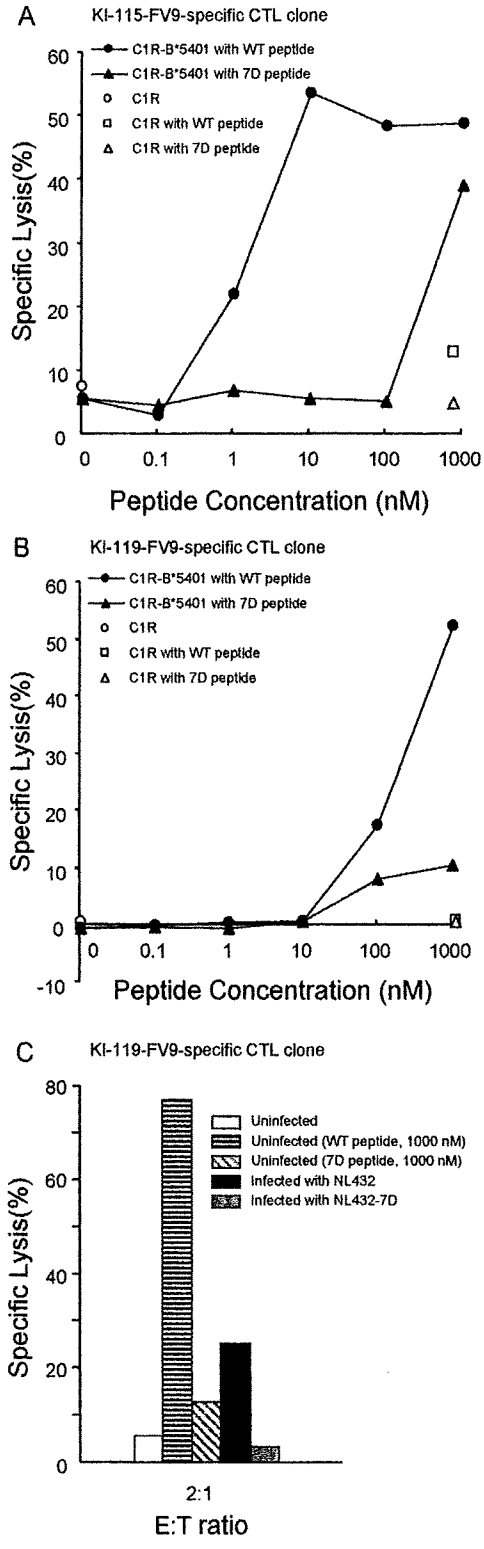

Fig. 2. Cytolytic activity of FV9-specific CTLs against target cells pulsed with the mutant (FV9-7D) peptide or those infected with the mutant virus (NL432-FV9-7D). A. Cytolytic activity of KI-115-FV9-specific CTL clone to kill C1R-B*5401 cells pulsed with the wild-type (FV9) or FV9-7D peptide. C1R-B*5401 cells were pulsed with various concentrations of the FV9 or FV9-7D peptide. B. Cytolytic activity of KI-119-FV9-specific CTL clone to kill C1R-B*5401 cells pulsed with the FV9 or FV9-7D peptide. C1R-B*5401 cells were pulsed with various concentrations of the FV9 or FV9-7D peptide. C. Cytolytic activity of KI-119-FV9-specific CTL clones against 721.221-CD4-B*5401 cells infected with the NL432 or NL432-FV9-7D virus. 721.221-CD4-B*5401 cells were used as target cells at an E:T ratio of 2:1. The percentages of p24 antigen-positive cells among target cells infected with NL432 and NL432-FV9-7D were 53.4 and 56.4%, respectively.

cultured in 1 ml of complete medium [21]. Aliquots (0.1 ml) of the culture were harvested every other day, and the volume removed was replaced with fresh medium. The concentration of p24 antigen was measured using an enzyme immunoassay (HIV-1 p24 antigen enzyme-linked immunosorbent assay kit; ZeptoMetrix, Buffalo, NY). Replication kinetics assays were performed in duplicate.

2.9. Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed for 2 hours to mixtures of paired virus preparations (300 blue cell-forming units each; NL432 vs NL432-FV9-7D) for examination of their replication ability, washed twice with PBS, and cultured as described previously [21]. On day 1, one third of the infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 passage). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells. The cells harvested at the end of each passage were then subjected to direct DNA sequencing of the HIV-1 RT gene. The change in viral population was determined from the relative peak height on sequencing electrograms. The persistence of the original amino acid substitution was confirmed for each infectious clone used in this assay.

3. Results

3.1. Association of an HLA-B*5401 allele with mutations among five HLA-B*5401-restricted HIV-1-specific CTL epitopes

To clarify whether HLA-B*5401-restricted HIV-1-specific CTLs select the escape mutant at the population level, we analyzed the sequences of 5 HLA-B*5401-restricted CTL epitopes (FPISPIETV; FV9, LPQGWKGSPA; LA10, HVASGYIEA; HA9, FPDWQNYTP; FP9, and VPVEPEKVEEA; VA11) in HIV-1 infected individuals. Among these 5 CTL epitopes, HLA-B*5401-associated mutations were reported only in the FV9 epitope. There were no HLA-B*5401-associated mutations within or at the flanking regions of the other 4 HLA-B*5401-restricted CTL epitopes (data not shown). The sequence analysis of the FV9 epitope and its flanking regions of HIV-1 from 23 HLA-B*5401$^+$ and 39 HLA-B*5,401$^-$ HIV-1-infected individuals exhibited several mutations at positions 6, 7, and 9 of the epitope (Fig. 1). The frequency of the substitution of Glu (E) to Asp (D) mutation at position 7 (FV9-7D) was significantly higher in HLA-B*5401$^+$ than in HLA-B*5401$^-$ HIV-1-infected individuals, whereas no significant differences were observed at other positions between HLA-B*5401$^+$ and HLA-B*5401$^-$ donors (Fig. 1). In the flanking regions of the FV9 epitope, there was also no significant difference among these donors (data not shown). These results suggest that the FV9-7D mutant was selected by FV9-specific CTLs.

3.2. FV9-7D is an escape mutant from FV9-specific CTLs

To confirm that FV9-7D was an escape mutant for FV9-specific CTLs, we investigated whether FV9-specific CTLs could recognize the FV9-7D mutant. First, we tested the activity of FV9-specific CTL clones to kill target cells pulsed with the FV9-7D mutant peptide. Two FV9-specific CTL clones, which were generated from two HLA-B*5401$^+$ HIV-1-infected individuals (KI-115 and KI-119), effectively killed target cells pulsed with the FV9 wild-type peptide. In contrast, both CTL clones showed a reduced ability to kill target cells pulsed with the FV9-7D mutant peptide (Fig. 2A, 2B).

To clarify whether FV9-specific CTLs failed to recognize target cells infected with HIV-1 mutant virus containing FV9-7D muta-

tion, we generated the HIV-1 mutant by introducing the FV9-7D mutation into NL432 (NL432-FV9-7D) and examined whether FV9-specific CTLs could kill target cells infected with the FV9-7D mutant virus. The FV9-specific CTL clone killed target cells infected with NL432 effectively but failed to kill those infected with NL432-FV9-7D (Fig. 2C). These results indicate that FV9-7D was indeed a mutant that had escaped from FV9-specific CTLs.

We performed longitudinal analysis of a sequence of the FV9 epitope in two HIV-1-infected HLA-B*5401$^+$ individuals, KI-091 and KI-160 (Table 1). In KI-091, the wild-type sequence was detected 11 months after the first visit in the early phase of the infection (September 10, 2001). Four months later, a FV9-7D mutation appeared and then remained stable over 3 years. In KI-160, the sequence of the FV9 epitope was the wild type at the first visit in the early phase of the infection (July 25, 2002) and then had changed to FV9-7D within the next 3 years. These results support the idea that FV9-7D was the escape mutant selected by FV9-specific CTLs.

3.3. FV9-7D mutation does not impact viral replication in vitro or in vivo

To examine the impact of the FV9-7D mutation in the FV9 epitope on viral replication, we investigated the replication kinetics for NL432 and NL432-FV9-7D. NL432-FV9-7D exhibited replication equivalent to that of NL432 (Fig. 3A). Moreover, NL432 and NL432-FV9-7D exhibited comparable replication efficiency in a competitive HIV-1 replication assay using H9 cells (Fig. 3B).

Finally, to examine whether the FV9-7D mutation was stable or reverted to the wild-type sequence in HLA-B*5401$^-$ HIV-1-infected individuals, we performed longitudinal analysis of the sequence of the FV9 epitope in HLA-B*5401$^-$ HIV-1-infected individuals, those who had the FV9-7D mutation (Table 2). Among four HLA-B*5401$^-$ HIV-1-infected individuals with the FV9-7D mutation, plasma samples from three of them (KI-060, KI-068, and KI-107) were available for this analysis. In all samples, the FV9-7D mutation had remained stable during the period tested (range: 17–71 months, median period: 41 months).

4. Discussion

In the present study we identified FV9-7D as an escape mutation among the five HLA-B*5401-restricted CTL epitopes. Although it is known that a restricted number of HIV-1-specific T cells select escape mutants, the factors selecting escape mutants remain unclear. Given that HLA-associated polymorphisms are predominantly driven by immunodominant CTL responses [22], the FV9 epitope is the immunodominant one among chronically HIV-1-infected HLA-B*5401$^+$ individuals. In fact, our previous study showed that FV9-specific memory CTLs could be detected more frequently in chronically HLA-B*5401$^+$ HIV-1-infected individuals than in the other four epitope-specific ones [20].

The FV9-7D mutation may be assumed to have little impact on viral fitness and not to revert *in vivo* for the following reasons: first, the FV9-7D mutant virus exhibited a replication capacity equivalent to that of the wild-type virus *in vitro*. Second, the FV9-7D mutation remained stable in HLA-B*5401$^-$ hosts in the present study. Reversion to wild-type sequence would likely occur if the escape mutant is transmitted to a non-HLA-matched recipient in the absence of CTL selective pressures and if the escape mutation is located in a region within the viral genome where an escape mutation is accompanied with a high replication fitness cost for the virus [23,24]. Consequently, the FV9-7D mutation is speculated to accumulate in our cohorts. However, compared with other escape mutations previously reported in Japanese cohorts [7,25], the accumulation rate of the FV9-7D mutation was not as high, with the mutant being reported in only about 10% of HLA-B*5401$^-$ HIV-1-infected individuals in the present study. Gag28-3R and Nef138-2F are escape mutants selected by HLA-A*2402-restricted HIV-1-specific CTLs, and those mutants were shown to accumulate among HLA-A*2402$^-$ hosts with an approximate frequency of 30–50% [7,25,26]. One explanation for this discrepancy in accumulation rates of the escape mutants is that the prevalence of the population with the relevant HLA-allele by which HIV-1-specific CTLs responses are restricted is different. HLA-B*5401 is reported in approximately 13% of the Japanese population, whereas HLA-A*2402 is expressed

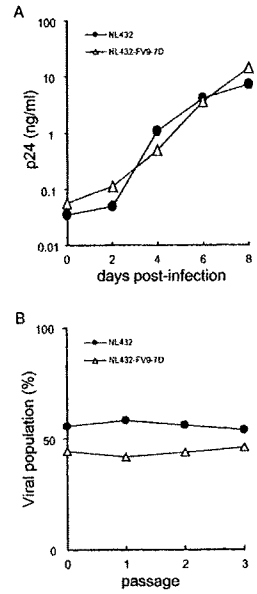

Fig. 3. Replication kinetics of HIV-1 clones. A. HIV-1 clones were propagated in MT-2 cells. The concentration of p24 in the culture medium was measured every other day. The assay was performed in duplicate, and the data represent the logarithmic mean values of p24 concentrations. B. Two infectious HIV-1 clones to be compared for their fitness (L432 vs NL432-FV9-7D) were mixed and used to infect H9 cells. The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells on day of the culture (0 passage) and at the end of each passage were subjected to nucleotide sequencing, and the proportion of Glu and Asp at position 7 of the FV9 epitope was determined. The black circle and white triangle indicate the population of Glu and Asp at position 7 of the FV9 epitope, respectively.

Table 1
Longitudinal analysis of the FV9 epitope sequence of HLA-B*5401$^+$ HIV-1-infected individuals

ID	Sample date (month/day/year)	Sample	Sequence*
KI-091	09/10/2001	Proviral DNA	----------
	01/09/2002	Proviral DNA	-------D--
	08/04/2005	RNA	-------D--
KI-160	07/25/2002	RNA	----------
	12/27/2005	RNA	-------D--

*The consensus sequence of the FV9 epitope in clade B is FPISPIETV.

Table 2
Longitudinal analysis of the FV9 epitope sequence of HLA-B*5401$^-$ HIV-1-infected individuals

ID	Sample date (month/day/year)	Sample	Sequence*
KI-060	08/18/2001	RNA	-------D--
	07/03/2007	RNA	-------D--
KI-068	09/27/2001	RNA	-------D--
	03/06/2003	RNA	-------D--
KI-107	06/12/2001	RNA	-------D--
	11/29/2004	RNA	-------D--

*The consensus sequence of the FV9 epitope in clade B is FPISPIETV.

in about 70%. Other possible explanations are variability in the frequency of CTL responses induced among the populations with relevant HLA-alleles and the ability of those epitope-specific CTLs to select escape mutants. An FV9-specific CTL response was detected in 50% of chronically HIV-1-infected HLA-B*5401$^+$ individuals [20], whereas Gag28- and Nef138-specific CTL responses were detected in 67 and 58% of chronically HIV-1-infected HLA-A*2402$^+$ individuals, respectively [7,25]. Furthermore, the FV9-7D mutation was selected by FV9-specific CTLs in 35% of HLA-B*5401$^+$ HIV-1-infected individuals, whereas both Gag28-3R and Nef138-2F mutations were selected by those epitope-specific CTLs in approximately 70% of HLA-A*2402$^+$ individuals. Together, these differences might influence the pace of accumulation of the HIV-1 mutants, including the FV9-7D mutation at the population level.

The appearance of the FV9-7D mutation in HIV-1-infected HLA-B*5401$^+$ individuals would not lead to a preferable outcome of the disease because escape mutations often undermine immune control. Taking into account that FV9-specific CTLs failed to recognize cells infected with FV9-7D mutant virus *in vitro*, those CTLs may fail to respond to the mutant virus in HLA-B*5401$^+$ HIV-1-infected hosts. By contrast, because it is well known that some escape mutants are associated with reduced viral replication capacity [18], they are sometimes advantageous for HIV-1-infected individuals [27]. Such a situation, however, might not be applicable to the FV9-7D mutation for HLA-B*5401$^+$ hosts because the FV9-7D mutation is not accompanied by low fitness. Taken together, the data indicate that this mutation ultimately might be disadvantageous for HLA-B*5401$^+$ HIV-1-infected individuals.

The present study demonstrated that the FV9-7D mutation had been selected as an escape mutation by FV9-specific CTLs among HIV-1-infected HLA-B*5401$^+$ individuals. Further studies of CTL escape mutations selected in Japanese and other Asian populations are necessary to understand the interaction between the hosts and HIV-1 in those populations.

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Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients

Atsuko Hachiya[a,b], Kazuki Shimane[c,d], Stefan G. Sarafianos[e], Eiichi N. Kodama[c,*,1], Yasuko Sakagami[c], Fujie Negishi[a], Hirokazu Koizumi[b], Hiroyuki Gatanaga[a,b], Masao Matsuoka[c], Masafumi Takiguchi[b], Shinichi Oka[a,b]

[a] AIDS Clinical Center, International Medical Center of Japan, Japan
[b] Center for AIDS Research, Kumamoto University, Japan
[c] Institute for Virus Research, Kyoto University, 53 Kawaramachi, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[d] Graduate School of Biostudies, Kyoto University, Japan
[e] Christopher Bond Life Sciences Center, Department of Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia, MO, USA

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RNase H domain

ABSTRACT

Some mutations in the connection subdomain of the polymerase domain and in the RNase H domain of HIV-1 reverse transcriptase (RT) have been shown to contribute to resistance to RT inhibitors. However, the clinical relevance of such mutations is not well understood. To address this point we determined the prevalence of such mutations in a cohort of antiretroviral treatment-naïve patients ($n = 123$) and assessed whether these substitutions are associated with drug resistance *in vitro* and *in vivo*. We report here significant differences in the prevalence of substitutions among subtype B, and non-subtype B HIV isolates. Specifically, the E312Q, G333E, G335D, V365I, A371V and A376S substitutions were present in 2–6% of subtype B, whereas the G335D and A371V substitutions were commonly observed in 69% and 75% of non-B HIV-1 isolates. We observed a significant decline in the viral loads of patients that were infected with HIV-1 carrying these substitutions and were subsequently treated with triple drug regimens, even in the case where zidovudine (AZT) was included in such regimens. We show here that, generally, such single substitutions at the connection subdomain or RNase H domain have no influence on drug susceptibility *in vitro* by themselves. Instead, they generally enhance AZT resistance in the presence of excision-enhancing mutations (EEMs, also known as thymidine analogue-associated mutations, TAMs). However, N348I, A376S and Q509L did confer varying amounts of nevirapine resistance by themselves, even in the absence of EEMs. Our studies indicate that several connection subdomain and RNase H domain substitutions typically act as pre-therapy polymorphisms.

1. Introduction

The zidovudine (AZT)-resistance mutations reside at the DNA polymerase domain of HIV-1 reverse transcriptase (RT). They are associated either with (a) the exclusion mechanism that enhances discrimination at the point of AZT monophosphate (AZT-MP) incorporation through a set of mutations at codons A62, V75, F77, F116 and Q151 of the polymerase domain (Deval et al., 2002; Ueno and Mitsuya, 1997), or with (b) the excision mechanism that involves selective removal of AZT-MP after it has been incorporated by RT into the viral DNA (Boyer et al., 2001; Meyer et al., 1999). The excision mechanism is associated with mutations at the polymerase domain, including M41L, D67N, K70R, L210W, T215F/Y and K219E/Q (excision-containing mutations [EEMs] also known as thymidine analogue-associated mutations [TAMs]).

Certain mutations in the connection subdomain (CD; codons 322–440) of the polymerase domain or in the RNase H domain (codons 441–560) of HIV-1 RT have recently been shown to be associated with resistance to AZT (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Yap et al., 2007). In some cases it appears that mutations that affect AZT resistance have different phenotypes, depending on the presence or absence of other resistance mutations. For example, the polymorphism G333D/E does not confer drug resistance by itself, but has been reported to contribute significantly to dual AZT-lamivudine (3TC) resistance when combined with EEMs and M184V (Caride et al., 2000; Gallego et al., 2002; Kemp et al., 1998; Zelina

* Corresponding author. Tel.: +81 22 717 8220; fax: +81 22 717 8221.
E-mail address: ekodama@virus.kyoto-u.ac.jp (E.N. Kodama).
[1] Present address: Emerging Infectious Diseases, Tohoku University School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8575, Japan.

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et al., 2008). Similarly, A371V and Q509L, which were selected in the background of D67N and K70R by high concentrations of AZT *in vitro*, show strong resistance to AZT and weak cross-resistance to 3TC, abacavir (ABC) and tenofovir (TNF/PMPA) in the presence of EEMs (Brehm et al., 2007). Santos et al. (2008) also recently reported that the A360V and A371V mutations are frequently observed in AZT-treated patients. In contrast, one of the connection subdomain mutations, N348I, is associated with resistance to both nucleoside RT inhibitors (NRTIs) and non-nucleoside RT inhibitors (NNRTIs) and appears to be induced by regimens containing AZT, didanosine (ddI) and/or nevirapine (NVP) (Hachiya et al., 2008; Yap et al., 2007). Recently, it has been shown that the N348I mutation decreases the efficiency of RNase H cleavage and increases excision of AZT from AZT-terminated primer/templates, in the presence of the pyrophosphate donor ATP (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008; Yap et al., 2007). The decreased degradation of the RNA template by the diminished RNase H cleavage has been proposed to provide additional time for RT to excise AZT-MP and hence result in the observed increased AZT resistance (Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008).

With the exception of N348I, the clinical relevance of these mutations remains to be clarified. A major obstacle to understanding the contribution of connection subdomain mutations to NRTI or NNRTI resistance has been the shortage of relevant sequencing data. This is because, until recently, the majority of commercially available genotypic and phenotypic assays have not been targeting this region of the enzyme. This is now changing, as more attention is being focused on such substitutions, following recent publications from us (Hachiya et al., 2008) and others (Yap et al., 2007) showing that at least one connection subdomain mutation, N348I, contributes to multi-class drug resistance. However, it has not yet been determined if the genotypic substitutions encountered in the connection subdomain of polymerase or in the RNase H domain of RT have any phenotypic impact or any effect on virologic response to subsequent therapies. Another important question is whether resistance testing now performed should include these mutations.

To ascertain whether some mutations at the connection subdomain or at the RNase H domain of RT that appear in the absence of known drug-resistance mutations of the polymerase domain are induced by reverse transcriptase inhibitor (RTI) treatment or are simply pre-existing polymorphisms, we determined the frequency of amino acid substitutions in antiretroviral treatment-naïve patients and assessed whether these substitutions at the reported sites (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) can cause drug resistance by themselves. We also explored whether these substitutions may have any effect on the virologic response to subsequent therapies.

2. Materials and methods

2.1. Patients

A total of 123 clinical isolates were obtained from fresh plasma of treatment-naïve HIV-infected patients using MAGIC-5 cells as described previously (Hachiya et al., 2001). Written informed consent was obtained from each patient under approval by the Institutional Review Board of the International Medical Center of Japan (IMCJ-H13-80). The clinical course and antiretroviral therapies used were reviewed retrospectively.

2.2. Recombinant molecular clones

Recombinant molecular clones were generated as described previously (Hachiya et al., 2008). Briefly, the pBS-RT_{WT} contains almost entire RT coding sequence (amino acid position 14–560) containing silent mutations for cloning (restriction enzyme sites, Xma I and Xba I at 5′- and 3′-end of DNA fragment, respectively). After site directed mutagenesis, the mutated RT was ligated into the corresponding restriction enzyme site of the HIV infectious clone pNL101 (Hachiya et al., 2008; Shimura et al., 2008).

2.3. Genotypic and phenotypic assays

For the genotypic assay, viral RNA was extracted from the culture supernatant of clinical isolates, amplified by nested RT-PCR, and then directly sequenced as described previously (Hachiya et al., 2008). For subtype classification, the RT sequences were analyzed using the 'Genotyping' software (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) which uses the BLAST algorithm. HIV-1 sequences in worldwide, treatment-naïve patients were obtained from the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu/index.html, accessed as late as 26 February 2008) and compared with our cohort. Prevalence of mutations at each codon were compared by the χ^2-test, or Fisher's exact test when the number of patients was smaller than 5.

For phenotypic assay, each clinical isolate was directly tested for drug susceptibility in triplicates, using the MAGIC-5 cell-based assay as described previously (Hachiya et al., 2001). Infectious viruses were obtained by transfection of 293T cells with individual HIV molecular clones containing the desired mutations that were introduced by site directed mutagenesis. Cells were subsequently harvested and examined with the MAGIC-5 cell based assay (Hachiya et al., 2001, 2008).

2.4. Measurements of HIV-1 viral load

To assess virologic outcome, HIV-1 viral loads in plasma were measured using the commercially available Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland). Mean change from 0 at weeks 4, 8, 12, 16, 20 and 24 were evaluated. The statistical significance of the longitudinal changes of HIV-1 viral load in plasma was assessed by the Mann–Whitney *U*-test.

2.5. Molecular modeling studies

The SYBYL and O programs were used to prepare molecular model of the complexes of HIV-1 RT in complex with RNA/DNA (Protein Data Bank code number 1HYS), and containing mutations A376S, N348I and Q509L that were introduced manually into the original 1HYS structure. After introduction of the mutations, the structure coordinates were optimized through 100 cycles of Coleman energy minimization protocol.

3. Results

3.1. Sequence analysis of RT region

We sequenced nearly the entire RT coding region (amino acid position 9–560) of 123 clinical isolates obtained from treatment-naïve patients. Among these isolates, six contained the known RTI-associated resistant mutations, D67N ($n=2$), K238S ($n=2$) (http://www.hiv.lanl.gov/content/index), V108I/K238S ($n=1$) and V106A/V108I/K238S ($n=1$), and thus were excluded from further analysis. The clinical isolates were obtained from six patients within 1 year of the diagnoses for HIV-1 infection. Prevalence of HIV-1 with drug-associated mutations in Japanese treatment-naïve patients is estimated at approximately 4% (Gatanaga et al., 2007) and in American and European patients at 8–27% (Descamps et al., 2005; Little et al., 2002; UK Collaborative Group on Monitoring the Transmission

of HIV Drug Resistance, 2001; Weinstock et al., 2004). Therefore, the prevalence in our cohort (4.8%) seems to be comparable or lower than in previous reports, suggesting that the six patients are treatment-naïve and newly infected from treated patients. The strong majority of the remaining samples in our cohort were of subtype B ($n = 101$ of a total of 117 isolates), while other subtypes were also identified (CRF01_AE, A, C and CRF12_BF, with12, 2, 1 and 1 isolates, respectively).

Substitutions at the connection subdomain and RNase H domain observed in this cohort and in previous reports (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) are shown in Table 1. In the treatment-naïve patients of our cohort that were infected with subtype B ($n = 101$), the frequencies of all mutations associated with AZT-resistance (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007) were comparable to those (treatment-naïve) deposited in the Stanford HIV Drug Resistance Database, except for the A360T mutation. The G335D and A371V substitutions were more prevalent in the non-B, rather than in the B isolates of our cohort. Moreover, the G335D/A371V combination was observed in 9 (56.3%) of the non-B isolates. Other polymorphisms, including E312A/D/N/T, G335E/N/S, A360S/T, A371T, A376T/V and Q509H, were widely observed in all subtypes in our cohort as well as in the Stanford HIV Drug Resistance Database. None of the clinical isolates of our cohort had the G333D, G335C, N348I, A360I/V, and Q509L mutations.

3.2. Phenotypic assay for clinical isolates

Phenotypically, all clinical isolates showed little resistance to tested drugs (Table 1). Isolates with the V365I substitution ($n = 4$ in subtype B) showed slightly reduced susceptibility to 3TC (2.3-fold). However, V365I may not be clinically relevant, since generally at least 3-fold resistance is required for assigning 3TC resistance *in vivo* (Parkin et al., 2004; Rhee et al., 2006). Furthermore, the prevalence of V365I in treated and untreated patients in the Stanford HIV Drug Resistance Database is comparable (3.7% and 3.6%, respectively). Notably, clinical isolates from treatment-naïve patients from our cohort with HIV carrying the E312N, G335E/N or A376V substitutions displayed rather enhanced susceptibility (over five-fold) to

Table 1
Drug susceptibilities of 117 clinical isolates obtained from *treatment-naïve patients*.

Amino acid substitutions	Frequency[a] % (n)		Median fold change in resistance[b]			
	Subtype B ($n = 101$)	Non-B ($n = 16$)	AZT	3TC	NVP	EFV
E312	84.1 (85)	18.8 (3)	1.2	1.3	1	1.1
Q[c]	**3(3)**	**0**	**1.3**	**1.4**	**1.2**	**1.1**
A	6.9 (7)	0	1.1	1.1	1.3	1
D	1(1)	6.3 (1)	1.7	1.2	1.7	1.1
N	0	6.3 (1)	0.1	1.3	0.2	1.2
T	5(5)	68.8 (11)[d]	0.8	1	1.1	1.1
G333	94.1 (95)	100(16)	1.1	1.3	1.1	1.1
D[c]	**0**	**0**	–	–	–	–
E[c]	**5.9 (6)**	**0**	**1.4**	**1.5**	**1**	**1.4**
G335	95(96)	25(4)	1.2	1.4	1	1.1
C[c]	**0**	**0**	–	–	–	–
D[c]	**2(2)**	**68.8 (11)[d]**	**0.7**	**0.9**	**1.1**	**1.1**
E	0	6.3 (1)	0.3	0.06	0.2	0.5
N	1(1)	0	0.2	0.2	0.8	1.5
S	2(2)	0	0.6	0.9	1.3	1.4
N348	100(101)	100(16)	1.1	1.3	1	1.1
I[c,e]	**0**	**0**	–	–	–	–
A360	79.2 (80)	87.5 (14)	1.1	1.3	1.1	1.1
I[c]	**0**	**0**	–	–	–	–
V[c]	**0**	**0**	–	–	–	–
S	0	6.3 (1)	0.7	1.2	1.5	0.8
T	20.8 (21)[f]	6.3 (1)	0.9	1.4	1	1.2
V365	96(97)	100(16)	1.1	1.2	1	1.1
I[c]	**4(4)**	**0**	**1**	**2.3**	**1.7**	**1.3**
A371	96(97)	25(4)	1.2	1.3	1.1	1.1
V[c]	**3(3)**	**75(12)[d]**	**0.7**	**0.9**	**0.9**	**1.1**
T	1(1)	0	0.5	1.3	0.7	0.7
A376	92.1 (93)	75(12)	1.1	1.3	1	1.1
S[c]	**3(3)**	**6.3 (1)**	**1.3**	**0.9**	**1**	**0.6**
T	5(5)	12.5 (2)	1.2	1	1.4	1.2
V	0	6.3 (1)	0.1	1.3	0.2	1.2
Q509	98(99)	100(16)	1.1	1.3	1.1	1.1
L[c]	**0**	**0**	–	–	–	–
H	2(2)	0	0.6	0.7	0.7	0.8

[a] Of 123 clinical isolates, six carried the known RTI-associated mutations and were excluded from this analysis.

[b] The drug susceptibility assay (Hachiya et al., 2001) was clinically accepted in Japan.

[c] Resistant mutations reported previously (Brehm et al., 2007; Delviks-Frankenberry et al., 2007, 2008; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Ntemgwa et al., 2007; Santos et al., 2008; Yap et al., 2007) are indicated in bold. Greater than three-fold increase of EC_{50} compared to that of NL4-3 was defined as resistance.

[d] The prevalence of these substitutions (E312T, G335D and 371V) is significantly difference among treatment-naïve patients between subtype B and non-B isolates ($p < 0.0001$).

[e] N348I confers cross-resistance to NRTIs and NNRTIs (Hachiya et al., 2008; Yap et al., 2007).

[f] The prevalence of A360T is significantly higher in our cohort compared to the Stanford HIV Drug Resistance Database (8.7%, $p = 0.0021$).

Table 2
Drug susceptibilities of molecular HIV-1 clones.

Mutation	EC_{50}, μM (fold increase)[a]				
	NRTI			NNRTI	
	AZT	3TC	TNF[b]	NVP	EFV
WT	0.026 ± 0.009	0.42 ± 0.04	6.2 ± 1.5	0.023 ± 0.01	0.0012 ± 0.0001
E312Q	0.037 ± 0.006 (1.4)	0.36 ± 0.05 (0.9)	4.1 ± 1.4 (0.7)	0.056 ± 0.007 (2.4)	0.0009 ± 0.0002 (0.8)
G333D	0.04 ± 0.01 (1.5)	0.28 ± 0.1 (0.7)	4.5 ± 1.8 (0.7)	0.055 ± 0.01 (2.4)	0.0017 ± 0.0003 (1.4)
G335C	0.04 ± 0.02 (1.5)	0.45 ± 0.1 (1.1)	7.7 ± 1.1 (1.2)	0.065 ± 0.02 (2.8)	0.0007 ± 0.00009 (0.6)
N348I	**0.14 ± 0.01 (5.4)**	0.56 ± 0.07 (1.3)	8.8 ± 1.9 (1.4)	**0.24 ± 0.04 (10)**	0.0032 ± 0.0005 (2.7)
A360I	0.037 ± 0.01 (1.4)	0.35 ± 0.1 (0.8)	7.1 ± 2.1 (1.1)	0.038 ± 0.01 (1.7)	0.0009 ± 0.00008 (0.8)
A360V	0.03 ± 0.002 (1.2)	0.28 ± 0.09 (0.7)	5.7 ± 2.3 (0.9)	0.051 ± 0.01 (2.2)	0.0016 ± 0.0006 (1.3)
V365I	0.045 ± 0.008 (1.7)	0.27 ± 0.06 (0.6)	6.1 ± 2.0 (1)	0.066 ± 0.02 (2.9)	0.0013 ± 0.0002 (1.1)
A376S	0.053 ± 0.02 (2)	0.3 ± 0.03 (0.7)	5.9 ± 1.6 (1)	0.084 ± **0.02 (3.7)**	0.0022 ± 0.0004 (1.8)
Q509L	0.072 ± 0.02 (2.8)	0.45 ± 0.1 (1.1)	8.1 ± 2.7 (1.3)	**0.21 ± 0.06 (9.1)**	0.0032 ± 0.0009 (2.7)

[a] Data means ± standard deviations from at least three independent experiments. The relative increase in the EC_{50} value compared with that in HIV-1_{WT} is given in parentheses. Bold indicates an increase in EC_{50} value greater than three-fold.

[b] TNF (PMPA) [(*R*)-9-(2-phosphonomethoxypropyl) adenine or tenofovir] is the active nucleotide of the clinical prodrug tenofovir disoproxil fumarate.

AZT and NVP, AZT, 3TC and NVP, and AZT and NVP, respectively (Table 1). In our cohort, in the absence of EEM mutations, A371V had no significant effect on drug resistance (Table 1). However, other studies have shown that combined with EEMs, A371V can confer strong resistance to AZT and A371V has also been recently reported to be associated with weak cross-resistance to 3TC, TNF/PMPA and ABC (Brehm et al., 2007). In our cohort, ABC inhibits efficiently the clinical isolates that contain the A371V substitution in the absence of EEMs ($n = 13$) either in a subtype B, or non-B background (median fold increase was 0.9-fold, data not shown). Further, the combination of A371V and G335D commonly observed in non-B isolates also showed no resistance to AZT, 3TC or ABC (0.7-, 1.0- and 1.1-fold increase in susceptibility as compared to wild-type HIV, respectively). These results demonstrate that none of the above substitutions that were observed in clinical isolates confer any significant resistance to NRTIs or NNRTIs in the absence of EEMs.

3.3. Phenotypic assay for molecular clones

To further expand our understanding of the role of substitutions in these RT regions on drug resistance we also prepared HIV-1 recombinant viruses with related mutations that have been reported previously in similar drug resistance studies (Brehm et al., 2007; Hachiya et al., 2008; Kemp et al., 1998; Nikolenko et al., 2007; Yap et al., 2007). The results shown in Table 2 confirm that in the absence of NRTI or NNRTI resistance mutations, most substitutions in the connection subdomain and RNase H domain (with the exception of N348I, A376S and Q509L) show no significant resistance to AZT, 3TC, TNF/PMPA, NVP or efavirenz (EFV) (less than three-fold), suggesting that these mutations act as secondary mutations and may enhance resistance that is caused by primary mutations and/or may somehow improve replication kinetics impaired by the primary mutations. Q509L, which has been reported to enhance

Table 3
Profiles of patients infected with HIV carrying connection subdomain substitutions, and initial therapies used in patient treatments.

Parameter	Combination for treatment-naïve patients infected HIV-1			
	With substitutions		Without substitutions	
	With AZT ($n = 8$)	Without AZT ($n = 13$)	With AZT ($n = 16$)	Without AZT ($n = 24$)
Male, *n* (%)	5(63)	10(77)	15(94)	23(96)
Median age (range)	37(27–60)	41(27–54)	36(24–55)	38(26–59)
Median baseline viral load, $\log_{10}$ copies/ml (range)	5.0(3.0–6.0)	5.0(4.2–5.8)	5.0(4.1–6.4)	5.2(4.2–6.3)
Median baseline CD4 cell count, cell/μl (range)	217(3–549)	110(3–332)	225(9–613)	170(4–760)
Substitutions in the connection subdomain, *n* (%)[a]				
E312Q	–	3(23)	–	–
G333E	2(25)	2(15)	–	–
G335D	3(38)	6(46)	–	–
V365I	2(25)	–	–	–
A371V	2(25)	5(38)	–	–
A376S	1(13)[b]	2(15)	–	–
Initial therapy, *n* (%)				
Zidovudine	8(100)	–	16(100)	–
Lamivudine	4(50)	11(85)	12(75)	24(100)
Stavudine	–	11(85)	–	20(83)
Didanosine	4(50)	–	4(25)	–
Abacavir	1(13)	1(8)	1(6)	3(13)
Tenofovir	–	1(8)	–	1(4)
Emtricitabine	–	1(8)	–	–
Nevirapine	–	–	–	3(13)
Efavirenz	2(25)	3(23)	9(56)	9(38)
One protease inhibitor (PI)	3(38)	7(54)	5(31)	7(29)
Dual-boosted PI	1(13)	2(15)	1(6)	5(21)

[a] E312Q, G333E, G335D, V365I, A371V and V376S were reported to be AZT-resistant mutations (Brehm et al., 2007; Kemp et al., 1998; Nikolenko et al., 2007).

[b] In this case, the viral load did not fall below the limits of detection.

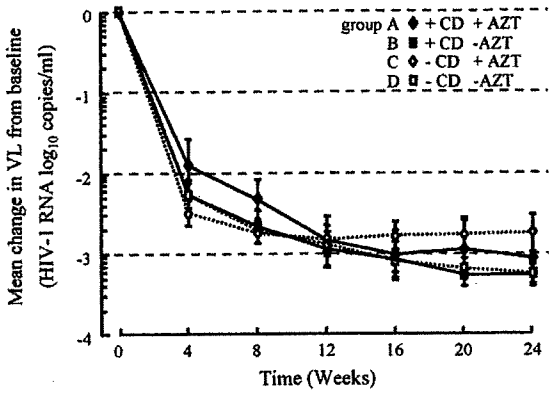

Fig. 1. Virological response up to 24 weeks after initiation of combination therapy. Mean (±standard error of the mean; S.E.M.) changes in plasma viral load (VL) were measured by Amplicor HIV-1 Monitor Test (Version 1.5, Roche Diagnostics K.K., Basel, Switzerland) from 0 to 24 weeks. Treatment-naive patients that were subsequently treated with combination therapy regimens are classified into four groups: patients that were infected with HIV-1 containing connection subdomain (CD) mutations and that subsequently received either combination therapy with AZT ($n = 8$, closed diamonds, group A) or without AZT ($n = 13$, closed squares, group B) and patients who were infected with HIV-1 with none of connection subdomain substitutions, and who subsequently received combination therapy with either AZT ($n = 16$, open diamonds with broken line, group C) or without AZT ($n = 24$, open squares with broken line, group D).

cross resistance to NRTIs in the presence of EEMs (Brehm et al., 2007), conferred little resistance to at least AZT, 3TC and TNF/PMPA in this study. Unlike N348I that confers dual resistance to NRTIs and NNRTIs, A376S and Q509L provided only NVP resistance.

3.4. Virological response after initiation of combination therapy

To further assess whether the CD substitutions at baseline are one of predictive factors of virologic outcome, we examined clinical samples from the treatment-naïve patients who subsequently received combination therapy through measuring virus load in plasma from 0 to 24 weeks (Table 3 and Fig. 1). The treatment-naïve patients were classified in four groups: (A) patients who were infected by HIV-1 that carried one or two of the CD substitutions E312Q, G333E, G335D, V365I, A371V or A376S and who subsequently received combination therapy that contained AZT ($n = 8$); (B) patients who were infected by HIV-1 that carried the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n = 13$); (C) patients who were infected by HIV-1 that did not carry any of the above CD substitutions and who subsequently received combination therapy containing AZT ($n = 16$); and (D) patients who were infected by HIV-1 that did not carry the above CD substitutions and who subsequently received combination therapy that did not contain AZT ($n = 24$). The mean change in viral load from baseline (week 0) to week 24 was from −2.76 to −3.28 $\log_{10}$ copies/ml among four groups. There were no significant differences in viral load changes up to 24 weeks among these groups (Fig. 1). Marginal viral suppression was observed in one patient who was infected by HIV-1 carrying A376S and who received combination therapy containing AZT. Any of the drug-associated resistant mutations were detected during the first 5 months of receiving combination therapy. However, HIV-1 protease mutations D30N and M36I that are responsible for resistance to NFV and HIV-1 RT D67N mutation that is responsible for AZT resistance eventually emerged. After switching to a new combination regimen (d4T/3TC/LPV), the viral load was successfully decreased. These results indicate that at least combination of two substitutions in the connection subdomain that are observed in treatment-naïve patients do not affect the virologic response of the ensuing combination therapy. Instead, they merely act as polymorphisms among the treatment-naïve patients.

4. Discussion

According to the crystal structure of HIV-1 RT in complex with RNA/DNA, some amino acids in the connection subdomain may affect binding to the RNA/DNA substrate (Sarafianos et al., 2001). It has been proposed that mutations at the connection subdomain may alter the binding affinity for nucleic acid at the connection subdomain and lead to enhanced resistance to AZT when combined with EEMs. This is thought to happen through a decrease in template RNA degradation which in turn provides additional time for RT to excise AZT-MP from the AZT-terminated template-primer$_{AZT\text{-}MP}$, thus causing resistance to AZT (Delviks-Frankenberry et al., 2007; Nikolenko et al., 2005, 2007). In our cohort, as well as in the Stanford HIV Drug Resistance Database, we observed a considerable number of treatment-naïve clinical samples containing substitutions (E312Q, G333E, G335D, V365I, A371V and A376S) that have been previously associated with AZT resistance. Our phenotypic studies with clinical isolates carrying mutations located in the connection subdomain of the polymerase or in the RNase H domain of RT revealed that in the absence of other known NRTI or NNRTI resistance mutations they do not cause by themselves significant resistance to the tested RTIs. Additionally, results from our cohort establish that the presence of G333E, G335D, V365I or A371V among treatment-naïve patients does not play any significant role in the virologic response after initiation of therapies that may, or may not, include AZT. We identified 25 isolates that have been deposited before 1986, prior to clinical trails for AZT in the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/content/index). Some of these isolates also contained E312V, V365I, A376S/T/P, indicating that at least these substitutions are polymorphisms that preceded any antiviral therapy.

None of the isolates in our cohort had the H539N or H549N substitutions which have been proposed to be associated with resistance to NRTIs due to decreasing the frequency of RT template-switching and the level of RNase H activity (Nikolenko et al., 2004; Roquebert and Marcelin, 2008). Furthermore, the G333D, G335C, N348I, A360I/V and Q509L substitutions were not observed in our cohort, and were also rarely observed among treatment-naïve patients (less than 1%) in the Stanford HIV Drug Resistance Database. Their increased incidence among NRTI-treated patients as compared to untreated patients (>3-fold, >40-fold and >12-fold increases for G333C, N348I, and A360V respectively [http://hivdb.stanford.edu/cgi-bin/RTPosMutSummary.cgi]) and in the case of Q509L reported by others (Brehm et al., 2007; Roquebert et al., 2007) suggests that they are associated with AZT resistance. However, site directed mutagenesis studies showed that G333D (Kemp et al., 1998), G335C (Nikolenko et al., 2007), A360I/V (Nikolenko et al., 2007) and Q509L (Brehm et al., 2007) did not confer significant AZT resistance in the absence of other AZT resistance mutations. At present, only N348I has been shown to be involved in resistance to multiple RTIs (Hachiya et al., 2008; Yap et al., 2007). HIV with a serine at codon 376 also exhibits some NVP resistance in the absence of other mutations (Table 2). However, clinical isolates harboring different residues at position 376 exhibited no significant changes in their drug susceptibilities (Table 1). This discrepancy may arise from strain-specific polymorphisms that are present in the clinical isolates or the reference virus used in this study that may influence NVP susceptibility positively or negatively, respectively. In fact, we observe several polymorphisms in the majority of these isolates and it is possible that they somehow affect drug resistance. For instance, V118I has been identified in 2% of treatment-naïve patients as one of strain-specific polymorphisms, but more frequently observed in RTI-treated patients

(Delaugerre et al., 2001). Although this mutation by itself confers no resistance, it has been reported to contribute to hypersusceptibility to NNRTI (Clark et al., 2006) as well as resistance to NRTI in the presence of E44A/D and/or EEMs (Romano et al., 2002). Therefore, it is possible that polymorphisms present in our clinical isolates may also affect drug-susceptibility leading to minor discrepancies with the results obtained with recombinant virus.

In this study, the reference clone has an A376T polymorphism that is observed in a wide range of subtypes. Therefore, it is unlikely that A376T affects NVP susceptibility. Q509L confers moderate (~9-fold) resistance to NVP (Table 2). Although Q509L was not observed in our cohort, this mutation was found in the pretreated patients of another survey ($n = 118$) (Roquebert et al., 2007). These results indicate that introduction of Q509L may alter virologic responses, especially for NVP, although so far the clinical relevance and virological response of Q509L among the antiretroviral-experienced patients remains to be elucidated by further experiments.

Analysis of the crystal structure of RT bound to RNA/DNA showed that residues 376 (of the p66 subunit) and 509 are located relatively close to the nucleic-acid binding cleft of RT, and residue 348 of the p66 subunit is located close to the hinge region of the thumb subdomain and to the NNRTI-binding pocket (Fig. 2). Recently, Abbondanzieri et al. demonstrated that binding of nevirapine to RT causes conformational changes to the enzyme, allowing it to somehow relax the grip on nucleic-acid substrate (Abbondanzieri et al., 2008; Arnold and Sarafianos, 2008). NVP acts as a rapid-equilibrium inhibitor, not a tight-binding inhibitor as EFV (Maga et al., 2000; Motakis and Parniak, 2002), and it might be more sensitive to changes in the interaction between RT and the nucleic acid substrate. Thus, changes in the interactions of RT with nucleic-acid substrate could also influence the interaction balance between polymerase and RNase H activity and consequently might lead to RTI resistance. Nevertheless, additional biochemical and structural studies are warranted to define the exact mechanisms by which these mutations in the connection subdomain and RNase H domains confer NVP resistance.

Several studies have reported a correlation between two distinct types of EEMs in various HIV subtypes (Kantor et al., 2005; Montes et al., 2004; Novitsky et al., 2007). The Type I EEMs (M41L, L210W, T215Y and occasionally the D67N mutation) appear twice as frequently as Type II EEMs (D67N, K70R, T215F and K219Q mutation) in subtype B (Marcelin et al., 2004), whereas Type II EEMs are mostly observed in non-B isolates (Montes et al., 2004; Novitsky et al., 2007). Type II EEMs confer lower levels of AZT and TNF/PMPA resistance, as compared to Type I EEMs (Cozzi-Lepri et al., 2005; Miller et al., 2004). Addition of A371V to Type II EEM background conferred cross-resistance to AZT and tenofovir (Brehm et al., 2007). A371V was observed in the majority of non-B isolates in our cohort (75%) and the Stanford HIV Drug Resistance Database (96% in CRF01_AE). Therefore, it is possible that in the background of non-B isolates, the majority of which contains drug resistance associated connection subdomain mutations, smaller number of EEMs, especially Type II EEMs, might be preferentially selected for AZT and TNF/PMPA resistance. In the absence of EEMs, mutations at the connection subdomain of non-subtype B HIV, such as E312N, G335E or A376V, appear to act as simple polymorphisms, because they either maintain or enhance drug susceptibility in non-subtypes B HIV (Table 1). However, the A376S polymorphism in samples of treatment-naive patients or in a recombinant virus used in this study conferred mild NVP resistance (Table 2). These mutations were stable even in the absence of any drug treatment, suggesting that viral fitness of these variants is likely to be comparable to wild type non-subtype B HIV.

In this study we report the prevalence of amino acid substitutions in the connection subdomain of the polymerase domain and in the RNase H domain of RT in a cohort of treatment-naïve patients. We also determined the phenotypic susceptibility of these mutants to various RTIs. Our results support the hypothesis that the substitutions observed among treatment-naïve patients have little impact on therapeutic outcome by themselves in the absence of AZT-associated mutations, although certain substitutions, such as N348I, A376S, and Q509L, are involved in drug resistance even by themselves. These results may help improve existing interpretation algorithms and analysis of drug resistance mutations.

Fig. 2. Structure of HIV-1 RT in complex with RNA/DNA. The fingers, palm, thumb, connection subdomains, and RNase H domain of the p66 subunit colored in blue, red, green, yellow and orange, respectively. The p51 subunit is shown in dark yellow. Residue 348 of the p66 subunit is shown as pink Van der Waals spheres, and located proximally to the hinge region of the thumb subdomain and to the NNRTI binding pocket. Residues 376 and 509 of the p66 subunit are also shown, and are located proximally to the nucleic acid binding cleft.

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