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Different Abilities of Escape Mutant-Specific Cytotoxic T Cells To Suppress Replication of Escape Mutant and Wild-Type Human Immunodeficiency Virus Type 1 in New Hosts▽

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There is much evidence that in human immunodeficiency virus type 1 (HIV-1)-infected individuals, strong cytotoxic T lymphocyte (CTL)-mediated immune pressure results in the selection of HIV-1 mutants that have escaped from wild-type-specific CTLs. If escape mutant-specific CTLs are not elicited in new hosts sharing donor HLA molecules, the transmission of these mutants results in the accumulation of escape mutants in the population. However, whether escape mutant-specific CTLs are definitively not elicited in new hosts sharing donor HLA molecules still remains unclear. A previous study showed that a Y-to-F substitution at the second position (2F) of the Nef138-10 epitope is significantly detected in HLA-A*2402$^+$ hemophilic donors. Presently, we confirmed that this 2F mutant was an escape mutant by demonstrating strong and weak abilities of Nef138-10-specific CTL clones to suppress replication of the wild-type and 2F mutant viruses, respectively. We demonstrated the existence of the 2F-specific CTLs in three new hosts who had been primarily infected with the 2F mutant. The 2F-specific CTL clones suppressed the replication of both wild-type and mutant viruses. However, the abilities of these clones to suppress replication of the 2F virus were much weaker than those of wild-type-specific and the 2F-specific ones to suppress replication of the wild-type virus. These findings indicate that the 2F mutant is conserved in HIV-1-infected donors having HLA-A*2402, because the 2F-specific CTLs failed to completely suppress the 2F mutant replication and effectively prevented viral reversion in new hosts carrying HLA-A*2402.

Cytotoxic T lymphocytes (CTLs) play an important role in the control of human immunodeficiency virus type 1 (HIV-1) replication during acute and chronic phases of HIV-1 infections (9, 28, 34). However, CTLs cannot completely eradicate HIV-1 because HIV-1 escapes from the host immune system by various mechanisms, including mutations of immunodominant CTL epitopes (10–12, 40). A substitution of one amino acid within CTL epitopes is crucial for binding to HLA class I molecules or for the interaction between the T-cell receptors (TCRs) of specific CTLs and the peptide-HLA class I complex. Both mechanisms result in the loss of CTL activities against target cells infected with HIV-1 and contribute to the selection of a virus able to escape from CTLs (10, 13, 23, 26, 35). There are many studies demonstrating that CTL-mediated immune pressure selects CTL escape variants during both acute and chronic HIV-1 and simian immunodeficiency virus (SIV) infections (2, 15, 31) and that selection of the escape mutants could result in the loss of immune control and disease progression (6, 16, 23). The escape of HIV-1 from CTL responses has been proposed to be an important obstacle for HIV-1 vaccine development (7, 16, 39).

HIV-1 mutations that allow escape from HIV-1-specific CTLs are HLA dependent because HIV-1-specific T-cell responses are restricted by HLA alleles. This means that an HIV-1 escape mutant can adapt in populations sharing some dominant HLA alleles (33). An escape mutant can be transmitted vertically from mother to child (21, 22) and horizontally between individuals through unprotected sexual intercourse (USI) (3, 20, 21, 29). A study on HIV-1 evolution has provided direct evidence that an escape mutation of an HLA-B57/5801-restricted CTL epitope is stable after transmission to individuals who did not share HLA-B57/5801 and suggested the accumulation of the escape mutation in the population (29). On the other hand, a recent study demonstrated that an escape mutant selected by the CTLs specific for the wild-type (WT) virus can elicit the escape mutant-specific CTLs in the same donors (4), suggesting the possibility that these escape mutant-specific CTLs are elicited in new donors carrying the same restriction allele. If these escape mutant-specific CTLs are elicited in the donors, it is likely that the escape mutant cannot adapt in them. However, it is well known that in both HIV-1 and SIV infections, common escape mutations are poorly recognized in new hosts who share the same HLA alleles with a donor (17, 32).

In a Japanese population infected with HIV-1 through USI, mutant viruses with Y-to-F substitutions at the second position (2F) in the HLA-A*2402-restricted, Nef138-10 WT CTL epitope (RYPLTFGWCF) were shown to accumulate in HLA-A*2402-positive and even HLA-A*2402-negative patients (20). Nef138-10-specific CTLs are frequently detected in

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chronically HIV-1-infected, HLA-A*2402-positive Japanese individuals (25), suggesting that the Nef138-10 CTL epitope is an immunodominant CTL epitope in the population. On the other hand, the 2F mutation of this epitope impaired the cytotoxic activity of the Nef138-10-specific CTLs, suggesting this mutation to be an escape one (20). We found that Nef138-10 WT tetramer$^+$CD8$^+$ T cells frequently exist, even in HLA-A*2402-positive Japanese patients with primary infections (unpublished data). As most of these Japanese patients were infected with the 2F mutant virus, we speculated that 2F-specific CTLs would be elicited in new hosts having HLA-A*2402.

The present study addressed the following three questions. Do Nef138-10-specific CTLs have strong abilities to suppress HIV-1 replication, but fail to suppress replication of the 2F mutant? Can the 2F escape mutant elicit 2F mutant-specific CTLs in a new host? Can the 2F-specific CTLs suppress replication of 2F mutant and WT viruses? The answers to these are expected to clarify the mechanisms of accumulation of escape mutants in the population.

MATERIALS AND METHODS

Patient samples. This study was approved by the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA types were determined by standard sequence-based genotyping. Donors with structured treatment interruption belonged to the clinical trial designed as a prospective study of the AIDS Clinical Center, International Medical Center of Japan.

Patients with early HIV infection, with or without acute retroviral symptoms, were recruited. Early HIV infection was confirmed within 6 months before recruitment by a documented history of seroconversion detected by enzyme-linked immunosorbent assay or by a longitudinal increase in band density on Western blots. Patients with active opportunistic infections or psychological disorders, or those treated with immunomodulatory agents, were excluded.

Sequence of autologous virus. Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (QIAGEN), and cDNA was synthesized from the RNA with SuperScript RNase H reverse transcriptase and random primer (Invitrogen). The Nef region was amplified by nested PCR using *Taq* DNA polymerase (Promega). Proviral DNA was extracted from HIV-1-infected patient PBMCs by using a QIAamp DNA blood mini kit (QIAGEN). We prepared the following *nef*-specific primer sets: 5′-AGCAGCAGATGGGGTGGGAGC-3′ and 5′-AGCATCTGAGGGA CGCCACTCCC-3′ for the first PCR primer set 1, 5′-TCGAGACCTGGAAA AACATGGAGC-3′ and 5′-AAAGTCCCCAGCGGAAAGTCCC-3′ for the second PCR primer set 1, 5′-TCGAGACCTGGAAAAACATGGAGC-3′ and 5′-TAACCAGAGAGACCCAGTACAGGC-3′ for the first PCR primer set 2, and 5′-GGCTAGAAGCACAAGAGGAGG-3′ and 5′-AGCATCTGAGGGAC GCCACTCCC-3′ for the second PCR primer set 2. The PCR cycling conditions were 94°C for 30 s, followed by 30 cycles of 30 s at 94°C, 40 s at 55 or 60°C, and 1 min at 72°C, with a final extension of 72°C for 7 min or 94°C for 1 min for the first PCR, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, with a final extension of 72°C for 7 min for the second PCR. The PCR products were then agarose gel purified and sequenced directly or cloned by the use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed by using a BigDye Terminator (version 1.1) cycle sequencing kit (Applied Biosystems) and an ABI PRISM 310 genetic analyzer.

HIV-1-specific CTL clones. For the generation of KI-158-derived and KI-144-derived CTL clones, PBMCs which were obtained at weeks 69 and 64, respectively, after the first visit were used. Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding cells of this population into U-bottom, 96-well microtiter plates together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human interleukin-2, irradiated allogeneic PBMCs from a healthy donor, and irradiated C1R-A*2402 cells prepulsed with the corresponding peptide), as previously described (25). Nef138-10-specific and Nef138-10-2F-specific CTLs were generated by using WT (RYPLTFGWCF) and 2F (RFPLT FGWCF) peptides, respectively. All CTL clones were cultured in RPMI 1640-10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

HIV-1 clones. Infectious proviral clones of HIV-1, pNL-432 and its Nef mutant pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), reported previously, were used (1). For pNL-432-10F, pNL-432-2F10F, and pNL-M20A-10F, mutations were introduced by site-directed mutagenesis based on overlap extension.

Flow cytometric analysis for surface expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells. HLA-A*2402-positive CD4$^+$ T cells infected with HIV-1 clone were stained to assess the expression of HLA class I in HIV-1-infected CD4$^+$ T cells, as previously described (38). Briefly, the cells were stained with anti-A11 and -A24 monoclonal antibody (MAb) A11.1 M, following staining with phycoerythrin (PE)-conjugated anti-mouse immunoglobulin (Ig) (Dako-Cytomation, Glostrup, Denmark) and thereafter were fixed and permeabilized for intracellular HIV-1 p24 staining with fluorescein isothiocyanate (FITC)-conjugated anti-p24 MAb KC-57 (Beckman Coulter, Miami, FL). The expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells was analyzed by using a flow cytometry.

Peptide-binding assay. The binding of peptides to HLA-A*2402 molecules was tested as previously described (25). HLA-A*2402 RMA-S transfectants, which were transfected with HLA-A*2402 and human β_2-microglobulin, lacked functional TAP2. They express a very low level of HLA class I molecules on the cell surfaces when they are cultured at 37°C, while empty HLA class I molecules are stably expressed if they are cultured at 26°C, because empty HLA class I molecules are unstable on cell surfaces at 37°C but stable at 26°C. The stabilization of HLA class I molecules is dependent on peptide binding affinity (24, 30, 36). Briefly, RMA-S-A*2402 cells were cultured at 26°C for 14 to 18 h. The cells were incubated at 26°C for 1 h with WT (RYPLTFGWCF) or 2F (RFPLTFG WCF) peptide at various concentrations and then at 37°C for 3 h. After two washes with phosphate-buffered saline (PBS) supplemented with 20% FCS (PBS-20% FCS), they were incubated for 30 min on ice with an appropriate dilution of MAb TP25.99. After two washes with PBS-20% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, they were washed three times with PBS-20% FCS, after which the fluorescence intensity was measured by using a flow cytometer (Becton Dickinson, Mountain View, CA).

HLA-peptide tetrameric complexes. The tetrameric complexes were synthesized as previously described (5). Briefly, an ectodomain of HLA class I proteins and human β_2 microglobulin, produced in *Escherichia coli* that had been transformed with the relevant expression plasmids, were first solubilized in denaturing buffer containing 8 M urea and refolded in refolding buffer in the presence of a synthesized peptide for 48 h at 4°C. The resultant 45-kDa complex was purified by size exclusion (Superdex G75; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England) and anion exchange chromatographies (MonoQ column; Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE-conjugated avidin (ExtrAvidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Tetramer analysis. CTL clones were first stained with either PE-conjugated WT or allophycocyanin (APC)-conjugated mutant (2F) tetramer (final concentration, 300 nM) at 37°C for 30 min. For the competitive assay, the clones were stained with WT and 2F tetramer (final concentration, 300 nM for each tetramer) at the same time at 37°C for 30 min. After two washes with RPMI 1640 medium supplemented with 10% FCS (RPMI 1640-10% FCS), the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min. For ex vivo analysis, thawed PBMCs were stained with PE-conjugated WT and APC-conjugated 2F tetramers (final concentration, 300 nM for each tetramer) at 37°C for 30 min. Following two washes with RPMI 1640-10% FCS, the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min and subsequently analyzed by using flow cytometry. The percentage of tetramer-positive cells among CD8-positive cells was then calculated.

Replication suppression assay. The abilities of HIV-1-specific CTLs to suppress HIV-1 replication were examined as previously described (37). Briefly, CD4$^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C. After three washes with RPMI 1640-10% FCS, the cells were cocultured with HIV-1-specific CTL clones. From day 3 to day 9 postinfection, 10 μl of culture supernatant was collected and the concentration of p24 antigen (Ag) was measured by use of an enzyme immunoassay (HIV-1 p24 Ag enzyme-linked immunosorbent assay kit; ZeptoMetrix Corporation, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1 − concentration of p24 Ag in the supernatant of HIV-1-infected CD4$^+$ T cells

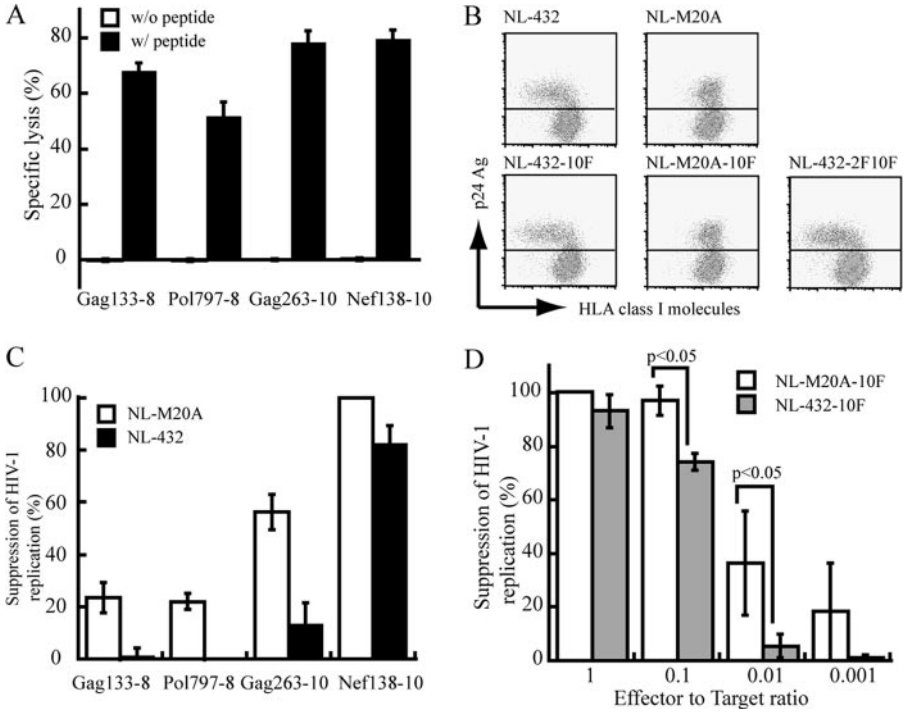


FIG. 1. Suppression of HIV-1 replication in HIV-1-infected $CD4^+$ T cells by HLA-A*2402-restricted HIV-1-specific CTLs. (A) Cytolytic activities of HLA-A*2402-restricted HIV-1-specific CTLs (four Gag133-8-specific, five Pol797-8-specific, four Gag263-10-specific, and four Nef138-10-specific CTL clones) toward HLA-A*2402^+ cells pulsed with (w) the indicated peptides (1,000 nM). w/o, without. The cells were tested at an E-to-T ratio of 2:1. Values represent averages ± standard deviations (SD) (error bars) of results from triplicate assays. (B) $CD4^+$ T cells derived from healthy donors were infected with NL-432, NL-M20A, NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cultured for 3 to 5 days. The cultured $CD4^+$ T cells were stained with anti-p24 and A11.1 M anti-A11 and anti-A24 MAbs. (C) Suppression of HIV-1 replication by HLA-A*2402-restricted CTLs. Cultured $CD4^+$ T cells derived from an HLA-A*2402^+ donor were infected with NL-432 or NL-M20A and then cocultured with HIV-1-specific CTLs at an E-to-T ratio of 1:1. HIV-1 p24 Ags in the supernatant were measured on day 6 postinfection by conducting an enzyme immunoassay. The percentage of suppression of HIV-1 replication was calculated. Values represent averages ± SD (error bars) for four Gag133-8-specific, three Pol797-8-specific, four Gag263-10-specific, and three Nef138-10-specific CTL clones. (D) The ability of Nef138-10-specific CTL clone 189 to suppress NL-432-10F and NL-M20A-10F replication in $CD4^+$ T cells. HIV-1-infected HLA-A*2402^+CD4^+ T cells were cocultured with Nef138-10-specific CTLs at various E-to-T ratios. Values represent averages ± SD (error bars) of results from two independent experiments. The *P* values were determined by nonparametric Mann-Whitney test.

cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured without the CTLs) × 100.

^{51}Cr release assay. The cytotoxicity of CTL clones against C1R-A*2402 pulsed with appropriate peptide at various concentrations or HIV-1-infected 221-$CD4^+$-A*2402 cells was determined as previously described (37, 38). Briefly, target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline. Effector cells were cocultured with target cells (2×10^3/well) for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release was determined by measuring the counts per minute (cpm) in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cmp max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% Triton X-100. Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the cpm in the supernatant in the wells containing both the target and effector cells at an effector-to-target ratio of 2:1. The peptide concentration providing half of the maximum percentage of specific lysis (LL_{50}) was calculated by using KaleidaGraph (Hulinks, Inc., Tokyo, Japan).

RESULTS

Complete suppression of HIV-1 replication by HLA-A*2402-restricted Nef138-10-specific CTLs. Since Nef138-10-specific CTLs are frequently detected in HLA-A*2402-positive HIV-1-infected individuals, it has been speculated that Nef138-10 (RY PLTFGWCY) is an immunodominant epitope and that these specific CTLs can strongly suppress HIV-1 replication. To clarify the abilities of Nef138-10-specific CTLs to suppress HIV-1 replication, we compared the abilities of four epitope-specific, HLA-A*2402-restricted CTL clones to suppress HIV-1 replication by assaying for the suppression of HIV-1 replication. All CTL clones specific for the Gag133-8, Pol797-8, Gag263-10, or Nef138-10 epitope showed similar cytotoxic activities against target cells prepulsed with the corresponding peptide (Fig. 1A). Gag133-8, Pol797-8, and Gag263-10 epitopes are conserved in both NL-432 and NL-M20A strains, the latter of which has the ability to down-regulate the expression of CD4 molecules, but not the expression of HLA class I molecules, on the cell surface, whereas the Nef138-10 epitope is not conserved in these strains. The Nef138-10 epitope has a tyrosine instead of phenylalanine at position 10, but Nef138-10 epitope-specific CTLs effectively recognized both peptides, RYPLTFGWCF and RYPLTFGWCY. We investigated HLA-A*2402 down-regulation on $CD4^+$ cells infected with NL-432 or NL-M20A. The down-regulation of HLA-A*2402 was found on $CD4^+$ cells infected with NL-432, but not on those infected with NL-M20A (Fig. 1B). We measured the abilities of these CTL clones to suppress HIV-1 replication in primary $CD4^+$ T cells infected with either HIV-1 clone NL-432 or HIV-1 clone NL-M20A. Nef138-10-specific CTL clones com-

pletely suppressed both NL-432 and NL-M20A replication at an effector-to-target (E-to-T) ratio of 1:1, whereas CTL clones specific for the Gag133-8, Pol797-8, or Gag263-10 epitope partially suppressed NL-M20A replication but failed to suppress NL-432 replication (Fig. 1C), indicating that Nef138-10-specific CTLs have strong abilities to suppress NL-432 replication. We generated mutant viruses, NL-432-10F and NL-M20A-10F, carrying the SF2 strain-derived Nef138-10 epitope because the CTL clones specific for the SF2 strain-derived Nef138-10 epitope (RYPLTF GWCF) had been established. Down-regulation of HLA-A*2402 was found on $CD4^+$ cells infected with NL-432-10F, whereas it did not occur on those infected with NL-M20A-10F (Fig. 1B). The strong ability of Nef138-10-specific CTL clone 189 to suppress the replication of both viruses was also found at an E-to-T ratio of 0.1:1 (Fig. 1D). These results indicate that Nef138-10-specific CTLs had strong abilities to suppress HIV-1 replication, regardless of Nef-mediated down-regulation of HLA class I molecules. A significant difference between the abilities of the Nef138-10-specific CTL clone to suppress NL-432-10F and NL-M20A-10F replication was found at E-to-T ratios of 0.1:1 and 0.01:1, but not at an E-to-T ratio of 1:1 (Fig. 1D), suggesting that the suppressive ability of this CTL clone was minimally affected by Nef-mediated HLA class I molecules.

A previous study demonstrated that the 2F substitution of this epitope is associated with HLA-A*2402$^+$ Japanese hemophiliacs (20). We therefore investigated HLA-A*2402-associated mutations of the Nef138-10 epitope in chronically HIV-1-infected Japanese hemophiliacs and nonhemophiliacs. We sequenced the Nef138-10 epitope and its flanking region from HIV-1 RNA from plasma samples from 41 HLA-A*2402-positive and 22 HLA-A*2402-negative Japanese patients (Fig. 2A). We found mainly three types of mutation at the epitope region: a Y-to-F substitution at the second position (2F), a T-to-C substitution at the fifth position, and an F-to-Y substitution at the tenth position. Only the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2B). Although we detected an I-to-T or I-to-V substitution at the −1 position in the N-terminal flanking region, we could not find any association with HLA-A*2402 or other HLA alleles (data not shown). We also analyzed the 2F substitution in two groups, hemophiliacs and nonhemophiliacs who had become infected through USI. In both groups, the 2F substitution was significantly associated with HLA-A*2402 (Fig. 2C). A previous study showed that the 2F substitution was significantly found in the HLA-A*2402-positive Japanese hemophiliacs but not in HLA-A*2402-positive patients infected through USI (20). Our results confirmed the association of the 2F substitution with HLA-A*2402 in Japanese hemophiliacs but suggested a different conclusion for the Japanese patients infected through USI. The frequency of 2F substitutions was much higher in HLA-A*2402$^-$ patients infected through USI than that in HLA-A*2402$^-$ hemophiliacs (31% versus 0%), suggesting that this mutation had accumulated in the HLA-A*2402$^-$ population.

Antiviral activities of Nef138-10-specific CTLs toward NL-432 and Nef138-10-2F mutant virus. To investigate the effect of the 2F substitution in the Nef138-10 epitope on the antiviral activities of Nef138-10-specific CTLs, we first measured the cytotoxic activity of these cells against WT or mutant epitope peptide-prepulsed cells (Fig. 3A). Four CTL clones derived from an HIV-1-infected Japanese patient (KI-158) showed

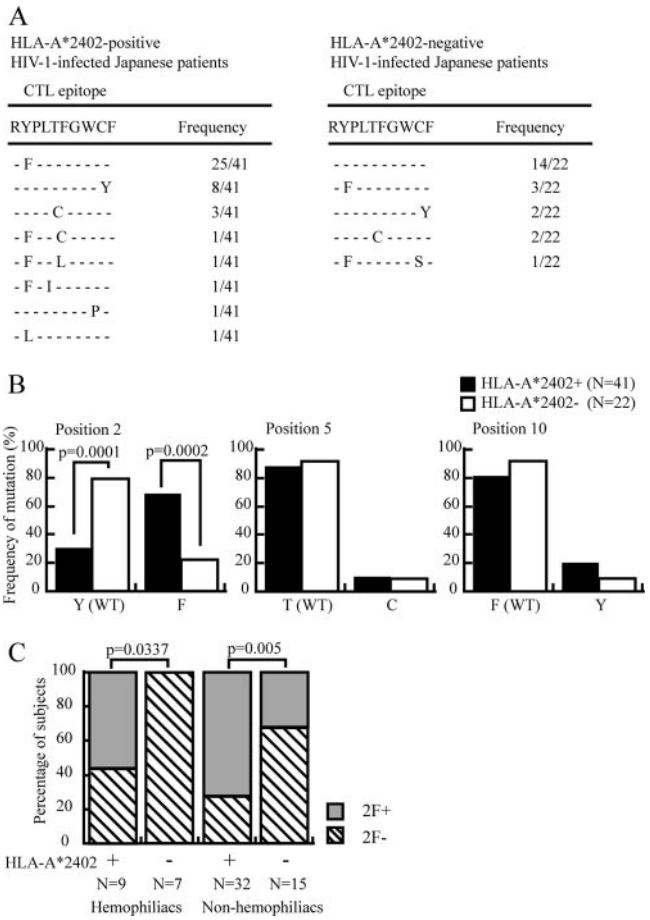


FIG. 2. Frequency of mutation in the Nef138-10 epitope and flanking region. (A) Alignment of the amino acid sequences from 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. (B) Frequency of the substitutions in the Nef138-10 epitope. The Y-to-F substitution at position 2 was more frequently found in the HIV-1-infected Japanese population expressing HLA-A*2402 ($P = 0.0002$). The vertical axis shows the frequency (%) of mutation among 41 HLA-A*2402$^+$ and 22 HLA-A*2402$^-$ patients. The horizontal axis shows the amino acid at each position. (C) Y-to-F substitutions between hemophiliacs and nonhemophiliacs (more than 2 years after infection). Bars indicate the percentages of individuals whose viral sequences are 2F (shaded part of bar) or not (striped part of bar) in subjects either expressing HLA-A*2402 (2F+) or not expressing it (2F−). The *P* values were determined by Fisher's exact test.

higher cytotoxic activities toward HLA-A*2402$^+$ target cells prepulsed with WT peptide (LL_{50}, 0.16 ± 0.07 nM) than toward those prepulsed with the mutant epitope peptide (LL_{50}, 5.03 ± 6.23 nM). The difference in CTL activity between the two targets varied among the four clones. The binding affinity of the WT peptide for HLA-A*2402 molecules was higher than that of the mutant peptide, but the difference between these peptides was minimal (Fig. 3B). These results indicate that TCRs of Nef138-10-specific CTLs could effectively recognize the 2F mutant epitope. To investigate the effects of the 2F substitution on the abilities of Nef138-10-specific CTLs to recognize target cells infected with HIV-1, we measured the activities of Nef138-10-specific CTL clones to kill HIV-1-infected cells and to suppress HIV-1 replication in HIV-1-infected

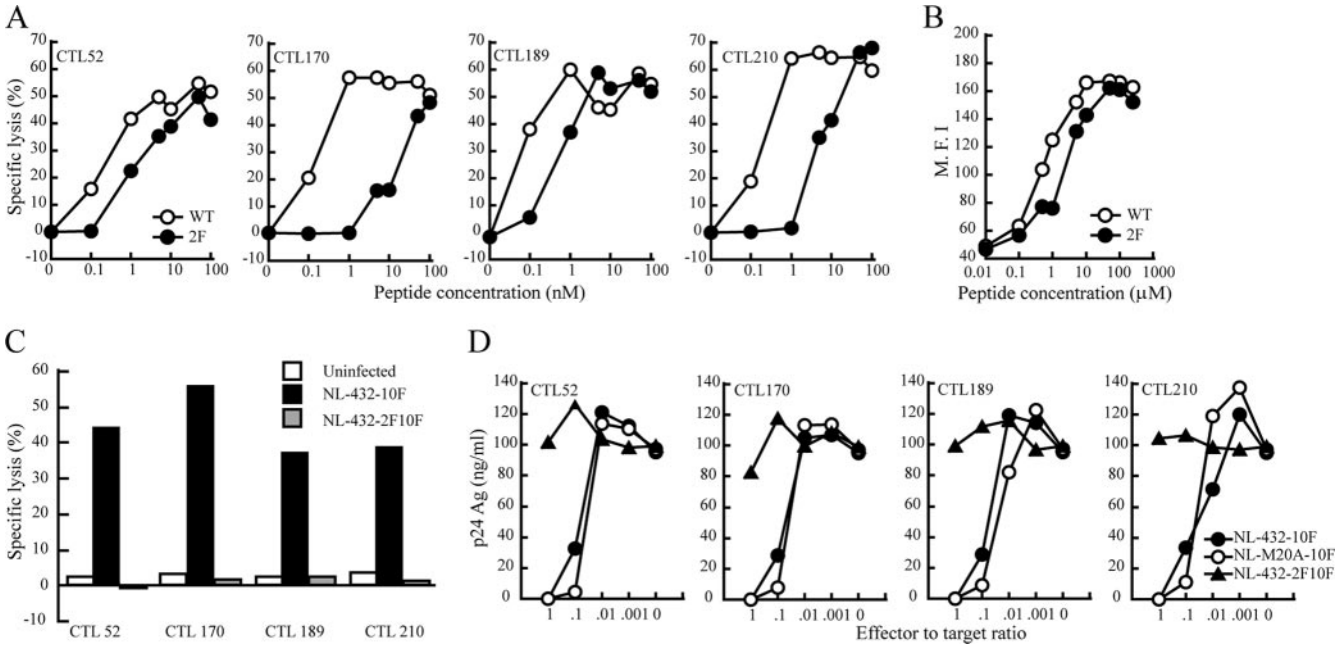


FIG. 3. Abilities of Nef138-10-specific CTLs to suppress replication of NL-432-2F10F. (A) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). They were tested at an E-to-T ratio of 2:1. (B) Binding of WT and 2F peptides to HLA-A*2402 molecules was quantified by the HLA-A*2402 stabilization assay. (C) Cytolytic activity of HLA-A*2402-restricted Nef138-10-specific CTLs toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (40% and 50% p24-positive cells, respectively) used as targets. Cytolytic activity of the CTLs was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with Nef138-10-specific CTLs at various E-to-T ratios. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by enzyme immunoassay.

CD4$^+$ T cells (Fig. 3C and D). For the assay, NL-432-2F10F, carrying a Y-to-F substitution at the second position, had been established. All four CTL clones strongly lysed NL-432-10F-infected cells but not NL-432-2F10F-infected cells (Fig. 3C). In addition, those CTL clones strongly suppressed the replication of both NL-432-10F and NL-M20A-10F, but not that of NL-432-2F10F (Fig. 3D). Taken together, these results indicate that the 2F substitution is a mutation permitting escape from Nef138-10-specific CTLs.

Antiviral response to the 2F mutant of CTLs having high-affinity TCRs for the 2F mutant epitope. Among the established Nef138-10-specific CTL clones, we found three CTL clones (from HIV-1-infected patient KI-144) to have much higher cytotoxic activity toward cells prepulsed with mutant peptide than toward those prepulsed with the WT peptide (Fig. 4A). The cytotoxic activities of these CTL clones toward the former were approximately 100-fold higher than those toward the latter (Fig. 4A), suggesting that these CTL clones carried TCRs that more effectively recognized the mutant epitope than that of the WT. To test this possibility, we generated HLA-A*2402 tetramers with Nef138-10 and its 2F mutant and then tested the abilities of these tetramers to bind to the CTL clones at different concentrations of the tetramers. Clone 82 exhibited stronger affinity for the 2F tetramer than for the WT tetramer, whereas clone 189 showed weaker affinity for the former tetramer than for the latter one (Fig. 4B). These results indicate that clone 82 had a TCR with higher affinity for the 2F mutant than for the WT.

It is speculated that CTLs carrying high-affinity TCRs for the 2F mutant would have the ability to recognize cells infected with the 2F virus. Therefore, we investigated the activities of clones 82, 98, and 108 against HIV-1-infected cells by measuring their cytotoxic activities toward HIV-1-infected cells. These CTL clones killed NL-432-2F10F-infected cells (Fig. 4C), indicating that the Nef138-2F mutant epitope was presented in HIV-1-infected cells. Interestingly, they killed NL-432-10F-infected cells more effectively than they did NL-432-2F10F-infected cells (Fig. 4C). This finding indicates that the presentation of the 2F epitope was much weaker than that of the WT one. The results may not reflect Ag presentation of the 2F mutant epitope in HIV-1-infected CD4$^+$ T cells in vivo, because 221 transfectants highly expressing HLA-A*2402 were used as target cells in this assay. We therefore tested the abilities of these CTL clones to suppress HIV-1 replication. They significantly suppressed the replication of NL-432-2F10F at an E-to-T ratio of 1:1 (Fig. 4D and E) and more effectively suppressed the replication of NL-432-10F and NL-M20A-10F than that of NL-432-2F10F (Fig. 4D), supporting the idea that the 2F mutant epitope was presented more weakly than the WT epitope was. Since KI-144-derived CTL clones had higher-affinity TCRs for the 2F mutant and suppressed the replication of NL-432-2F10F, we assume that the 2F mutant-specific CTLs had been elicited by priming with the mutant epitope in this patient.

Ex vivo analysis of the 2F mutant-specific CTLs in HIV-1-infected individuals who had been infected with the 2F mutant virus. Since the 2F mutant-specific CTL clones were established from patient KI-144, we assumed that the 2F mutant-

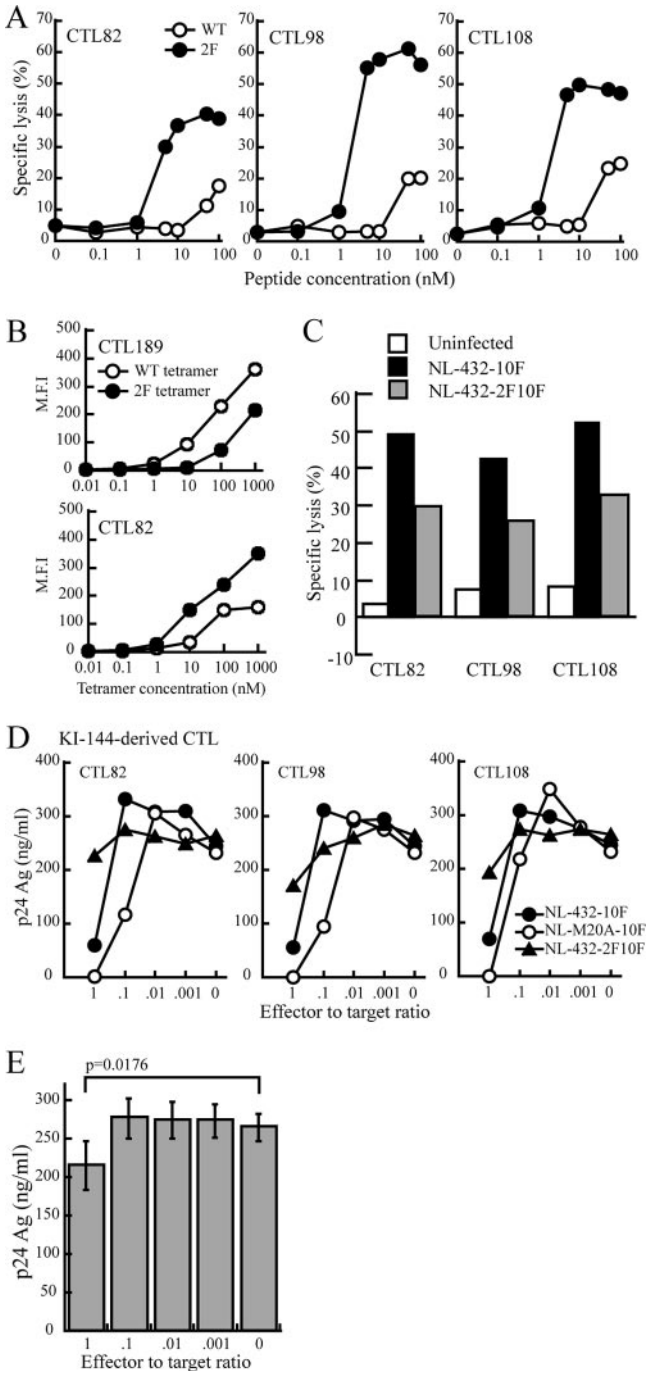
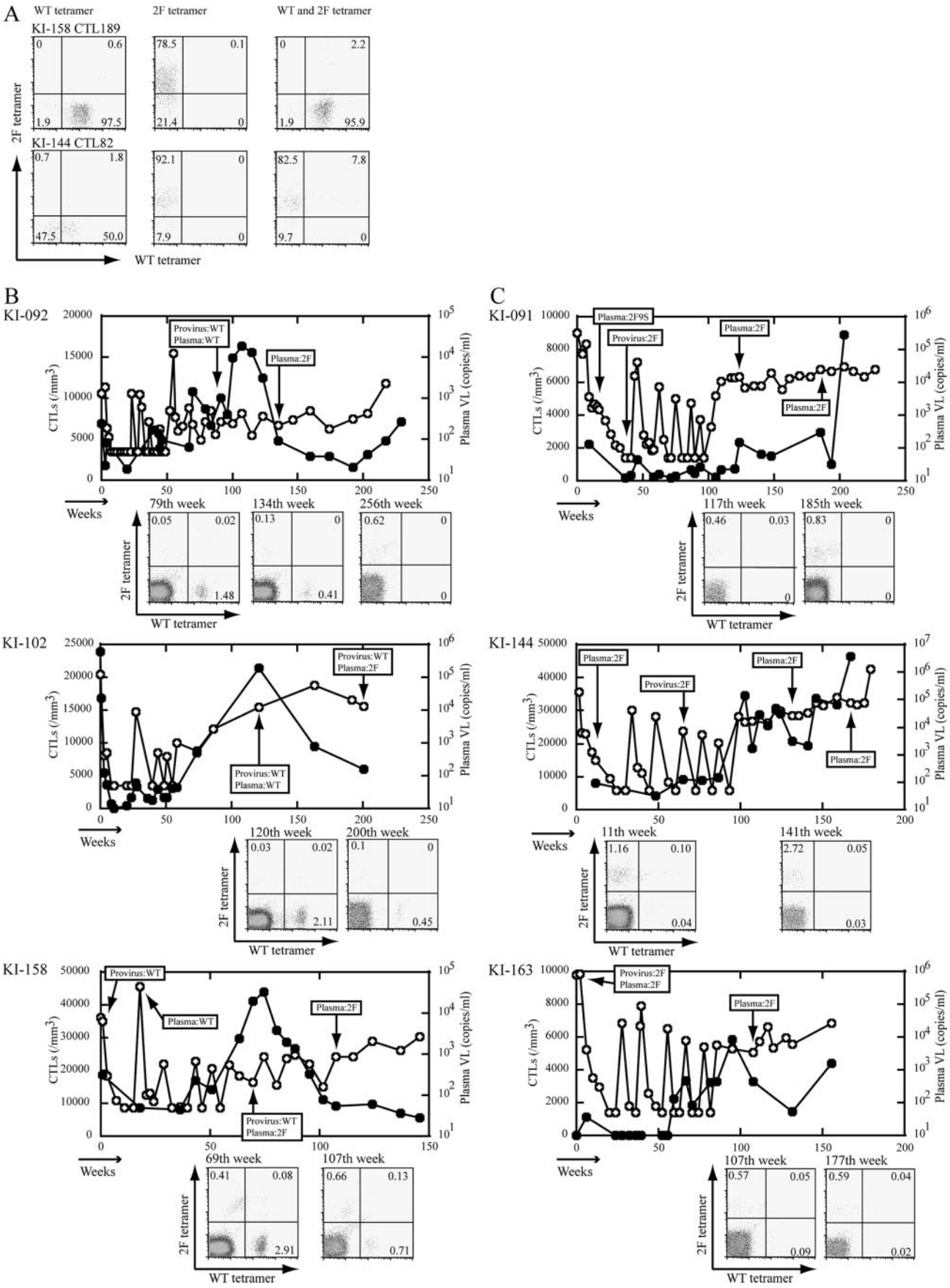


FIG. 4. Distinct antiviral activities toward NL-432-2F10F of KI-144-derived Nef138-10-specific CTL clones. (A) Cytolytic activity of KI-144-derived HLA-A*2402-restricted Nef138-10-specific CTL clones toward HLA-A*2402$^+$ cells pulsed with WT or mutant (2F) peptides (0.1 to 100 nM). The clones were tested at an E-to-T ratio of 2:1. (B) WT and 2F tetramer binding activities of CTL clones 189 (top) and 82 (bottom) clones were determined. (C) Cytolytic activity of clones in panel A toward NL-432-10F-infected or NL-432-2F10F-infected HLA-A*2402$^+$ cells (45% and 55% p24-positive cells, respectively) used as target cells. Cytolytic activity was tested at an E-to-T ratio of 2:1. (D) Abilities of HIV-1-specific CTLs to suppress NL-432-2F10F replication in CD4$^+$ T cells. Cultured CD4$^+$ T cells derived from an HLA-A*2402$^+$ donor were infected with NL-432-10F, NL-M20A-10F, or NL-432-2F10F and then cocultured with KI-144-derived CTLs dominantly binding 2F tetramers. The various E-to-T ratios are indicated. The HIV-1 p24 Ag level in the supernatant was measured on day 6 postinfection by using the enzyme immunoassay. (E) Replication of NL-432-2F10F at each E-to-T ratio. Values represent the averages for the three CTL clones ± standard deviations (error bars). Significant differences between cultures with and without CTLs are shown (nonparametric Mann-Whitney test).

specific CTLs were effectively elicited in this patient. To clarify this point, we investigated when and how many 2F-specific CTLs were elicited in this patient. First we established a competitive tetramer binding assay using two HLA-A*2402 tetramers carrying the 2F and the WT peptides to detect the respective 2F-specific and the WT-specific CD8$^+$ T cells. When the CTL clones were stained with either tetramer at different concentrations, clone 82 from patient KI-144 exhibited stronger affinity for the 2F tetramer than for the WT one, whereas clone 189 from patient KI-158 showed weaker affinity for the former tetramer than for the latter (Fig. 4B). Therefore, we expected that each CTL clone would bind to only its higher-affinity tetramer when both tetramers were used at the same concentration. Indeed, when clones 189 and 82 were stained using these tetramers, clones 189 and 82 bound to only the WT and the 2F tetramers, respectively (Fig. 5A). By employing this assay, we investigated the appearance of Nef138-10-specific or 2F-specific CTLs in patients with early HIV-1 infection (Fig. 5B and C). Ex vivo analysis of PBMCs from three patients, KI-092, KI-102, and KI-158, who had been infected with the WT virus, showed that they had mainly the WT-specific CTLs when the WT was found, and then 2F-specific CTLs became predominant after the 2F mutant took the place of the WT-specific CTLs (Fig. 5B). WT virus was found in KI-092 and KI-102 for approximately 2 years after infection, indicating that the 2F mutant had been slowly selected by the specific CTLs. On the other hand, ex vivo analysis of PBMCs from patients KI-091, KI-144, and KI-163 revealed that they had the 2F-specific CTLs only (Fig. 5C). In addition, these patients had 2F sequences in both plasma RNA and proviral DNA throughout their clinical course (Fig. 5C). These findings strongly suggest that these patients had been infected with the 2F mutant. Thus, the 2F mutant-specific CTLs were effectively elicited in donors who had been primarily infected with the 2F mutant virus. However, it remains possible that the number of WT-specific CTLs was too low to be detected by the ex vivo tetramer binding assay. To exclude this possibility, we investigated whether the patients had the WT-specific memory CD8$^+$ T cells. We stimulated their PBMCs with either WT or mutant peptide and cultured the PBMCs for 2 weeks. The WT tetramer-binding CD8$^+$ T cells were then counted by using the competitive tetramer binding assay. The WT-specific CD8$^+$ T cells were not detected among the cultured cells (data not shown), indicating that these patients did not have WT-specific CTLs. These results indicate that when the 2F mutant virus infects a new HLA-A*2402$^+$ host, this host recognizes the 2F mutant epitope and elicits the 2F mutant-specific CTLs.

DISCUSSION

A previous study showed that the 2F mutant was frequently detected in chronically HIV-1-infected individuals having



HLA-A*2402 and that Nef138-10-specific CTLs failed to kill target cells infected with HIV-1 recombinant Sendai virus containing the 2F mutant; data suggested that the 2F is a mutation for escape from the specific CTLs (20). However, the question remained as to whether Nef138-10-specific CTLs can mediate strong immune pressure on HIV-1 replication so that they select the 2F mutant in vivo. In the present study, we clarified this question by investigating the abilities of Nef138-10-specific CTL clones to suppress replication of the WT and the 2F mutant viruses. Each Nef138-10-specific CTL clone exhibited a strong ability to suppress replication of Nef^+ HIV-1 at E-to-T ratios of 1:1 and 0.1:1. This ability is much stronger than that of most HIV-1-specific CTLs (19, 37, 38), suggesting that these CTLs can mediate strong immune pressure in vivo. In addition, our study using the HIV-1 2F mutant showed that Nef138-10-specific CTLs failed to kill target cells infected with the 2F mutant and to suppress replication of the mutant, confirming that 2F is an escape mutant.

Escape mutations occur at sites within CTL epitopes, where the substitution of an amino acid abrogates HLA binding, reduces the recognition of the TCR, and/or interferes with efficient Ag processing (14, 41). The 2F mutant peptide bound to HLA-A*2402 molecules with an efficiency similar to that of the WT peptide. Both the WT and the 2F tetramers bound to Nef138-10-specific CTL clones. In addition, Nef138-10-specific CTL clones killed target cells prepulsed with Nef138-10-2F peptide. These findings suggest that the escape mechanism of the 2F mutant involves the disruption of cellular processing of the 2F mutant peptide. However, since Nef138-10-2F-specific CTL clones effectively killed the target cells infected with the 2F mutant virus and suppressed the replication of the mutant virus, the 2F peptide can be naturally processed and presented by HLA-A*2402. The 2F-specific CTL clones could recognize the 2F peptide much more effectively than the WT peptide, whereas the clones showed much stronger abilities to suppress replication of the WT virus than that of the 2F mutant virus. These observations indicate that the change from Tyr to Phe remarkably reduced the presentation of the epitope peptides in Ag processing but that the 2F mutant could still be presented in the cells infected with the mutant.

It is well known that in both HIV-1 and SIV infections, escape mutations are poorly recognized in new hosts who share the same HLA alleles with donors (17, 32). If escape mutant peptides fail to bind to HLA class I restriction molecules or the mutation critically affects the Ag processing, these escape mutants are hardly recognized and fail to elicit the specific CTLs in new hosts sharing the same HLA alleles. On the other hand, if escape mutant peptides can bind to HLA class I restriction molecules and can be processed and presented, it remains possible that the mutant epitope is recognized in new hosts. The 2F mutant peptide effectively bound to HLA-A*2402 (Fig. 3B), suggesting the possibility that the 2F mutant peptide is presented by HLA-A*2402. We therefore selected the three donors who were infected with the 2F mutant virus at an early phase (within 10 weeks before the first visit) and investigated whether the 2F-specific $CD8^+$ T cells were elicited in these donors. It was strongly suggested that these patients had been infected with the 2F virus, since in the donors who had been infected with the WT virus, the 2F mutation was selected approximately 2 years after infection. The 2F-specific $CD8^+$ T cells were elicited in these three donors, although this mutant epitope was very weakly presented by HLA-A*2402. Thus, escape mutant-specific CTLs can be elicited in new hosts even if the mutant epitope peptide is very weakly presented.

The reversion of a CTL escape mutation to the WT occurs when the mutant virus is transmitted to a new host not sharing HLA class I alleles (18, 29) and even to a new host sharing HLA class I alleles with the monkey donors before the specific CTL is elicited (8, 27). Although the reversion of the 2F epitope to the WT one was reported for chronically HIV-1-infected individuals having no HLA-A*2402, the rate of reversion was very low (20), suggesting that the Y-to-F substitution does not inflict a large fitness cost on HIV-1. A previous study showed that the 2F mutant was still detectable in 56% of HLA-A*2402^- Japanese patients infected through USI (20). In contrast, in the present study, it was found in only 31% of the patients. This difference between these two studies may have resulted from the difference in the time when the sequence was analyzed after the infection. Although the frequency of the 2F mutant in HLA-A*2402^- Japanese individuals is different between the two studies, the studies indicate that 2F mutant did accumulate in HLA-A*2402^- Japanese individuals infected through USI. The reversion of this epitope should occur but may be very slow in HLA-A*2402^- donors. It is thought that the reversion does not occur in HLA-A*2402^+ individuals, because the 2F-specific CTLs can strongly suppress replication of the WT virus. Indeed, the reversion was not found in three patients who had been primarily infected with the 2F mutant virus and monitored for 2 to 3 years. Thus, the 2F mutant is accumulating in the Japanese population, of which 70% carry HLA-A*2402.

The competitive tetramer binding assay using the two tetramers could distinguish $CD8^+$ T cells carrying high-affinity TCRs for the WT epitope from those carrying high-affinity ones for the 2F epitope. By using this assay, we found that patients who had been infected with the WT virus first produced WT-specific $CD8^+$ T cells and then 2F-specific $CD8^+$ T cells approximately 6 to 12 months after the 2F mutant had become predominant. In those patients, the 2F mutant virus appeared more than 12 months after the WT virus infection. These findings support our contention that 2F is an escape mutant and that the three donors who had 2F sequences in

FIG. 5. Detection of Nef138-10-2F-specific CTLs in HIV-1-infected patients who had been infected with 2F mutant virus. (A) Tetramer binding of Nef138-10-specific CTLs. KI-158-derived CTL clone 189 (top) and KI-144-derived CTL clone 82 (bottom) were stained with either WT (left panels) or 2F (middle panels) tetramers or both (right panels). The percentage of tetramer-positive cells among $CD8^+$ cells was measured. (B and C) Ex vivo analysis of Nef138-10-specific and Nef138-10-2F-specific CTLs. Nef138-10-specific CTLs in PBMCs derived from HLA-A*2402^+ HIV-1-infected individuals were measured by using both WT and mutant (2F) tetramers. Symbols: ●, WT tetramer-positive cells; ○, plasma viral loads. The epitope sequences from viral RNA (plasma) or provirus DNA (PBMCs) during the clinical course are shown. The *x* axis represents weekly course from the first visit. The frequency of $CD8^+$ cells positive for each or both tetramers is given in the quadrants below the graphs. VL, viral load.

their plasma RNA and proviral DNA during the early phase were primarily infected with 2F virus.

In the present study, we demonstrated that new hosts could effectively produce the 2F escape mutant-specific CTLs, even though the 2F mutant epitope was very weakly presented by HLA-A*2402 in HIV-1-infected cells. The 2F-specific CTLs could suppress replication of the 2F mutant virus, but this ability was much weaker toward the 2F mutant than toward the WT virus. The reversion from 2F to WT was not found in the three patients who had been infected primarily with the 2F mutant virus and monitored for 2 to 3 years. This lack of reversion is explained by the fact that the 2F-specific CTLs could effectively suppress replication of the WT virus. This mutant accumulated in HLA-A*2402$^-$ USI patients. Since HLA-A*2402 is a common allele found in approximately 70% of the Japanese population, the 2F mutant can accumulate in the Japanese AIDS population.

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