HLA class I-restricted recognition of an HIV-derived epitope peptide by a human T cell receptor α chain having a Vδ1 variable segment

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A subset of human peripheral αβ T cells have been shown to express TCR α chains containing Vδ1 segments, although what antigens the Vδ1$^+$ αβ T cells recognize via these TCR is not known yet. We eventually established a human CD8 T cell clone that expressed αβ TCR and Vδ1 antigens. Corroboratively, a unique in-frame Vδ1.1JαCα transcript was found in the clone. The clone showed cytotoxic activity and IFN-γ production towards cells expressing HLA-B*3501 pulsed with an HIV Pol-derived epitope peptide (IPLTEEAEL). By flow-cytometric analysis *ex vivo* using an HLA-B*3501 tetramer, a fraction of Vδ1$^+$CD8$^+$tetramer$^+$ cells was found in peripheral lymphocytes of an HIV-infected patient, indicating the existence of HLA-restricted and HIV-specific Vδ1$^+$ CD8 T cells *in vivo*. Moreover, retrovirus-mediated transfer of the TCR-encoding genes into TCR-negative hybridoma cells showed that the transduced cells were stained by the tetramer and were activated in response to the Pol peptide, further confirming the ligand specificity of the TCR. Together, these results clearly demonstrate that Vδ1$^+$ αβ TCR are restricted to engaging peptide antigens in the context of classical MHC class I molecules, highlighting the difference in the ligand specificity between Vδ1$^+$ αβ TCR and Vδ1$^+$ γδ TCR.

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1 Introduction

The mammalian immune system includes three types of cells, B cells, αβ T cells, and γδ T cells that use somatic gene rearrangement to produce genes encoding their defining surface receptors. Among them, αβ T cells are restricted to engaging antigen in the context of a major histocompatibility complex (MHC) molecule, whereas γδ T cells recognize small molecules and intact proteins without the requirement for antigen processing [1]. Most γδ T cells bear a tissue-specific, restricted set of Vγ and Vδ domains. For example, human γδ T cells bearing Vδ1-encoded TCR account for the vast majority of γδ T cells in tissues such as the spleen and intestine [2]. Although the antigens recognized by the Vδ1$^+$ γδ T cells remain enigmatic, recent studies have demonstrated that a subset of Vδ1$^+$ γδ T cells can recognize stress-inducible MHC class I-like MIC molecules expressed by epithelial cells via specific TCR interactions [3]. In addition, the major tissue population of γδ T cells expressing Vδ1 TCR have been shown to recognize CD1c, one member of a family of nonpolymorphic CD1 molecules expressed on the surface of dendritic cells and B cells [4].

TCR genes are assembled from separate variable (V), diversity (D), and joining (J) elements by a site-specific recombination mechanism termed V(D)J recombination. The TCR α and δ genes form a single complex locus, spanning approximately one mega base on chromosome 14 in humans. Despite the δ locus being embedded in the α locus in the chromosome, the α and δ genes encode proteins participating exclusively in the αβ or γδ TCR complexes, which in turn are expressed in the two separate lineages of αβ and γδ T cells, respectively (see reference [5] for reviews). It has been reported, however, that some Vδ genes are rearranged either to Jα or to Jδ genes and can therefore be used in the synthesis of TCR-α or δ chains, which are expressed in the αβ and γδ T cells, respectively. For example, a subset of human peripheral T cells expressing Vδ1 antigens on their surface were found in both αβ and γδ T cell lineages as

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revealed by multi-color flow cytometry [6]. Sequencing analysis of TCR genes revealed the existence of Vδ1JαCα transcripts encoded by a unique Vδ1Jα rearrangement in the Vδ1-expressing αβ T cells [7]. However, what antigen or ligand the Vδ1-expressing αβ T cells recognize via the TCR has not been directly investigated yet. In addition, functional properties of such Vδ1-expressing αβ T cells in the periphery have yet to be examined.

In the present study, we clearly demonstrated that a Vδ1⁺ αβ T cell clone established from an HIV-infected patient could kill cells pulsed with an HIV Pol-derived epitope peptide presented by syngeneic HLA-B*3501. In addition, the finding that the Vδ1-bearing αβ TCR could recognize an engaging peptide as antigen in the context of a classical HLA class I molecule highlights the difference in the ligand specificity between Vδ1-expressing αβ and γδ TCR.

2 Results and discussions

2.1 Generation of a T cell clone and its functional characterization

An HIV Pol peptide (HIV $Pol_{448-456}$; IPLTEEAEL) has been shown to be a T cell epitope endogenously processed and presented by HLA-B*3501 [8]. We established CD8 T cell clones from an HIV-infected patient (carrying HLA-B*3501) following stimulation of peripheral lymphocytes from the patient with the Pol peptide. Among them, the T cell clone, designated as CTL 55, showed substantial cytotoxic activity toward T2 cells expressing HLA-B*3501 pulsed with the Pol peptide even at 0.1 nM concentration (Fig. 1A), which clone showed potency comparable to that of some other T cell clones with the same antigen specificity [8]. CTL 55 also showed significant IFN-γ production in response to the peptide-pulsed.221 cells expressing HLA-B*3501 as revealed by intracellular flow cytometry (Fig. 1B). These functional assays indicated that CTL 55 could recognize the Pol peptide presented by HLA-B*3501.

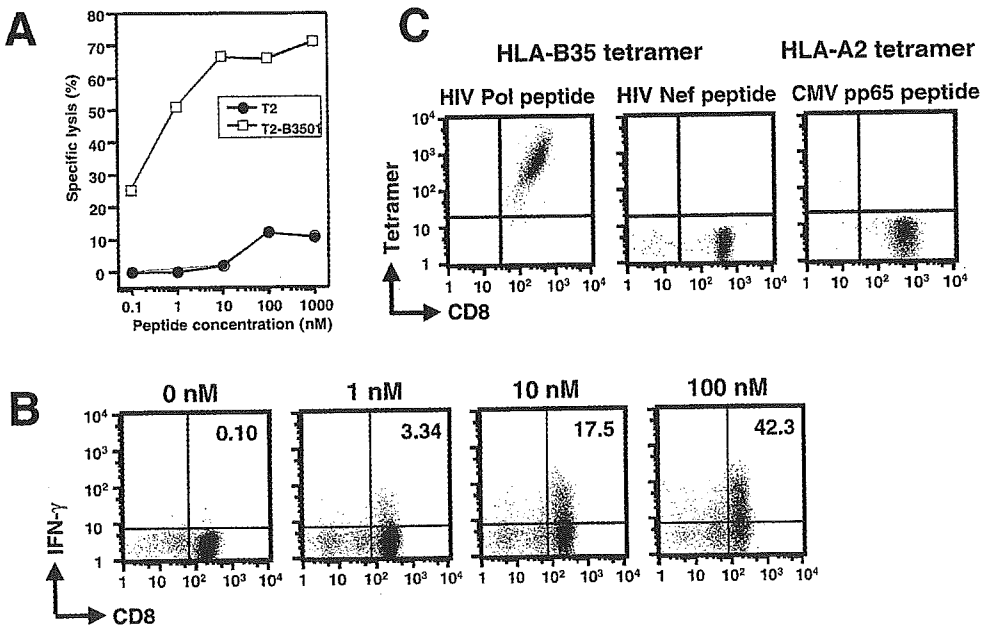

Fig. 1. Functional analysis of CTL 55 in response to the Pol peptide. (A) Cytotoxic activity of CTL 55 was examined by a standard Cr^{51}-release assay against T2 cells or a transfectant expressing HLA-B*3501 pulsed with the indicated concentrations of Pol peptide at effector to target ratio of 2:1. (B) IFN-γ production activity of CTL 55 was examined by an intracellular flow cytometric assay against.221 transfectants expressing HLA-B*3501 pulsed with the indicated concentrations of the Pol peptide at effector to target ratio of 1:1. Per cent CD8⁺IFN-γ⁺ cells is shown in each panel. (C) CTL 55 were stained with the HLA-B*3501 tetramer in complex with the HIV Pol peptide (IPLTEEAEL) or the HIV Nef peptide (RPQVPLRPMTY), or the HLA-A*0201 tetramer in complex with the human cytomegalovirus pp65 peptide (NLVPMVATV). Cells were then stained with the anti-CD8 mAb and analyzed by flow cytometry.

We next prepared an HLA-B*3501 tetramer in complex with the Pol peptide, and tested the ability of CTL 55 to bind the tetramer. As clearly shown in Fig. 1C, virtually all fractions of CTL 55 (>98%) were stained by the HLA-B35 tetramer in complex with the Pol peptide, whereas no fractions of CTL 55 (<0.1%) bound the HLA-B35 tetramer in complex with an HIV Nef peptide (RPQVPLRPMTY). Nor was stained CTL 55 by the HLA-A2 tetramer in complex with a human cytomegalovirus peptide (pp65, NLVPMVATV) as shown in Fig. 1C. These data confirmed the HLA-B35-restricted and HIV Pol epitope-specific antigen recognition property of CTL 55.

2.2 Analysis of TCR-encoding genes of CTL 55

Next we analyzed V-region segments of the TCR on CTL 55 by cloning the TCR-encoding genes by using the rapid amplification of 5'-cDNA ends protocol. When we used an anti-sense primer specific for Cα transcripts, the genes amplified by the PCR contained a Vδ1 gene segment followed by a JαCα-encoded gene segment (Table 1 and Fig. 2). This result confirmed the previous findings by Miossec et al. [6, 7] that a small subset (<0.5%) of human peripheral T cells expressed Vδ1-encoded variable segments on αβ T cells. Using a Cβ-specific antisense primer, we isolated TCR-β cDNA containing a Vβ13.3-encoded gene segment (Table 1), suggesting that the Vδ1.1-bearing TCR-α chain was paired with TCR-β chain on the surface of CTL 55.

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        10         20         30         40         50         60
atgctgtttctccagcctgctgtgtgtatttgtggccttcagctactctggatcaagtgtg
M  L  F  S  S  L  L  C  V  F  V  A  F  S  Y  S  G  S  S  V

        70         80         90        100        110        120
gcccagaaggttactcaagcccagtcatcagtatccatgccagtgaggaaagcagtcacc
A  Q  K  V  T  Q  A  Q  S  S  V  S  M  P  V  R  K  A  V  T

       130        140        150        160        170        180
ctgaactgcctgtatgaaacaagttggtggtcatattatatttttttggtacaagcaactt
L  N  C  L  Y  E  T  S  W  W  S  Y  Y  I  F  W  Y  K  Q  L

       190        200        210        220        230        240
cccagcaaagagatgattttccttattcgccagggttctgatgaacagaatgcaaaaagt
P  S  K  E  M  I  F  L  I  R  Q  G  S  D  E  Q  N  A  K  S

       250        260        270        280        290        300
ggtcgctattctgtcaacttcaagaaagcagcgaaatccgtcgccttaaccatttcagcc
G  R  Y  S  V  N  F  K  K  A  A  K  S  V  A  L  T  I  S  A

       310        320        330        340        350        360
ttacagctagaagattcagcaaagtacttttgtgctcttggggaaggggggagcccagaag
L  Q  L  E  D  S  A  K  Y  F  C  A  L  G  E  G  G  A  Q  K

       370        380        390        400        410
ctggtatttggccaaggaaccaggctgactatcaacccaaatatccagaat
L  V  F  G  Q  G  T  R  L  T  I  N  P  N  I  Q  N
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Fig. 2. Nucleotide and amino acid sequence of TCR-α chain of CTL 55. TCR-α transcript of CTL 55 encoded by Vδ1.1JαCα gene segment is shown (see also Table 1).

Table 1. TCR-encoding genes isolated from CTL 55[a)]

	V gene	J segment	CDR3 sequence
Vδ1.1	TRDV1*01	TRAJ54*01	CALGEGGAQKLVF
Vβ13.3	TRBV6-1*01	TRBJ2-7*01	CASRTGGTLIEQYF

a) Alignment of variable and joining regions of the TCR genes were analyzed by IMGT, the international ImMunoGeneTics database (home page at http://imgt.cines.fr/). Complementary determining region 3 (CDR3) sequences for α and β TCR are shown.

2.3 Flow-cytometric analysis of CTL 55 and peripheral lymphocytes

Phenotypic analysis showed CTL 55 were CD3$^+$ (clone: UCHT1) lymphocytes that were positive for αβ TCR (clone: BMA031) and negative for γδ TCR (clone: B1.1, Fig. 3). In addition, mAb A13 [9], specific for an epitope encoded by Vδ1 gene segments transcribed along with either JαCα or JδCδ [6] reacted with CTL 55 (Fig. 3A). These results indicate that CTL 55 expressed αβ TCR complexes including a Vδ1 variable segment on their surface. In contrast, another Vδ1-specific mAb (clone: δTCS1) did not react with CTL 55 (data not shown), consistent with the previous observations that the δTCS1 mAb recognized the epitope spanning Vδ1 and Jδ1 domains [10].

We then examined the presence of the antigen-specific Vδ1$^+$ T lymphocytes in the periphery *in vivo*. Peripheral lymphocytes of an HIV-infected patient were directly stained by the HLA-B35 tetramer in complex with the Pol peptide, anti-CD8 mAb and anti-Vδ1 mAb, and were analyzed by flow cytometry. As shown in the Fig. 3B, a subset of CD8$^+$ tetramer$^+$ cells (0.09% in total CD8$^+$ cells) was gated and the surface expression of the Vδ1 antigen was determined. More than 70% cells in the CD8$^+$ tetramer$^+$ subset were positive for the Vδ1 antigen (Fig. 3B). In contrast, virtually no fractions of CD8$^+$ tetramer$^+$ cells (less than 0.02% in total CD8$^+$ cells) were found in peripheral lymphocytes of HIV-negative donors (Fig. 3C). These data clearly indicated the existence of the HLA-B35-restricted and HIV Pol-specific Vδ1$^+$ T cells in the peripheral lymphocytes of the HIV-infected patient.

It is conceivable that Vδ1$^+$ αβ T cells constitute a dominant repertoire of HIV Pol-specific HLA-B35-restricted T cells among HIV-infected patients carrying HLA-B35, and that the frequency of HIV Pol-specific Vδ1$^+$ T cells is correlated with disease progression, anti-retroviral treatment, or CTL escape. In this regard, we have been testing this hypothesis by examining the frequency of HIV Pol-specific Vδ1$^+$ T cells of the same patient at several

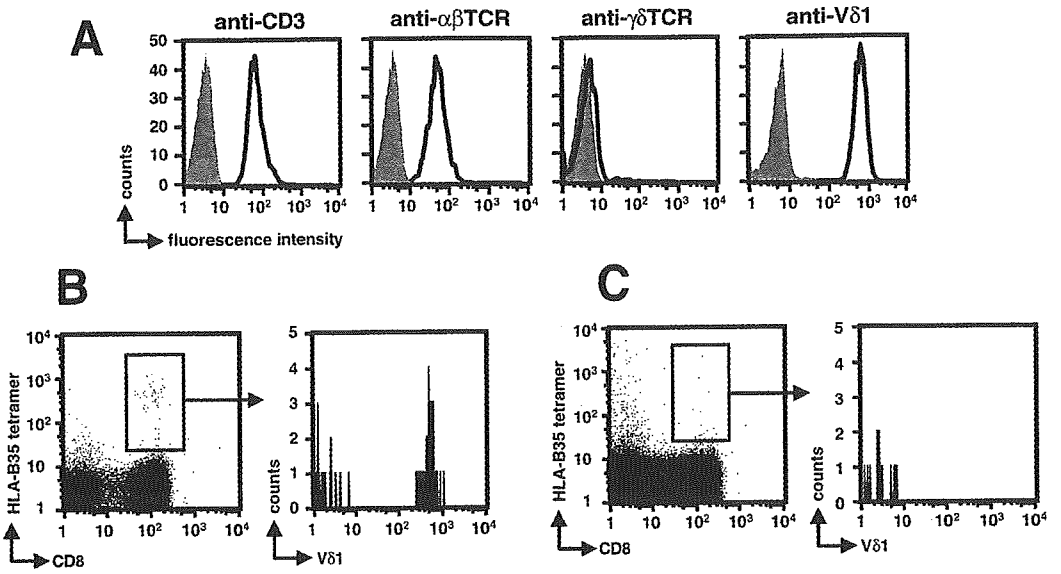

Fig. 3. Flow-cytometric analysis of antigen-specific Vδ1⁺ T lymphocytes. (A) CTL 55 was stained (solid lines) or not (shaded areas) with mAb and analyzed by flow cytometry. The mAb tested were anti-human CD3 (clone UCHT1), anti-human αβ TCR (clone BMA031), anti-human γδ TCR (clone B1.1), and anti-human Vδ1 TCR (clone A13). (B) HIV-infected patient's peripheral lymphocytes (1×10^6) were stained by the HLA-B35 tetramer, anti-CD8 mAb, and anti-Vδ1 mAb, and were analyzed by flow cytometry. A subset of CD8⁺ tetramer⁺ cells were gated (left panel) and the Vδ1 antigen expressed on the surface of gated cells was analyzed (right panel). (C) Peripheral lymphocytes (4×10^6) of an HIV-negative donor carrying HLA-B*3501 were stained and analyzed as above. The data show one representative of four different HIV-negative donors.

time points during the course of HIV infection: the frequency of Vδ1⁺ T cells in the CD8⁺ tetramer⁺ subset appeared to vary between 5 to 80% (T. Ueno, unpublished data). Further studies focusing on the association between the frequency of HIV-specific Vδ1⁺ αβ T cells and the disease progression to AIDS as well as functional properties of HIV-specific Vδ1⁺ αβ T cells toward HIV-infected cells *in vivo* are intriguing.

2.4 Reconstruction of the TCR complex on TCR-negative T cell hybridoma

Despite the allelic exclusion system operating on TCR genes, as many as 30 and 1% of human peripheral αβ T cells have been reported to coexpress two different α or β chains on their surface, respectively [11–13]. In addition, γδ T cells expressing two different γ or δ chains have also been reported [14–16]. We therefore cannot simply exclude the possibility that different TCR chains expressed on the surface of CTL 55 played a role in apparent antigen specificity, although only a unique set of α and β transcripts was identified in CTL 55.

Thus, in order to address the ligand specificity of the Vδ1.1/Vβ13.3 TCR, we used the retrovirus-mediated system for transduction of both TCR genes into TG40/CD8 cells [17], a human CD8α-expressing mouse T cell hybridoma cell line deficient in αβ TCR expression. The TCR-transduced TG40/CD8 cells showed the surface expression of CD3ε and Vδ1 antigens (Fig. 4A), indicating that the Vδ1.1/Vβ13.3 TCR had been successfully reconstituted on the surface of the transduced TG40/CD8 cells.

We then tested the TCR-transduced cells for their ability to recognize the Pol peptide presented by HLA-B*3501. The Vδ1.1/Vβ13.3 TCR-transduced cells showed specific reactivity with the tetramer, whereas mock-transduced cells did not (Fig. 4A). Furthermore, the TCR-transduced cells, but not the mock-transduced ones, secreted IL-2 in response to the HLA-B*3501-expressing cells pulsed with even a very low 10 nM concentration of the Pol peptide (Fig. 4B). These results provide direct evidence that the Vδ1.1/Vβ13.3 TCR was solely responsible for the peptide-specific response in the context of HLA-B*3501.

In summary, employing functional and phenotypic analysis including a tetramer staining technology toward the human T cell clone and human peripheral lymphocytes as well as the TCR-transduced cell, we provided evidence here that the Vδ1-bearing αβ TCR recognized a peptide presented by HLA class I molecules, as do other

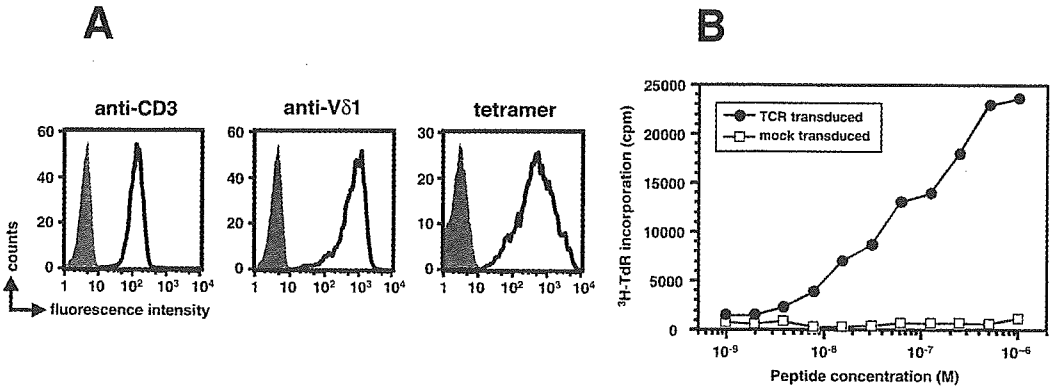

Fig. 4. Phenotypic and functional analysis of the Vδ1.1/Vβ13.3 TCR in TG40/CD8 cells following transduction with TCR-encoding genes. (A) TG40/CD8 cells transduced with the TCR-encoding genes were stained with anti-CD3ε mAb, anti-Vδ1 mAb, or HLA-B35 tetramer and were analyzed by flow cytometry (solid lines). Mock-transduced cells stained by the same reagents are shown as shaded areas. (B) The TCR- or mock-transduced TG40/CD8 cells (2×10^4) were incubated with C1R transfectants expressing HLA-B*3501 (1×10^4) in the absence or the presence of the indicated concentrations of the Pol peptide. The amounts of IL-2 secreted by TG40/CD8 cells were determined by assaying the proliferation activity of CTLL-2 cells.

Vα-bearing αβ TCR. The findings suggest that the V gene, whether comes from the α or δ locus, once assembled with JαCα as a result of somatic gene arrangements becomes a part of αβ TCR by pairing with a TCR-β chain, and thus plays a role in recognizing peptide antigens in the context of classical MHC class I molecules.

3 Materials and methods

3.1 Cell lines and antibodies

C1R, T2, and.221 transfectants expressing HLA-B*3501 (C1R-B*3501, T2-B*3501, and.221-B*3501, respectively) were previously generated [8, 18]. The TCR-negative mouse T cell hybridoma TG40 (kindly provided by T, Saito, Chiba University, Chiba, Japan) transduced with a human CD8α gene (TG40/CD8) was previously generated [17].

The following antibodies were used: phycoerythrin (PE)-conjugated anti-human IFN-γ mAb, anti-mouse CD3ε mAb (2C11), anti-human CD3 mAb (clone UCHT1), and anti-human γδ TCR mAb (clone B1.1) from PharMingen (San Diego, CA); fluorescein-5-isothiocyanate (FITC)-conjugated anti-human CD8 mAb and PE-conjugated anti-mouse immunoglobulins from DAKO Corporation (Glostrup, Denmark); and PE-conjugated anti-human TCR Pan α/β mAb (clone BMA031) from Immunotech (Marseilles Cedex, France). Anti-Vδ1 mAb A13 [9] was kindly provided by L. Moretta (Istituto di Istologia ed Embriologia Generale, Genova, Italy).

3.2 Preparation of tetrameric peptide-MHC complex

A tetrameric complex of peptide, β2-microglobulin (β2M), and HLA-B*3501 was prepared as described [17]. Briefly, an ectodomain of HLA-B35 (15 mg) and β2M (100 mg) produced in *Escherichia coli* as inclusion bodies were first solubilized in denaturing buffer containing 8 M urea and then refolded in refolding buffer (100 mM Tris-HCl, pH 8.0, containing 400 mM arginine, 2 mM ethylenediaminetetraacetic acid, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.1 mM phenylmethylsulfonyl fluoride) in the presence of 5 mg of a chemically synthesized peptide (Sawady Technologies, Inc., Tokyo) for 48 h at 4°C. The resultant 45-kDa ternary complex was purified by size-exclusion and anion-exchange chromatographies. Purified complexes were enzymatically biotinylated at a birA recognition sequence located at the C terminus of the heavy chain, and were mixed with PE- or allophycocyanin-conjugated avidin (Molecular Probes, Inc., Eugene, OR) at a molar ratio of 4:1 to give the HLA-tetramer complex.

3.3 Generation of antigen-specific CTL clone

CTL clones were established from an HIV-infected patient (HLA-A*2402/A*2601, HLA-B*3501/B*5101) following stimulation of peripheral lymphocytes with an HIV Pol-derived synthetic epitope peptide (HIV $Pol_{448-456}$; H-IPLTEEAEL-OH) as described [8]. Briefly, a bulk CTL culture was seeded at a density of 0.8 cells/well in U-bottom 96-well microtiter plate with a cloning mixture (5×10^5 irradiated allogeneic peripheral lymphocytes from a healthy donor and 5×10^4 irradiated T2-B*3501 cells prepulsed with a 1 μM concentration of the Pol peptide in RPMI1640 supplemented with 10% fetal calf

serum and 100 U/ml human rIL-2). Two weeks later, cells positive for growth were tested for cytolytic activity by the ^{51}Cr-release assay described below. Positive clones were maintained in RPMI1640 and 10% fetal calf serum supplemented with 100 units/ml rIL-2 and were stimulated weekly with irradiated T2-B*3501 cells pulsed with the Pol peptide.

3.4 Cytotoxic assay

The cytotoxic activity of the CTL clone was determined by using a standard ^{51}Cr-release assay as described [8]. T2 cells, a human lymphoblastoid line deficient in peptide transport, or transfectants expressing HLA-B*3501 were first incubated with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham Pharmacia) in saline for 1 h at 37°C, and then washed three times with culture medium. The ^{51}Cr-labeled T2 cells (2×10^3 cells/well) were plated in a 96-well round-bottom microtiter plate in the absence or presence of various concentrations of the peptide. After 1 h, CTL clones (2×10^3 cells/well) were added and the plate was incubated for an additional 4 h at 37°C. To determine maximum and spontaneous ^{51}Cr release, we also added 5% Triton X-100 or culture medium alone, respectively, to different wells. A portion of the culture supernatant (100 μl) was then removed from each well and analyzed by a γ counter. The percent specific lysis was calculated as described [8].

3.5 IFN-γ secretion assay

The IFN-γ production activity of the CTL clone was determined by an intracellular flow cytometry as described [19]. Briefly, CTL clones were stimulated by .221-B*3501 cells pulsed with various concentrations of the peptide for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Cells were collected and stained by anti-CD8 mAb. After having been treated with paraformaldehyde solution, cells were permeabilized in a permeabilization buffer (0.1% saponin and 20% newborn bovine serum in phosphate-buffered saline) at 4°C for 10 min and stained with anti-IFN-γ mAb. After thorough washing with the permeabilization buffer, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

3.6 Flow-cytometric analysis *ex vivo*

Cryopreserved peripheral lymphocytes of an individual (HLA-A*2402/2601, HLA-B*3501/5101) with chronic HIV-1 infection (1×10^6 cells) or of HIV-negative individuals (4×10^6 cells) were stained first by the tetramer at 37°C for 15 min. Cells were subsequently stained by anti-Vδ1 (A13), anti-mouse Ig-PE, and anti-CD8-PerCP antibodies at 4°C for 15 min. Cells were then washed twice with a washing buffer (10% newborn bovine serum in phosphate-buffered saline), and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

3.7 Cloning and sequencing analysis of TCR-encoding genes

Total RNA was prepared from the T cell clone (approximately 1×10^5 cells) by using a total RNA isolation kit (QIAGEN GmbH, Hilden, Germany). cDNA clones encoding α and β TCR were obtained by reverse transcription using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA), isolated total RNA (50 ng), and a primer specific for Cα (5'-actggatttagagtctctcagctggtaca-3') or Cβ (5'-ttgggtgtgggagatctctgcttctgatg-3'), respectively. cDNA was then amplified by polymerase chain reaction (PCR) using a highly specific Taq DNA polymerase (AccuPrime SuperMix I; Invitrogen Corp., Carlsbad, CA) and was cloned into a plasmid. The DNA sequence of the α and β TCR-encoding genes was determined by using a Genetic Analyzer 310 (PE Biosystems). Alignment of V and J regions of the TCR genes was analyzed by use of the International ImMunoGeneTics database (http://imgt.cnusc.fr:8104) created by M.-P Lefranc [20].

3.8 Construction of retroviral vectors and gene transfer

Retrovirus-mediated gene transfer was used to reconstruct αβ TCR complexes on TG40/CD8 cells as previously described [17]. Briefly, the genes encoding full-length α and β TCR were subcloned into the retroviral vector pMX [21], which was kindly provided by T. Kitamura (Tokyo University, Tokyo, Japan). The ecotropic virus packaging cell line Plat-E (also a gift from T. Kitamura, Tokyo University, Tokyo, Japan) was first transfected with the resulting constructs by using the transfection reagent Lipofectamine 2000 (Invitrogen Corp.). Two days later, the culture supernatant containing recombinant virus was collected and then incubated with TG40/CD8 cells in the presence of 10 μg/ml polybrene for 6 h. Transduced TG40/CD8 cells showing bright staining by anti-mouse CD3ε mAb were selected by fluorescence-activated cell sorting and cloned for use in further functional assays.

3.9 Interleukin-2 assay for cellular activation

C1R transfectants expressing HLA-B*3501 (10^4 cells/well) were preincubated in a 96-well microtiter plate for 30 min at 37°C in the absence or presence of various concentrations of the Pol peptide. The mock- or TCR-transduced TG40/CD8 cells (2×10^4 cells/well) were added to the culture medium containing RPMI1640 and 10% fetal calf serum in a total volume of 200 μl. A portion of the culture supernatant (100 μl) was removed from each well after a 48-h incubation at 37°C, transferred to another culture plate, and frozen at –20°C. The amount of IL-2 produced by the TG40/CD8 cells was determined by analyzing the proliferative activity of the IL-2 indicator cell line CTLL-2. CTLL-2 cells (2×10^4/well) were incubated with the prepared culture supernatants for 18 h at 37°C. Then 0.5 μCi of [^{3}H]thymidine (TdR; Amersham Pharmacia) was added, and the cells were subsequently

incubated for an additional 6 h before being harvested onto glass fiber filters. After thorough washing of the filters with distilled water, the amount of [^{3}H]TdR incorporated by CTLL-2 cells that was retained on the filters was determined with a scintillation counter (MicroBeta Trilux; Warrac Oy, Turku, Finland).

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Impaired Processing and Presentation of Cytotoxic-T-Lymphocyte (CTL) Epitopes Are Major Escape Mechanisms from CTL Immune Pressure in Human Immunodeficiency Virus Type 1 Infection

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Investigating escape mechanisms of human immunodeficiency virus type 1 (HIV-1) from cytotoxic T lymphocytes (CTLs) is essential for understanding the pathogenesis of HIV-1 infection and developing effective vaccines. To study the processing and presentation of known CTL epitopes, we prepared Epstein-Barr virus-transformed B cells that endogenously express the *gag* gene of six field isolates by adopting an *env/nef*-deletion HIV-1 vector pseudotyped with vesicular stomatitis virus G protein and then tested them for the recognition by Gag epitope-specific CTL lines or clones. We observed that two field variants, SLFNTVAVL and SVYNTV ATL, of an A*0201-restricted Gag CTL epitope SLYNTVATL, and three field variants, KYRLKHLVW, QYRLKHIVW, and RYRLKHLVW, of an A24-restricted Gag CTL epitope KYKLKHIVW escaped from being killed by the CTL lines, despite the fact that they were recognized when the synthetic peptides corresponding to these variant sequences were exogenously loaded onto the target cells. Thus, their escape is likely due to the changes that occur during the processing and presentation of epitopes in the infected cells. Mutations responsible for this mode of escape were located within the epitope regions rather than the flanking regions, and such mutations did not influence the virus replication. The results suggest that the impaired antigen processing and presentation often occur in HIV-1 field isolates and thus are one of the major mechanisms that enable HIV-1 to escape from CTL recognition. We emphasize the importance of testing HIV-1 variants in an endogenous expression system.

Accumulated evidence has indicated a critical role of cytotoxic T lymphocytes (CTLs) in controlling human immunodeficiency virus (HIV) replication during acute and chronic infection (16). Eliciting HIV type 1 (HIV-1)-specific CTLs has been thought to be crucial for effective HIV/AIDS vaccines (15). However, despite the presence of CTLs, the majority of HIV-1-infected cases eventually progress to AIDS, probably as a consequence of the emergence of escape mutants from CTLs (8, 20). Among immunized monkeys, which developed strong cellular immune responses against HIV-1, eventual vaccine failure occurs by viral escape from CTLs (2). Thus, investigating the mechanisms of CTL failure to control the virus is essential to understanding the pathogenesis of HIV-1 infection and to develop HIV/AIDS vaccines.

The high rate of HIV-1 replication in vivo indicates that HIV-1 has tremendous ability to mutate swiftly (9, 30) and to make a dynamic adaptation to host-immune environments (3, 14, 18, 21, 31). Several mutations have been described in CTL epitopes in HIV-1-infected individuals, which result in either a lack of binding to the MHC class I molecule or nonrecognition by T-cell receptor (TCR) (3, 8, 12, 20, 21). Consequently, the virus escapes from CTL recognition. There are other mutations that do not lead to either escape effects (12); very little is known about the influence of these mutations on CTL recognition. CTL antigens are processed and presented on the cell surface in a very complex manner. Peptides are cleaved from endogenously synthesized proteins by proteasome in the cytoplasm and transported into the endoplasmic reticulum by the transporter of antigen presentation. Amino-terminal extended peptides are trimmed to the right size of peptides by aminopeptidases, which exist in both the cytoplasm and the endoplasmic reticulum (23). These steps have various degrees of substrate sequence specificity (17). The generated peptides should have sufficient affinity to bind to a major histocompatibility complex (MHC) class I molecule in the presence of various other peptides derived from host proteins and to maintain the stability of peptide-MHC complexes until they are presented on the cell surface (28). Thus, it is plausible that some amino acid substitutions in the epitope and its flanking regions have a significant influence on antigen processing and presentation. In the present study, we hypothesized that such mutations often enable HIV-1 to escape from CTL recognition.

Conventionally, the intracellular HIV-1 antigen processing and presentation has been studied with recombinant vaccinia viruses expressing an HIV-1 gene (3, 4, 11, 20, 26). Several

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TABLE 1. Characteristics of five HIV-1-infected donors[a]

Donor	HLA type			CD4 count/μl	Virus load (copies/ml)	No. of isolated clones
	A	B	Cw			
IMS1	*0201/2402	52/75	3	286	<400	3
IMS2	*0201/31	27/5101	2	797	<400	2
IMS4	*0207/2402	46/52	1	448	<400	2
IMS6	2402/26	7/5101	7	368	3.6×10^5	3
IMS7	1/−	37/−	6	544	1.3×10^3	3

[a] HLA alleles, CD4 count, viral load, and the number of isolated clones from each donor's sample are shown.

studies have addressed this issue in the context of HIV-infected T cells (4, 29, 32, 33). Most studies, however, have only evaluated a single or a few laboratory-established strains. The CTL recognition of HIV-1 clinical isolates has been evaluated, in most cases, by exogenously applying synthetic variant peptides to the cell surface to replace MHC-bound peptides (8, 12, 20, 21). Very little is known about how the antigenic products of HIV-1 clinical isolates are processed and presented in the infected cells. To address this issue, we prepared CTL target cells that endogenously express the *gag* gene derived from HIV-1 clinical isolates by adopting an *env/nef*-deletion HIV-1-based vector pseudotyped with vesicular stomatitis virus protein G (VSV-G) proteins. Here, we show evidence that HIV-1 escapes from CTL recognition often via the impairment of antigen processing and presentation.

MATERIALS AND METHODS

Subjects. Peripheral blood mononuclear cells (PBMC) were collected from five HIV-1-infected individuals from the HIV clinic affiliated with the Institute of Medical Science, University of Tokyo. Two individuals (IMS1 and IMS2) had no therapy; one individual (IMS6) was off drugs but had received treatment (zidovudine alone) 2 years prior to blood sampling; two individuals (IMS4 and IMS7) had received therapy (zidovudine-lamivudine-indinavir and stavudine-lamivudine-nelfinavir, respectively) but for less than 3 months. CD4 count, viral load, and HLA type of the recruited individuals are shown in Table 1. HLA class I typing was initially performed by serology. Subtyping of HLA-A2 was done by a PCR–sequence-specific primer method (Dynal Classic SSP HLA-A2; Dynal A.S., Oslo, Norway).

Isolation and cloning of full-length *gag*. Full-length *gag* was amplified from proviral DNA extracted from the PBMC by nested PCR with *Pfu* DNA polymerase (Strategene, La Jolla, Calif.) and oligonucleotides specific for HIV-1 long terminal repeat (LTR) and reverse transcription (RT) regions. Four oligonucleotides were mixed as outer primers: the sense primers 1U5AS-S (5′-ACTCTG GTADCTAGAGATCCCTCA-3′; the position in HXB2 being 578 to 601) and TAR-2 (5′-TGAGCCTGGGAGCTCTCTGGCT-3′; 478-499) and the antisense primers RT7A-A (5′-TATGTTGAYAGGTGTAGGTC-3′; 2485 to 2504) and RT18A-A (5′-CTACYARTACTGTACCTATAG-3′; 2464 to 2484). Two oligonucleotides were used as inner primers: the sense primer TPBS1-S (5′-AAAA TCTCTAGCAGTGGCGCCCGAACAGG-3′; the position in HXB2 being 622 to 650) and the antisense inner primer PRO6A (5′-ACTGTATCATCTGCTCC TGTRTCTAA-3′; 2322 to 2347). The thermocycling conditions were 95°C for 45 s, 50°C for 45 s, and 72°C for 210 s (30 cycles) and 72°C for 7 min for both primary and secondary PCR. The PCR products were purified by using spin columns (QIAquick PCR purification kit; Qiagen, Santa Clarita, Calif.) and cloned into PT7Blue3 vector by using a commercial cloning kit (Perfectly Blunt cloning kit; Novagen, Dedham, Mass.). Two to three clones for each individual were sequenced by an automated sequencer (ABI Prism 377 automated DNA sequencer; Perkin-Elmer, Norwalk, Conn.) with BigDye terminators (PE Applied Biosystems, Foster City, Calif.). The sequences of *gag* clones that were used in the present study are available under GenBank Accession numbers as follows: AB074049 (IMS1-28), AB074050 (IMS1-29), AB074052 (IMS2-5), AB074058 (IMS4-24), AB074061 (IMS6-34), and AB074064 (IMS7-11).

Construction of HIV-1 vector. The design of HIV-1 vector, pCTLpac, is shown in Fig. 1. The backbone of the vector is derived from an infectious molecular clone, HXB2Ecogpt (22), which lacks the function of *vpr*, *vpu*, and *nef* genes. We deleted a 1.5-kb portion from the *env*-coding region but kept the function of Rev responsive element, Tat, and Rev. The *nef* gene was replaced with the puromycin *N*-acetyltransferase (*pac*) gene (pPUR; BD Biosciences Clontech, Palo Alto, Calif.) by using *Xho*I and *Cla*I sites where the *Cla*I site was introduced by site-directed mutagenesis. *Sbf*I and *Swa*I sites were introduced by site-directed mutagenesis in the upstream of the *gag* (nucleotide 788) and in the *pol* (nucleotide 3717), respectively. The fragment from *Spe*I in the gag (nucleotide 1507) to the *Swa*I was then replaced with that of a previously published vector, pHXB2cv (25), which has a *Not*I site but lacks an *Sbf*I site in the *pol* gene. Consequently, the final construct carries the single *Sbf*I site (nucleotide 788) and the *Not*I site (nucleotide 2275) that corresponds to the 10th codon of protease. These sites were used for incorporating the *gag* clones derived from clinical isolates into the pCTLpac vector. We confirmed that the expected variant sequences were inserted in the vector by sequencing.

Generation of VSV-G pseudotype virus. Subconfluent COS7 cells in 25-cm^2 T flasks (Becton Dickinson, Lincoln Park, N.J.) were cotransfected with 4 μg of pCTLpac and 2 μg of pVSVG (BD Biosciences Clontech), which expresses VSV-G protein, by lipofection (FuGENE6; Roche Molecular Biochemicals, Mannheim, Germany) and then incubated for 48 to 60 h. The supernatant, which contains pseudotype viruses carrying the HIV-1 vector with VSV-G envelope proteins, was harvested, filtered through a 0.45-μm (pore-size) Millex filter (Millipore, Bedford, Mass.), and used as pseudotype virus stocks, some of which were stored at −80°C before use. The amount of p24 antigen in the stocks was measured by p24 antigen capture enzyme-linked immunosorbent assay (ELISA; RETRO-TEK; Zeptometrix Corp., Buffalo, N.Y.). The range of the p24 antigen yield was 40 to 100 ng/ml.

Preparation of target cells by using VSV-G pseudotyped HIV-1 vector. Epstein-Barr virus-transformed B-lymphocyte lines (B-LCLs) were infected with pseudotype virus stocks for 6 h at 37°C. The medium was then replaced with fresh RPMI 1640 (Sigma-Aldrich, St. Louis, Mo.) supplemented with 10% fetal bovine serum (R10; HyClone, Logan, Utah), and the cells were incubated for an additional 36 h. Subsequently, 0.5 μg of puromycin (BD Biosciences Clontech)/ml was added to the R10 medium to select transduced cells. The culture was maintained until the number of transduced cells became sufficient for CTL experiments. When 10^6 B-LCLs were infected with 1 ml of pseudotype virus stocks, the transduction efficiency was 20 to 30%. Usually, more than 10^7 transduced cells were generated within 2 weeks and used as CTL target cells.

To standardize the expression level of Gag protein in target cells, we quantified the amount of extracellular p24 antigen that 10^6 cells per ml of target cells had produced in 24 h. The supernatant was harvested before (supernatant A) and after (supernatant B) the 24 h of culture for the measurement of p24 antigen by p24 antigen capture ELISA (Zeptometrix Corp.). The level of p24 antigen production was defined by the difference in the concentration of p24 antigen between supernatants A and B. If the target cells produced p24 antigen that was >1 ng/ml in 24 h, they were used for CTL experiments, since the specific percent lysis did not significantly differ among target cells producing Gag protein above

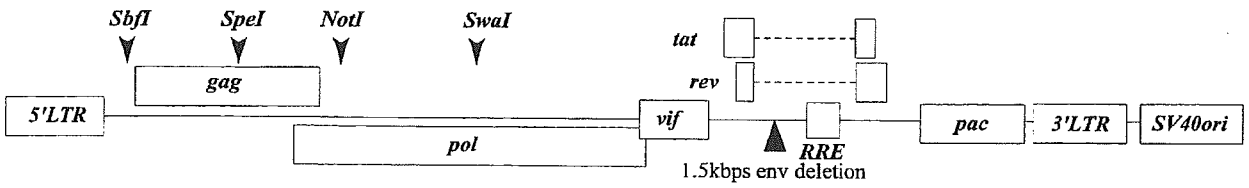

FIG. 1. Structure of pCTLpac. A 1.5-kbp portion of *env* was deleted (▲). Puromycin *N*-acetyltransferase gene (*pac*) was inserted in the *nef* region. The locations of restriction enzyme sites are indicated (▼). RRE, Rev responsive element.

this level (data not shown). We also investigated the level and pattern of protein expression of *gag* variants by Western blot analysis, as previously described (25).

^{51}Cr release experiments with HLA-class I-mismatched target cells in parallel with HLA-class I matched target cells of different donors confirmed that these target cells were recognized by CTLs in an HLA-restricted manner (data not shown). Repeated experiments showed that specific lysis of blank controls was equivalent to that of cells expressing *gag* variants that are known to escape either from TCR recognition or MHC binding. Some examples appear in the Results section below: specific lysis against IMS2-5 (Fig. 3a), IMS4-24 (Fig. 3c), IMS6-34 (Fig. 3e), and HXB2-wild (Fig. 5b). Thus, we regarded the blank control as a negative control.

Preparation of target cells by using recombinant vaccinia viruses. Recombinant vaccinia viruses used in the experiment shown in Fig. 3b were made as previously described (10). HLA-matched B-LCLs were infected with recombinant vaccinia viruses at a multiplicity of infection of 3:1 overnight before being tested in a ^{51}Cr release assay.

Effector cells. Peptide-specific CTL lines were induced from PBMC of HIV-1-infected donors. Half of the PBMC were stimulated with phytohemagglutinin (2 μg/ml) for 24 h and then pulsed with corresponding peptides at 100 μM for 1 h and irradiated before being added to the other half of the PBMC. A total of 3×10^5 cells in each well of a 96-well U-bottom plate, with at least 10 wells for each sample, were cultured in R10; 10% Lymphocult T (Biotest, Dreieich, Germany) was added to the medium on day 3 of culture. The CTL lines were maintained by adding fresh R10 medium containing 10% Lymphocult T every 3 to 4 days and splitting the well accordingly. Assays were performed on day 14 to 28 of culture.

Synthetic peptides. Peptides were manufactured at the Takara Shuzo Co., Ltd. (Shiga, Japan). The purity of peptides was >99% as determined by high-pressure liquid chromatography, and the identity of peptides was confirmed by matrix-assisted laser desorption ionization–mass spectrometry. Lyophilized peptides were dissolved in dimethyl sulfoxide and diluted in phosphate-buffered saline to make a stock concentration (2 mM). Further dilution was made in RPMI 1640 to make working concentrations of 200 μM for the induction of CTLs and of 20 μM for the preparation of target cells.

^{51}Cr release assay. In 96-well U-bottom plates, target cells were divided into aliquots at 5,000 per well. Effector cells were added to target cells at different effector/target (E:T) ratios. The amount of ^{51}Cr release in the culture supernatants was quantified after 6 h of incubation, and the percent specific lysis was determined by using the following formula: $[(E - M)/(D - M)] \times 100$, where E is the experimental ^{51}Cr release, M is the ^{51}Cr released in the presence of culture medium (which ranged between 15 and 25% of total release), and D is the total ^{51}Cr released in the presence of 5% Triton X-100 detergent. The results were regarded as positive when recognition of the HIV target was >10% above the control. The SD_{50} is the peptide concentration giving 50% of maximal specific lysis of target cells pulsed with 10 μM synthetic peptide (28).

Replication kinetics assay. Subconfluent 293T cells in Falcon 25-cm^2 T flasks (Becton Dickinson) were transfected by lipofection (Roche Molecular Biochemicals), with 2 μg of HXB2cv replication-competent HIV-1 plasmids, in which various mutations were introduced. After 60 h of culture, the supernatant was harvested, filtered through a 0.45-μm-pore-size filter, and used as mutant virus stocks. Two million Jurkat cells or eight million H9 cells were infected with an equivalent of 40 ng of p24 antigen of mutant viruses in 2 ml of R10 for 1 h. Cells were washed three times with 10 ml of R10, resuspended with 5 ml of R10, and cultured in a 12.5-cm^2 T flask at 37°C in 5% CO_2. Every 2 or 3 days, 1.5 ml of supernatant was harvested and replaced with fresh R10. The concentration of p24 was measured by using a p24 ELISA kit (Zeptometrix Corp.).

This study was approved by the Ethics Committee of the University of Tokyo.

RESULTS

Full-length *gag* clones of field isolates. We used 6 of 13 full-length *gag* clones that were isolated from the five infected individuals (Fig. 2). All of the clones did not have any stop codons. In the present study, we focused on the processing and presentation of three CTL epitopes: the HLA-A*0201-restricted epitope SLYNTVATL, the A24-restricted epitope KYKLKHIVW in p17 matrix protein (MA), and the HLA-B*5101-restricted epitope NANPDCKTI in p24 capsid protein (CA) (11, 26, 27). Amino acid sequences within the three epitope regions and the N- and C-terminal 15-amino-acid residues flanking each epitope were analyzed; the six clones were selected to maximize the diversity of amino acid sequences in the epitopes and its flanking regions.

The A*0201-restricted epitope and its flanking regions were highly variable. However, we did not observe a previously recognized variation in the flanking region, Arg (R) to Lys (K) at position 76 in our clones (4). In contrast, the B*5101-restricted epitope and its flanking regions were conserved except for clones IMS2-5 and IMS4-24. In the A24-restricted epitope and its flanking regions, variations were seen almost exclusively within the epitope region with two exceptions, a Lys (K)-to-Ser (S) mutation at position 26 (K26S) in clone IMS4-24 and an Arg (R)-to-Lys (K) at position 15 in clone IMS2-5. The Lys (K)-to-Arg (R) mutation at position 30 within the A24-restricted epitope was seen more frequently than any other sequences; none of the 13 clones had the wild-type sequence of KYKLKHIVW. We incorporated the six *gag* clones into the HIV-1 vector with *env* and *nef* deleted, pCTLpac (Fig. 1), to make target cells expressing *gag* genes of these filed isolates.

CTL recognition of target cells endogenously expressing *gag* genes of clinical isolates. We generated A*0201-restricted SLYNTVATL (wild type) epitope-specific oligoclonal CTL lines from one HIV-1-infected individual (IMS1) with A*0201 and used the lines to test the killing of the six different *gag* clones expressed on A*0201-matched B-LCLs by a conventional ^{51}Cr release assay. The A*0201-restricted CTLs efficiently recognized target cells expressing *gag* clones IMS1-29, IMS1-28, and IMS6-34, which encode either wild type or the SLYNTIATL sequence in the CTL epitope region. In contrast, the same CTLs did not recognize cells expressing *gag* clones IMS2-5, IMS4-24, and IMS7-11, which encode SLYNLVATL, SLFNTVAVL, and SVYNTVATL, respectively, indicating that these clones escaped from A*0201-restricted CTL recognition (Fig. 3a).

CTL recognition of IMS1-29 and IMS6-34 was also tested with recombinant vaccinia viruses expressing the *gag* gene of these variants in parallel with the VSV-G-pseudotyped HIV-1 vectors. The HIV-1 vector method demonstrated the CTL killing as well or slightly better than the vaccinia method did (Fig. 3b).

We used three B*5101-restricted NANPDCKTI -specific CTL clones to test the CTL recognition of five representative *gag* clones. The CTL clones recognized four *gag* clones, which convey the wild-type B*5101-restricted epitope sequence; they also recognized IMS2-5 that had a substitution in the flanking region. None of the clones recognized the IMS4-24 clone, which had the variant sequence NSNPDCKNI in the epitope region (Fig. 3c).

A24-restricted KYKLKHIVW (wild type) specific-CTL lines did not recognize synthetic peptides of the most common sequence, KYRLKHIVW (3R mutant type), and the other variant, RYRLKHIVW (Fig. 3d). These two variants were shown to bind to the A*2402 MHC class I molecule in a binding assay (data not shown). We screened eight A24-positive individuals for the presence of CTL activities against the 3R mutant epitopes and found one individual who carried CTLs recognizing the 3R mutant peptide. A24-restricted 3R mutant-reactive CTL lines were induced from this A24-positive individual and used for the remaining experiments. The 3R mutant-reactive CTL lines recognized target cells expressing IMS1-29 and IMS4-24 *gag* clones, both of which carry the 3R mutant sequence, but did not recognize any

(1) A*0201-restricted epitope (amino acid 62-100)

origin	62								70								80								90								100
HXB2	G	Q	L	Q	P	S	L	Q	T	G	S	E	L	E	R	S	L	Y	N	T	V	A	T	L	Y	C	V	H	Q	R	I	E	I	K	D	T	K	E	A
IMS 1-28	E	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
IMS 1-29	E	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
IMS 2-5	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	V	R	-	-	-
IMS 4-24	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	V	-	-	-	-	-	-	-	-	-	K	-	-	V	-	-	-	-
IMS 6-34	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
IMS 7-11	A	-	-	H	-	A	-	K	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(2) B51 restricted epitope (amino acid 310-348)

origin	310								320								330								340								348
HXB2	S	Q	E	V	K	N	W	M	T	E	T	L	L	V	Q	N	A	N	P	D	C	K	T	I	L	K	A	L	G	P	A	A	T	L	E	E	M	M	T
IMS 1-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 2-5	-	-	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 4-24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 6-34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 7-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(3) A24-restricted epitope (amino acid 13-51)

origin	13								20								30								40								51
HXB2	L	D	R	W	E	K	I	R	L	R	P	G	G	K	K	K	Y	K	L	K	H	I	V	W	A	S	R	E	L	E	R	F	A	V	N	P	G	L	L
IMS 1-28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 1-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 2-5	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 4-24	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 6-34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IMS 7-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	R	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIG. 2. Sequence variation in three CTL epitopes and their flanking regions. The amino acid sequences of six *gag* clones are shown. The reference sequence is derived from HXB2, and the differences are indicated. The numbering is done according to the HIV sequence database, Los Alamos National Laboratory, Los Alamos, N.Mex. The CTL epitope regions are boxed.

other target cells expressing different variants (Fig. 3e). Interestingly, IMS4-24 with Lys (K)-to-Ser (S) mutation at position 26 outside the epitope region was less well recognized than IMS1-29. We consistently observed this phenomenon in repeated experiments (data not shown).

CTL recognition of exogenously loaded variant peptides. To investigate whether the above findings of escape phenomenon from CTL killing were due to either loss of peptide binding to the MHC class I molecule or to the lack of TCR recognition, we prepared synthetic peptides that represented the variant epitopes and tested them for cross-recognition of the peptides in peptide titration assays by using the same CTL lines or clones that were used in experiments described for Fig. 3. To our surprise, A*0201-restricted CTL lines recognized the peptides of two A*0201-restricted CTL epitope variants, SVYNT VATL and SLFNTVAVL, which were not recognized by the CTLs when expressed endogenously. They recognized the SLFNTVAVL peptide less efficiently, with an SD_{50} of >100 nM (Fig. 4a). Target cells pulsed with SLYNLVATL peptide representing clone IMS 2-5 were not cross-recognized even at a saturated concentration (10 μM) (data not shown).

We also obtained similar discordant results in experiments of A24-restricted CTL epitope variants. A24-restricted 3R mutant-specific CTL lines recognized peptides of three variant s—KYRLKHLVW, RYRLKHLVW, and QYRLKHIVW—that were not recognized by the CTLs when they were expressed endogenously. In fact, the CTLs recognized QYRLKHIVW peptide even better than the 3R mutant peptide but did not cross-recognize the QYKLKHIVW peptide (Fig. 4b).

We tested one B*5101 variant peptide, NSNPDCKNI, in a peptide titration assay. This variant was not cross-recognized by any of the CTL clones even at a high concentration (1 μM) (Fig. 4c). The two amino acid mutations in this epitope coincided with two anchor residues to the MHC biding, suggesting that the lack of recognition of this variant was likely due to loss of peptide binding.

Mutations responsible for impairing the epitope processing and presentation. The discrepancies seen above between the CTL recognition of endogenously expressed and exogenously loaded antigen indicate that some mutations have caused the impairment of epitope processing and presentation. To locate specific variations that were responsible for the poor recognition of endogenously expressed HIV-1 *gag* variants, we constructed four different target vectors: an HXB2 *gag* sequence with A*0201-restricted epitope variations (SLFNTVAVL [HXB2-3F8V] or SVYNTVATL [HXB2-2V]) and IMS4-24- or IMS7-11-derived *gag* sequence with the wild-type A*0201 epitope sequence (IMS4-24-wild or IMS7-11-wild, respectively). The replacement of the variant epitope region with the

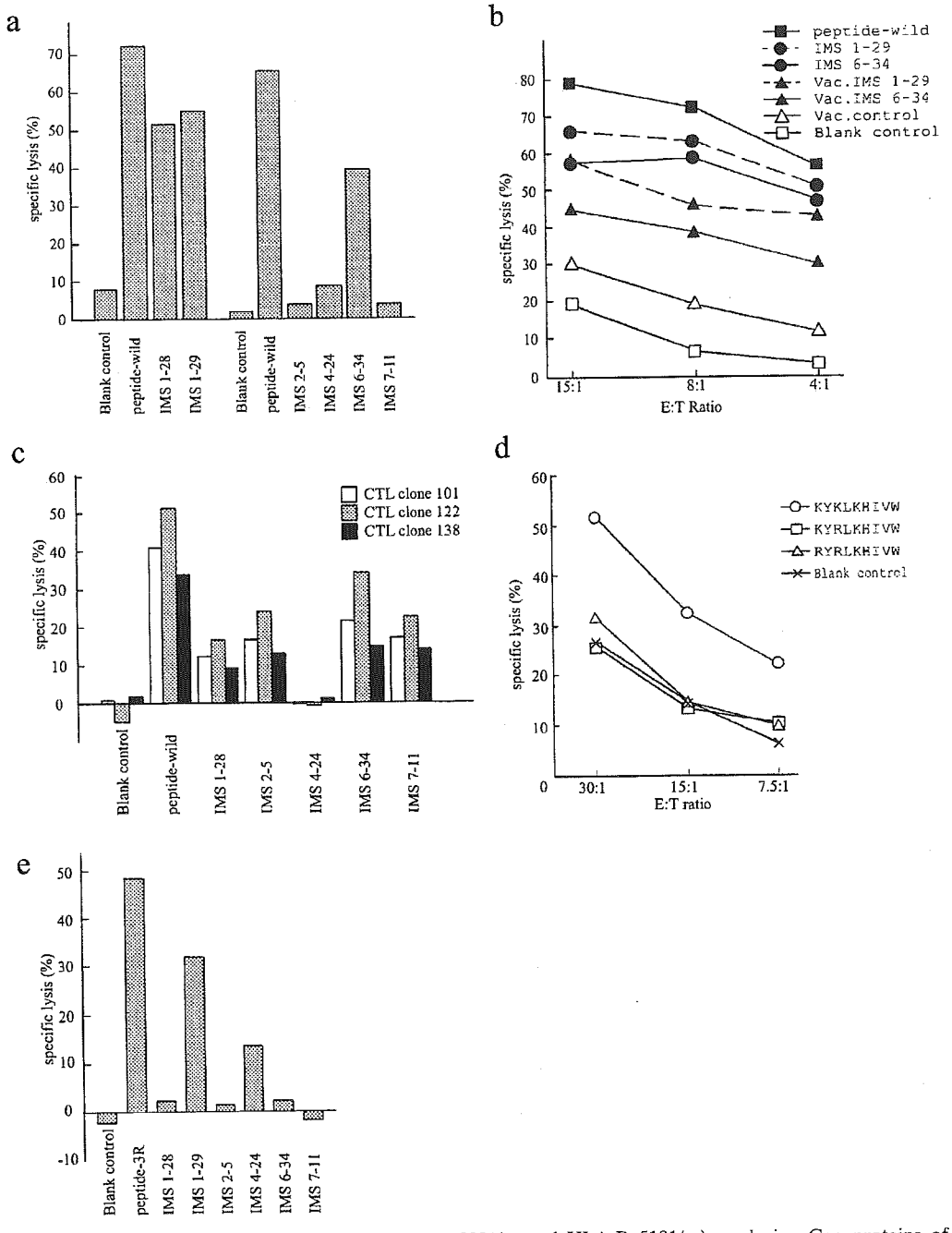

FIG. 3. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) producing Gag proteins of clinical isolates. Peptide target cells were pulsed with the A*0201 wild-type peptide, SLYNTVATL (10 μM). A*0201-restricted SLYNTVATL -specific CTL lines were induced from a single donor (IMS1). The E:T ratio was 10:1. This experiment was repeated, with a different B-LCLs (HLA-A*0201/31 and HLA-B27/*5101), giving the same pattern of recognition (data not shown). (b) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) expressing *gag* clones of two clinical isolates with the VSV-G-pseudotyped HIV-1 vector versus recombinant vaccinia viruses. Recombinant vaccinia virus expressing the human CD4 gene was used as a vaccinia virus control (1). The effector and peptide target cells were prepared as described for panel a. (c) Specific lysis of B*5101-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) producing the Gag proteins of five clones. Three B*5101-restricted NANPDCKTI -specific CTL clones were used as effector cells at an E:T ratio of 2:1 (23). The peptide target was pulsed with the B51 wild-type peptide NANPDCKI (1μM). (d) Specific lysis of A24-matched B-LCLs (HLA-A24/− and HLA-B46/52) pulsed with the peptides KYKLKHIVW, KYRLKHIVW, and RYRLKHIVW at 10 μM. A24-restricted, KYKLKHIVW -specific CTL lines were induced from one A24-positive donor. (e) Specific lysis of A24-matched B-LCLs (HLA-A24/− and HLA-B46/52) producing variant Gag proteins. A24-restricted KYRLKHIVW (3R)-specific CTL lines were induced from another A24-positive donor. The peptide target was pulsed with 3R mutant type peptide (10 μM). The E:T ratio was 20:1. The lysis of target cells without any peptide pulsing is shown as a blank control.

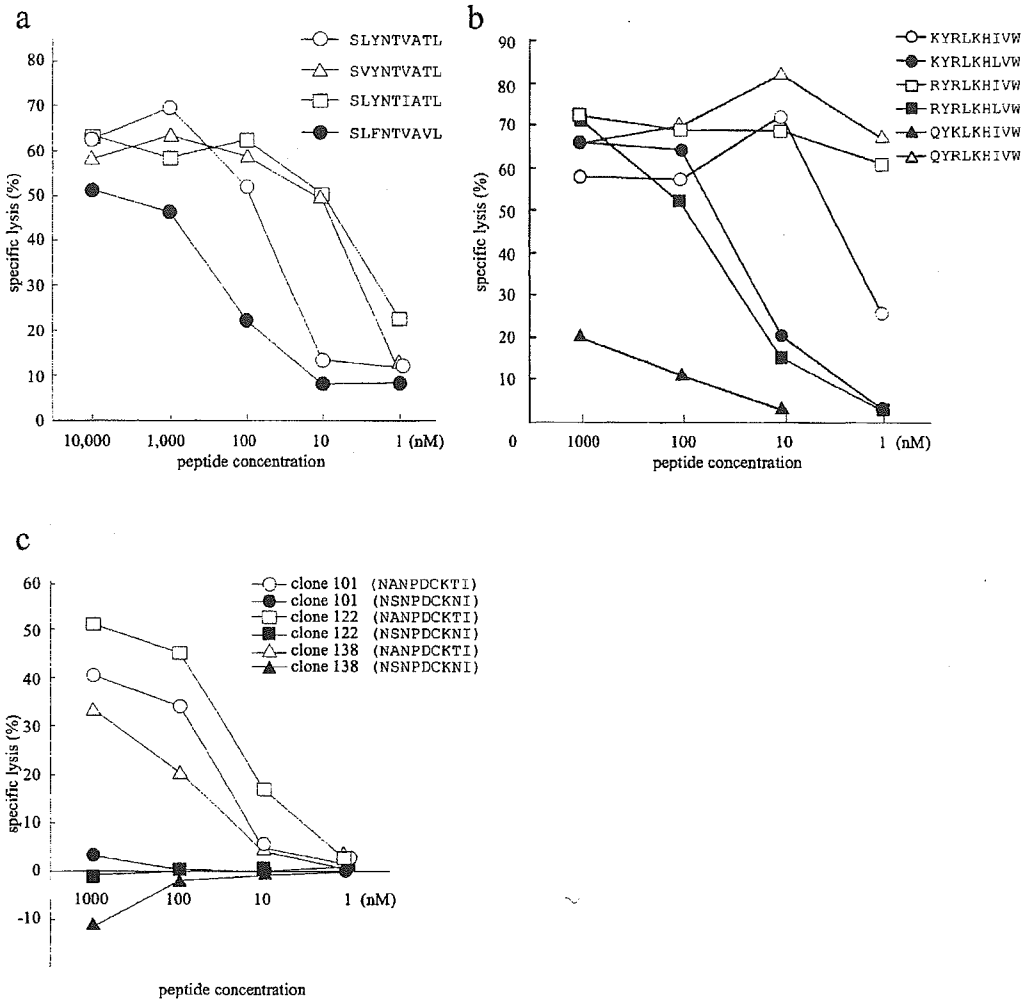

FIG. 4. Peptide titration assays. (a) Specific lysis of A*0201-matched B-LCLs pulsed with A*0201 variant peptides by A*0201-restricted CTLs at an E:T ratio of 20:1. (b) Specific lysis of A24-matched B-LCLs pulsed with 3R and its variant peptides by A24-restricted 3R mutant reactive CTLs at an E:T ratio of 20:1. (c) Specific lysis of B51-matched B-LCLs pulsed with B51 variant peptides by B51-restricted CTL clones at an E:T ratio of 2:1. The same effector and target cells were used as for Fig. 3. The percent lysis of the blank control has been subtracted.

wild-type epitope sequence restored CTL recognition of the escape variants, whereas replacement of the wild-type epitope with the two variant epitopes resulted in no CTL recognition of HXB2 Gag (Fig. 5a). The levels and patterns of Gag protein expression in target cells were analyzed by Western blot experiments (Fig. 5b). The expression levels of p55 Gag precursor and p24 CA did not significantly differ between the mutants and the wild type. The p17 MA band was not clear in HXB2-2V, IMS7-11-wild, and IMS4-24-wild, but the appearance of this band did not correlate with CTL killing. These results indicate that amino acid substitutions within the A*0201-restricted epitope region, rather than those in the flanking regions, have caused the inhibition of CTL recognition in our endogenous expression system.

To further investigate the effect of amino acid substitutions within the A24-restricted epitope on antigen processing and presentation, we introduced various point mutations into the wild-type HXB2 vector, pCTLpac, and tested them for the recognition by A24-restricted 3R mutant-reactive CTL lines. The A24-restricted 3R mutant-specific CTLs did not cross-recognize the wild-type peptide and the wild-type HXB2 vector but did recognize HXB2 with a 3R mutation (HXB2-1R). The substitution of Lys (K) with Arg (R) at position 28 (HXB2-1R3R) did not affect the A24-restricted 3R mutant-specific CTL recognition, but a Lys (K)-to-Gln (Q) substitution at position 28 (HXB2-1Q3R) or an Ile (I)-to-Leu (L) substitution at position 34 (HXB2-3R7L) resulted in the escape from CTL killing (Fig. 5c).

Replication kinetics of HIV-1 mutant viruses. We analyzed the replication kinetics of recombinant viruses carrying mutations that have affected the epitope processing and presentation by infecting H9 or Jurkat cells. All mutants were found to replicate to equivalent levels, suggesting that these mutations do not have a significant influence on HIV-1 replication (Fig. 6).

DISCUSSION

The present study focused on three Gag CTL epitopes restricted by three common HLA alleles in Japanese people (24).

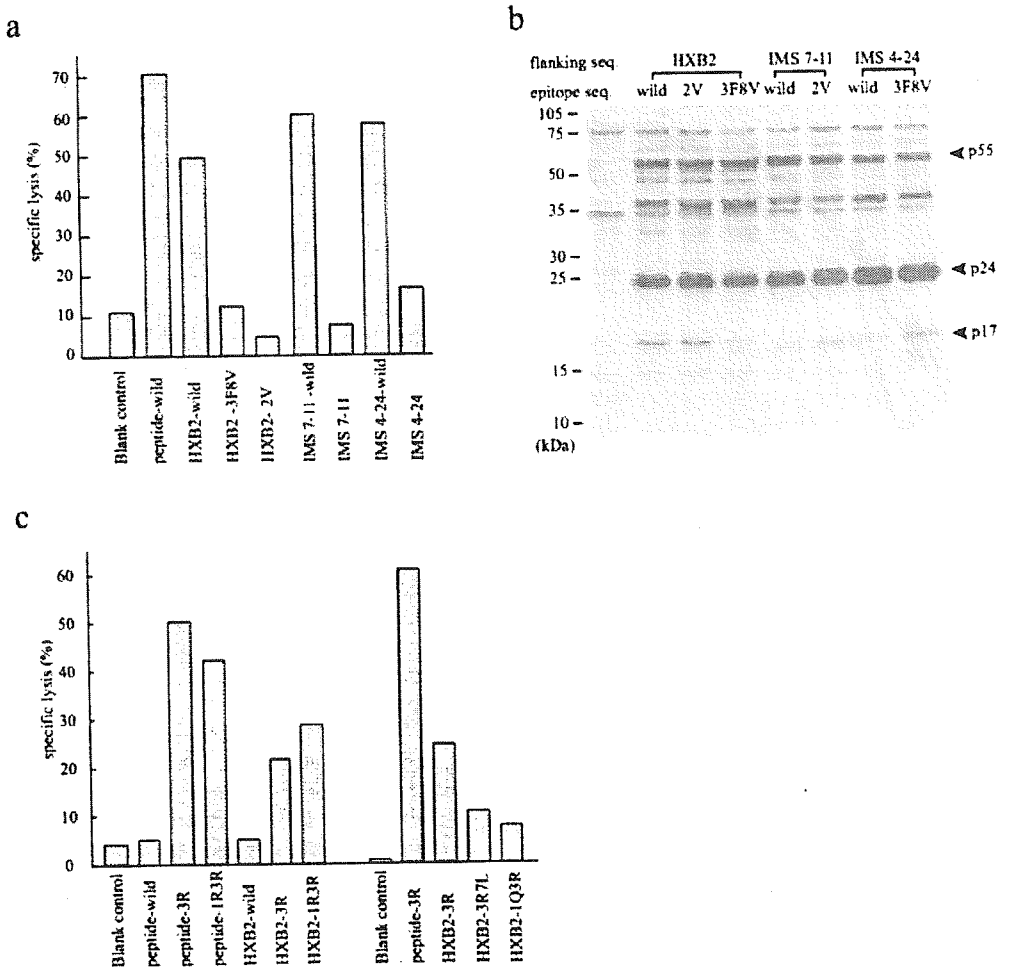

FIG. 5. (a) Specific lysis of A*0201-matched B-LCLs (HLA-A*0201/− and HLA-B*5101/−) that endogenously express chimeric *gag* clones bearing the variant CTL epitopes SLFNTVAVL and SVYNTVATL in the frame of HXB2 *gag* (HXB2-3F8V and HXB2-2V, respectively) or bearing the wild-type epitope in the frame of IMS7-11 and IMS4-24 Gag (IMS7-11-wild and IMS4-24-wild, respectively). A*0201-restricted SLYNTVATL CTL lines were induced from the same donor as for Fig. 3. Specific lysis of target cells expressing HXB2, IMS7-11, or IMS4-24 *gag* clones and being pulsed with the A*0201 wild-type peptide (10 μM) is shown in parallel. The E:T ratio was 20:1. (b) Levels and patterns of HIV-1 protein expression in target cells used in the experiments described for panel a. The Western blot was reacted with the serum from an HIV-1-infected individual. (c) Specific lysis of A24-matched B-LCLs (HLA A24/− and HLA-B46/52 or HLA-A24/26 and HLA-B51/52) that express *gag* clones with various point mutations. Point mutations were inserted into the A24-restricted CTL epitope region in the frame of wild-type HXB2 Gag (HXB2-wild): amino acid substitutions of Lys to Arg at position 30 (HXB2-3R) with Lys to Arg at position 28 (HXB2-1R3R), Ile to Lue at position 34 (HXB2-3R7L), or Lys to Gln at position 28 (HXB2-1Q3R). Peptide target cells were pulsed with either the KYRLKHIVW (3R) or the RYRLKHIVW (1R3R) mutant peptide at 10 μM. The effector cells were A24-restricted 3R mutant-specific CTL lines from the same donor as in the Fig. 3e experiment. The E:T ratio was 20:1.

The Gag protein is most commonly targeted by CTL-inducing HIV/AIDS vaccines (15). In our endogenous expression system, three A*0201-restricted epitope variants and one B*5101-restricted epitope variant escaped from the wild-type CTL recognition, and four A24-restricted epitope variants escaped from the A24-restricted 3R mutant-reactive CTL recognition. Intriguingly, two A*0201-restricted variants and three A24-restricted variants escaped from CTL killing when the *gag* clones were expressed endogenously in the target cells by the HIV-1 vector, despite the fact that the synthetic variant peptides were well recognized by the CTLs when loaded onto the MHC class I molecule exogenously. The peptide titration experiments have revealed that the strength of these variant peptides' recognition was almost equivalent to that of the A*0201-restricted wild-type peptide or the A24-restricted 3R mutant peptide. The results were not likely due to differences in the pattern of Gag protein expression, as shown in the Western blot experiments. All target cells were confirmed to express a sufficient level of Gag protein by p24 antigen production. Therefore, we believe that the escape mechanism of these variants resides in the antigen processing and presentation, as has been observed in a mouse model with murine leukemia virus infection (19). The observation of such phenomenon in two epitopes restricted by different alleles implies that this finding is not unique to a particular epitope-MHC pair.

Since all variants investigated here were derived from clinical samples and those mutations did not affect the virus replication, our observations are relevant for discussing what may be going on in HIV-infected individuals. Our results indicate

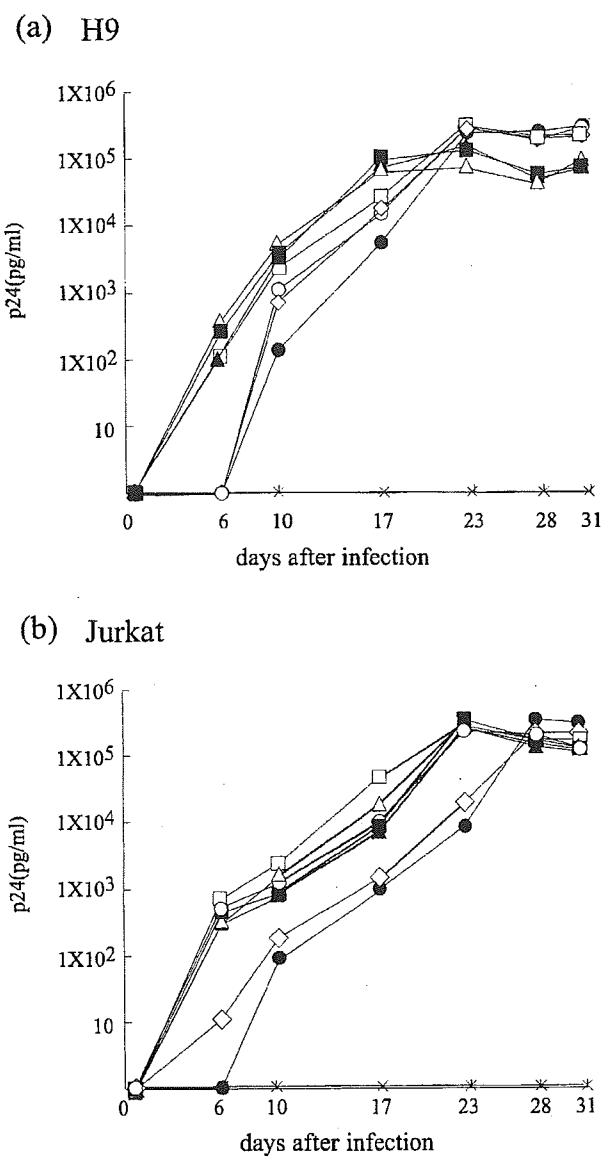

FIG. 6. Replication of HIV-1 clones with mutations that impaired the processing and presentation of A*0201 or A24 CTL epitopes in H9 (a) and Jurkat (b) cells. The kinetics of each recombinant virus replication were monitored as the production of p24 antigen by p24 ELISA. Symbols: ○, wild-type; □, A24-3R; ■, A24-K26S+3R; △, A24-3R7L; ▲, A24-1Q3R; ●, A*0201-3F8V; ◇, A*0201-2V; ×, mock.

that the impaired antigen processing and presentation often occurs in HIV-1 field isolates and thus is one of the major mechanisms that enable HIV-1 to escape from the CTL recognition. To understand further the significance of this escape mechanism, it is important to evaluate an accumulation of such escape variants in infected hosts in a longitudinal study or at a population level. A previous report using a vaccinia virus expression system did not reveal that any mutations in the A*0201-restricted p17 epitope of HIV-1 and its flanking region altered the processing and presentation of its variant epitope (4). However, that study did not investigate A*0201-restricted 2V and 3F8V variants, which we found affected epitope processing and presentation.

Experiments with chimeric genes, as well as point mutations, showed that escapes from epitope processing and presentation were mostly attributable to mutations within the epitope regions rather than its flanking regions. In the present study, we demonstrated that point mutations of Lys (L) to Gln (Q) at position 28 and of Ile to Leu at position 34 drastically impaired the processing and presentation of the A24-restricted CTL epitope. Moreover, the experiment with HXB2 clone carrying IMS 7-11 variant of A*0201-restricted CTL epitope indicates that a substitution of Leu (L) to Val (V) at position 78 was responsible for the impaired processing and presentation of the epitope. These mutations in the epitope region may have induced a proteasome cleavage site within the epitope (19). On the other hand, we observed that the variations in the 15 amino acids up- and downstream of the epitope did not affect CTL recognition. An exception was a Lys (L)-to-Ser (S) substitution (−2S) at position 26, which is only two amino acids adjacent to the N terminus of the A24-restricted epitope. However, this −2S substitution did not void the A24-restricted 3R mutant-reactive CTL recognition completely. One possible explanation is that the −2S substitution shifted the optimal proteasome cleavage site, resulting in the generation of a larger peptide, which has a lower affinity to the MHC class I molecule.

We have first attempted to investigate the antigen processing and presentation by the conventional recombinant vaccinia virus method for all variants before we established this VSV-G-pseudotyped HIV-1 vector method. Soon, we realized that preparing recombinant vaccinia viruses was much more laborious and time-consuming. Early experiments of comparing two methods by using the first available recombinant vacinia viruses concluded that the HIV-1 vector method demonstrated CTL killing better than did the recombinant vaccinia virus method (Fig. 3b). In the recombinant vaccinia virus expression system, the massive production of vaccinia virus proteins inevitably takes place, along with the expression of an HIV-1 gene and sometimes causes a high background lysis. The expression manner and the production ratio to non-HIV proteins may also influence antigen processing and presentation (27, 34). Thus, we thought that the antigen processing and presentation in the HIV-1 vector expression system is more physiological than the recombinant vaccinia virus expression system and that continuing vaccinia virus experiments would not be significantly beneficial to address the issue of antigen processing and presentation. Nevertheless, there remains a concern that there might be a potential difference in the antigen processing and presentation between immortalized B cells that were used here and primary $CD4^+$ T cells (32, 33). Perhaps it is important to reevaluate the interaction of CTLs and these variants in experiments with variant HIV-1-infected T cells. Our HIV-1 vector carries neither the *nef* gene nor the *vpu* gene, which significantly affect antigen presentation by downregulating MHC class I cell surface expression (5, 13). From this point of view, one might expect that more variants would escape from the CTL recognition in the actual HIV-1 infection than what is shown in our experiments. However, we think that our system is suited to identify a specific association between a certain mutation and the escape from antigen processing and presentation. To prove the existence of this mode of escape mecha-

nism, we may need a new system that can directly detect a trace of specific epitopes that are eluted from MHC class I molecules of HIV-1 antigen-producing cells.

Although the structure analysis of MHC class I molecules and its binding motif has facilitated the prediction of CTL epitopes from the primary amino acid sequence data of HIV-1 (6, 11, 26), it remains difficult to envisage the efficiency of epitope processing and presentation. Enormous diversity realized in HIV-1 field isolates causes a further complexity (7). Our data emphasize the importance of testing HIV-1 variants in an endogenous expression system. Detailed analysis of epitope processing and presentation among HIV-1 field isolates, particularly of non-B subtypes circulating in the vaccine trial fields, is essential, since such information allows us to forecast which virus may elude the immunity elicited by vaccines, thus providing a clue for a rational design for effective HIV/AIDS vaccines.

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Functionally Impaired HIV-Specific CD8 T Cells Show High Affinity TCR-Ligand Interactions[1]

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We eventually isolated two different clonotypic CD8 T cell subsets recognizing an HIV Pol-derived epitope peptide (IPLTEEAEL) in association with HLA-B35 from a chronic HIV-infected patient. By kinetic analysis experiments, the subsets showed a >3-fold difference in half-lives for the HLA tetramer in complex with the Pol peptide. In functional assays in vitro and ex vivo, both subsets showed substantial functional avidity toward peptide-loaded cells. However, the high affinity subset did not show cytolytic activity, cytokine production, or proliferation activity toward HIV-infected cells, whereas the moderate affinity one showed potent activities. Furthermore, using ectopic expression of each of the TCR genes into primary human CD8 T cells, the CD8 T cells transduced with the high affinity TCR showed greater binding activity toward the tetramer and impaired cytotoxic activity toward HIV-infected cells, corroborating the results obtained with parental CD8 T cells. Taken together, these data indicate that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions, providing us further insight into the immune evasion mechanisms by HIV. *The Journal of Immunology,* **2004, 173: 5451–5457.**

Our understanding of how HIV avoids control by the human immune system remains incomplete. Although $CD8^+$ CTL are believed to have an important role in the immunopathogenesis of HIV-1 infection, it is not completely clear why viral replication persists and progressive immunodeficiency generally ensues (see recent reviews, 1–5). The findings of several studies show that HIV-specific CTL taken ex vivo can have functional defects that could undermine their control of the virus. For example, whereas most HIV-specific CD8 T cells in patients with chronic HIV disease produced antiviral cytokines on contact with cognate Ag, these cells showed diminished perforin expression and capacity for proliferation compared with CMV-specific T cells (6) and T cells in long term nonprogressors (7), respectively. Such different functional outcomes in T cells can be caused by the quality of T cell activation, such as the strength of TCR engagement and costimulatory or inhibitory interactions (8–10). The kinetics and affinity of interaction between TCR and peptide-MHC complex (pMHC)[3] are the basis of T cell activation. For the most part, longer half-lives of TCR-pMHC interaction correspond to higher T cell activation (11–14). However, in the case of some peptide variants as well as mutations in MHC and/or TCR, a longer half-life was reported to weaken T cell reactivity (15–18). Moreover, it remains unclear what are the functional roles of peripheral T cell subsets that bear TCR with high affinity for a MHC ligand in association with a foreign peptide, because T cells with high affinity for a foreign pMHC appear to be negatively selected in the thymus and not exported to the periphery (19).

In contrast, we and others have generated many CTL lines and clones from HIV-infected patients that were cytotoxic toward HIV-infected cells in vitro in the course of experiments to identify HIV-derived CTL epitopes in previous studies (20). Given that only T cells that were positive for epitope-specific cytolytic activity were reported in these studies, we hypothesized that T cells with negative cytolytic activity toward HIV-infected cells, which may reflect the loss of antiviral effector functions of HIV-specific CTLs in vivo, were concurrently generated, but not further examined, due to their negative activity. Therefore, to examine cell-based mechanisms involved with impaired functions of HIV-specific CD8 T cells, we have again been testing CD8 T cell clones isolated from HIV-infected patients for their lack of killing activity toward HIV-infected cells even though they retained their specificity toward HIV Ags.

In the present study we focused on remarkable functional differences in two different CD8 $\alpha\beta$ T cell subsets (TCR $V\alpha12^+$ and $V\delta1^+$) specific for an HIV Pol-derived epitope peptide (IPLTEEAEL) from a chronic HIV-infected patient. Interestingly, the subsets showed a >3-fold difference in binding activity toward the HLA tetramer in complex with the Pol peptide. The high affinity subset ($V\delta1^+$) showed impaired reactivity toward HIV-infected cells in vitro and ex vivo, whereas the moderate affinity subset ($V\alpha12^+$) had potent reactivity. Additional genetic transfer of each of these TCR genes into human primary CD8 T cells clearly indicated that impaired responsiveness of T cells toward HIV-infected cells can occur at the level of TCR-ligand interactions.

Materials and Methods

Tetramer binding assay

The CTL lines (5×10^4 cells; >60% $tetramer^+$ $CD8^+$ cells) generated by repeated stimulation of the patient's lymphocytes (*HLA-A**2402/*A**2601, *HLA-B**3501/*B**5101) with the Pol peptide (IPLTEEAEL) were first stained with various concentrations of the tetramer at 4 or 37°C for 15 min. The cells were subsequently stained at 4°C for 15 min with anti-CD8-PerCP (BD Pharmingen, San Diego, CA), FITC-conjugated anti-$V\alpha12$

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[3] Abbreviations used in this paper: pMHC, peptide-MHC complex; IRES, internal ribosome entry site; MFI, mean fluorescence intensity.

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TCR (Serotec, Oxford, U.K.), and anti-Vδ1 TCR mAbs. The anti-Vδ1 TCR mAb (A13; provided by L. Moretta, Istituto di Istologia ed Embriologia Generale, Genova, Italy) (21) had been labeled with PE-conjugated Fab specific for the Fc portion of mouse IgG1 (Molecular Probes, Eugene, OR). For the kinetic analysis of tetramer binding, the CTL line was first incubated with 5 μM tetramer at 4°C. A portion of the reaction was removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with anti-CD8 and anti-TCR mAbs as described above. For kinetic analysis of the tetramer dissociation, a CTL line was stained with 5 μM tetramer for 60 min at 4°C. Then cells were rapidly washed twice and suspended in 1.5 ml of a buffer (2% BSA in PBS) supplemented with a blocking Ab. A portion of the reaction was then removed periodically (2, 5, 10, 15, 30, and 60 min), and the cells were subsequently stained with the anti-CD8 and anti-TCR Abs. For the flow cytometric analysis, $V\alpha12^+$ or $V\delta1^+$ $CD8^+$ cells were gated and then assessed for their tetramer binding level.

Cytotoxic assay

The cytotoxic activity of CTL clones generated previously (22, 23) was determined by a standard ^{51}Cr release assay as previously described (22). For Pol peptide-pulsed target cells, ^{51}Cr-labeled C1R-B*3501 cells were pulsed with the peptide for 1 h, then incubated with the effector T cells for an additional 4 h at 37°C. For virus-infected target cells, C1R-B*3501 cells or .221-B*3501 cells expressing human CD4 Ag were infected with HIV-1 GagPol-expressing vaccinia virus, HIV-1 LAI, or vesicular stomatitis virus envelope glycoprotein-pseudotyped HIV-1 HXB2D. Note that all these viruses have the same epitope sequence as that used for synthetic Pol peptide (IPLTEEAEL). The cells were subsequently labeled with ^{51}Cr and incubated with the effector T cells for 6 h at 37°C. It should be noted that >70% cells expressed the p24 Gag Ag, as revealed by intracellular flow cytometric analysis of target cells.

Cytokine secretion assay

CTL clones were cocultured with .221-CD4-B*3501 cells, either pulsed with various concentrations of the Pol peptide or infected with HIV-1 LAI for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. Then the cells were permeabilized, stained with anti-IFN-γ and TNF-α mAbs (BD Pharmingen), and analyzed by flow cytometry as previously described (23).

Ex vivo activation assay

Cryopreserved PBMC of HIV-positive (1×10^6) or negative donors (5×10^6) were stained with the tetramer at 37°C for 15 min, followed by anti-CD8 and anti-TCR Abs at 4°C for 15 min. The cryopreserved PBMC of the HIV-positive patient were stimulated, or not, with irradiated .221-CD4-B*3501 cells, either pulsed with 100 nM Pol peptide or infected with HIV-1 LAI (>70% p24 Gag^+). The cells were cultured at 37°C for 12 days in RPMI 1640 supplemented with 10% FCS and 200 U of IL-2. A portion of the stimulated cells (2×10^5) was stained as described above.

Construction of retroviral vectors and gene transfer

The genes encoding full-length α and β TCR of CTL 55 (23) and 589 (22) were subcloned into the pGC-based retroviral vector (pGCDNsap[MSCV]; provided by M. Onodera, Tsukuba University, Ibaragi, Japan) (24). The sequence data of the TCR genes are available from DDBJ under accession numbers AB164056, AB164057, AB164620, and AB164621. The genes encoding a murine heat-stable Ag (CD24) or a GFP were also incorporated into the constructs with an internal ribosome entry site (IRES) following the α or β TCR gene to facilitate monitoring of the expression of the α or β TCR gene, respectively, in the transduced cells.

Human primary CD8 T cells were isolated from PBMC of an HIV-negative healthy donor with *HLA-B*3501* using anti-CD8 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant CD8 T cells were activated over a 3-day period by anti-CD3 mAb (OKT3) coated on the culture dish, then transferred to recombinant fibronectin-coated plates (Takara Shuzo, Otsu, Japan) and incubated for 72 h with the retrovirus supernatant containing the TCR α-IRES-CD24 gene. Transduced T cells expressing CD24 Ags were isolated using PE-labeled anti-CD24 mAb (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec). The isolated cells (>80% of the cells were $CD8^+$ $CD24^+$) were subsequently transduced with another construct containing TCR β-IRES-GFP as described above.

Results

Functional difference in CTL clones in response to HIV-infected cells in vitro

An HIV Pol peptide (IPLTEEAEL) is a CTL epitope endogenously presented by HLA-B*3501 (20). In Pol peptide-stimulated lymphocytes from a patient with chronic HIV infection, we generated two $CD8^+$ αβ T cell clones, designated CTL 55 and 589, that were shown to express TCR Vδ1.1/Vβ13.3 and Vα12.1/Vβ5.6, respectively, on their cell surface (22, 23). It is of note that the genes encoding a Vδ1 variable segment are expressed in ~0.5% of peripheral $CD8^+$ αβ T cells in human healthy individuals and that the Vδ1-bearing TCR αβ recognizes a peptide presented by HLA class I molecules (21, 23). The functional properties of both T cell clones were first tested for their cytotoxic and cytokine production activities in response to cells either pulsed with the Pol peptide or infected with viruses expressing HIV Pol proteins. Peptide titration experiments showed that both clones had substantial cytotoxic activities (Fig. 1*A*). It should be noted that in repeated experiments, CTL 589 appeared to

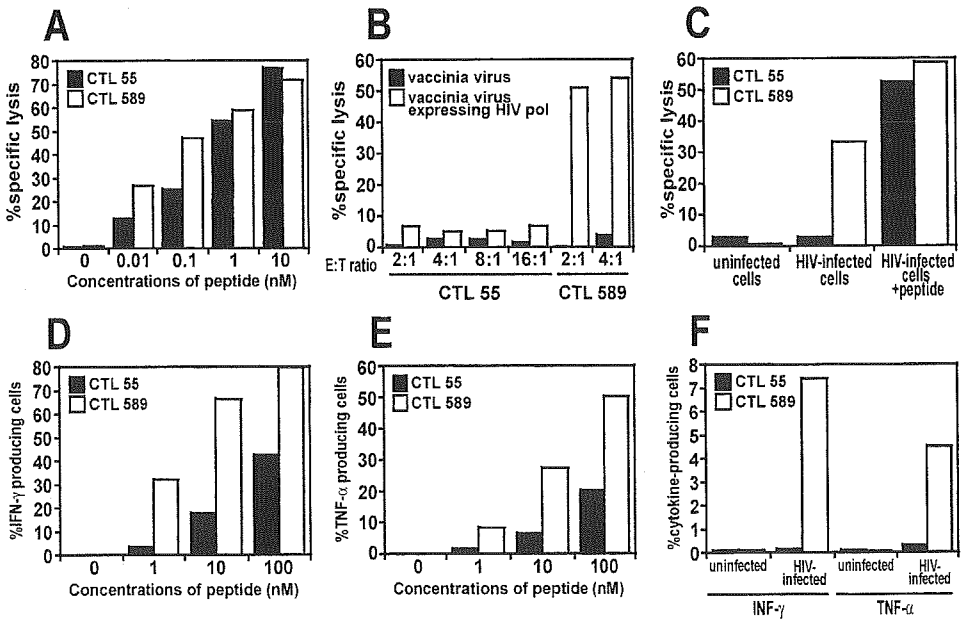

FIGURE 1. Analysis of effector functions of the CTL clones. *A* and *B*, Cytotoxic activity of CTL 55 and 589 toward C1R-B*3501 cells either pulsed with the indicated concentrations of the Pol peptide (*A*) or infected with vaccinia virus expressing HIV-1 GagPol polyproteins at the indicated E:T cell ratios (*B*). *C*, Cytotoxic activity of CTL 55 and 589 toward .221-B*3501 cells uninfected or infected with HIV-1 in the absence or the presence of 100 nM Pol peptide at an E:T cell ratio of 2:1. *D–F*, Intracellular staining for IFN-γ and TNF-α of CTL 55 and 589 in response to .221-B3501 cells either pulsed with the indicated concentrations of the Pol peptide (*D* and *E*, respectively) or infected with HIV-1 at an E:T cell ratio of 1:1 (*F*). Data are shown as the means of duplicate assays for at least three independent experiments.

have more profound cytotoxic activity at low concentrations of the Pol peptide than CTL 55, although the difference was modest under the assay conditions tested (Fig. 1*A*). CTL 589 also showed substantial cytotoxic activities toward cells infected with vaccinia virus expressing HIV-1 Gag-Pol polyproteins (Fig. 1*B*) as well as toward those infected with HIV-1 (Fig. 1*C*). In sharp contrast, CTL 55 was not cytotoxic toward cells infected with either viruses, even at increased E:T cell ratios (Fig. 1, *B* and *C*). Addition of the Pol peptide to the culture medium restored the cytotoxic activity of CTL 55 toward target cells (Fig. 1*C*), indicating the cytotoxic potential of CTL 55 during the assay.

As observed in cytotoxic assays, intracellular cytokine staining experiments showed that both clones had substantial ability to produce IFN-γ (Fig. 1*D*) and TNF-α (Fig. 1*E*) in response to the Pol peptide-loaded cells, although CTL 55 responded to a lesser extent than CTL 589. In response to HIV-infected cells, CTL 55 did not show production of IFN-γ or TNF-α (Fig. 1*F*), whereas CTL 589 produced both cytokines, confirming the impaired responsiveness of CTL 55 toward HIV-infected cells. Moreover, the other Pol peptide-specific CTL clones, 349 and 562, that had TCRs identical with CTL 55 and 589, respectively, showed a similar pattern of functional differences as that observed for CTL 55 and 589 (data not shown), suggesting that the impaired responsiveness toward HIV-infected cells was an inherent property of certain T cell subsets specific to the Pol peptide, rather than of a particular CTL clone.

Different proliferation capacities between the tetramer$^+$ CD8 T cell subsets in response to HIV-infected cells ex vivo

To further investigate the functional difference between these CD8$^+$ T cells toward HIV-infected cells, we examined the ex vivo proliferation capacity of these CD8 T cells in response to HIV-infected cells, because the Pol peptide-specific T cell clonotypes corresponding to CTL 55 and 589 can be exclusively stained by anti-TCR Vδ1 (21) and Vα12 Abs in association with the tetramer. In addition, TCR analysis of CD8$^+$tetramer$^+$ cells of the patient, followed by cell sorting, revealed that all TCR α transcripts that carried either Vδ1- or Vα12-encoded region had the CDR 3α sequence identical with that of TCR 55α or 589α, respectively (data not shown), confirming the T cell clonality and Ag specificity of tetramer$^+$ cells in the flow cytometric analysis.

Firstly, direct analysis of unstimulated peripheral lymphocytes of the HIV-infected patient ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.1% of the total CD8 T cell population and that the Vδ1$^+$ and Vα12$^+$ subsets were 75 and 8%, respectively, within this tetramer$^+$ fraction (Fig. 2*B* and Table I), whereas analysis of peripheral lymphocytes of the HIV-negative donors ex vivo revealed that the frequency of tetramer$^+$ CD8 T cells was ~0.02% of the total CD8 T cell population, and that the Vδ1$^+$ and Vα12$^+$ subsets were <5% within this tetramer$^+$ fraction (Fig. 2*A*).

Peripheral lymphocytes of the HIV-infected patient were then stimulated with cells that had been either pulsed with the Pol peptide or infected with HIV-1 and cultured for 12 days. When stimulated with the Pol peptide, frequencies of the Vδ1$^+$ and Vα12$^+$tetramer$^+$ CD8 T cell subsets in the total CD8 T cells were increased 7- and 30-fold (Table I and Fig. 2*D*), respectively, indicating that both subsets had substantial proliferation capacity in response to the Pol peptide-loaded cells. This observation was consistent with our previous finding that CTL 55 and 589 had been generated by repeated stimulation of the patient's lymphocytes by the Pol peptide. In contrast, when stimulated with HIV-infected cells, the frequency of the Vα12$^+$tetramer$^+$ CD8 T cell subset was increased >70-fold, whereas the frequency of the Vδ1$^+$tetramer$^+$ CD8 T cell subset was decreased (Fig. 2*E* and Table I), indicating that the Vδ1$^+$tetramer$^+$ CD8 T cell subset could not respond to HIV-infected cells. Noticeably, the frequency of the tetramer$^+$ CD8 T cell subset, without any Ag stimulation, was virtually constant after a 12-day culture period (Fig. 2*C* and Table I), confirming the Ag-specific proliferation response of the Vα12$^+$tetramer$^+$ subset. It is also of interest that the Vα12$^+$ cells showed more profound proliferation when stimulated by cells infected with HIV than when pulsed with the Pol peptide (Fig. 2 and Table I), suggesting a qualitative difference in Ag presentation to T cells between infected cells and peptide-loaded cells.

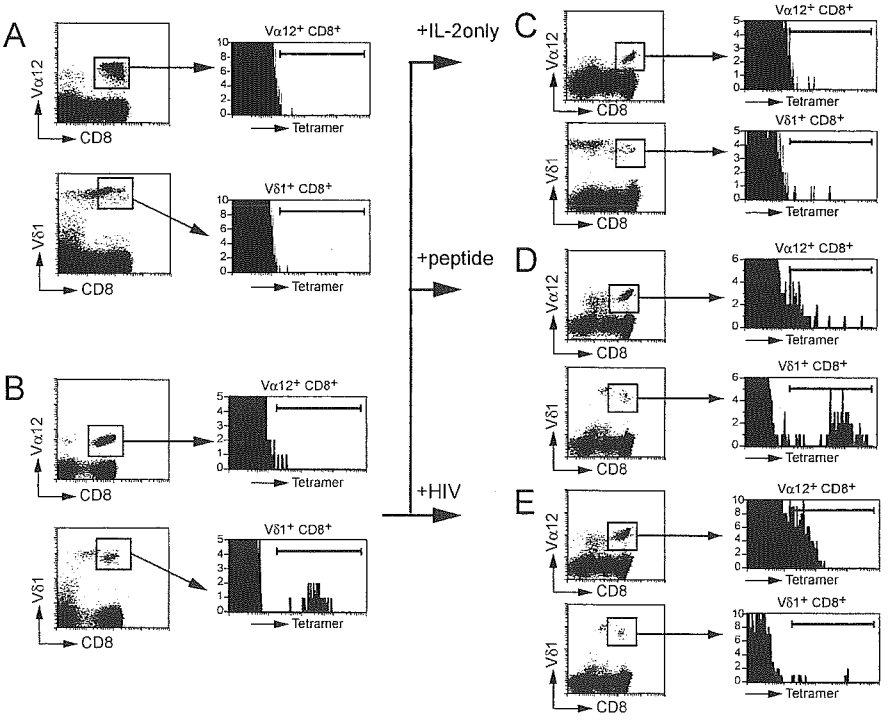

FIGURE 2. Ex vivo analysis of the tetramer$^+$ CD8 T cells in the peripheral lymphocytes in response to Ag stimulation. Cryopreserved PBMC of a representative HIV-negative donor (5×10^6; *A*) and the HIV-infected patient (1×10^6; *B*) were stained with the tetramer and anti-CD8 and anti-TCR Abs. The PBMC of the HIV-infected patient after stimulation with IL-2 alone (*C*) or with .221-B*3501 cells either pulsed with the Pol peptide (*D*) or infected with HIV-1 (*E*) were stained as described above. The Vδ1$^+$ and Vα12$^+$ CD8$^+$ subsets were gated, then analyzed for their levels of tetramer binding. Similar data were obtained in a separate independent experiment. The frequencies and MFI values of the tetramer$^+$ cells in each subset of the HIV-infected patient are summarized in Table I.

Table I. *Summary of ex vivo flow cytometric analysis of tetramer$^+$ CD8$^+$ cells*[a]

Subset	Before Stimulation		+IL-2 Only		+Peptide-Pulsed Cells		+HIV-Infected Cells	
	Cell number	MFI	Cell number	MFI	Cell number	MFI	Cell number	MFI
CD8$^+$Tet$^+$	113	173	46	83.9	1025	525	863	128
CD8$^+$Tet$^+$Vα12$^+$	10	50.9	13	51.9	310	123	755	97.2
CD8$^+$Tet$^+$Vδ1$^+$	86	291	20	105	502	710	12	705

[a] Cell number is the number of cells in the indicated subset per 1×10^5 total CD8 T cells. Mean fluorescence intensity (MFI) for the tetramer in each tetramer$^+$ (Tet$^+$) subset is also shown.

Distinct binding kinetics in HIV-specific CD8 T cell subsets to tetramer

During the flow cytometric analysis, we noticed that the Vα12$^+$ and Vδ1$^+$ subsets had different binding activities toward the tetramer (Fig. 2*D*, for example). Staining CTL clones with the tetramer showed that Vδ1$^+$ CTL 55 had >2-fold greater binding activity than Vα12$^+$ CTL 589 (Fig. 3), whereas both clones showed comparable surface expression of the CD3 Ags (Fig. 3), indicating that the apparent difference in tetramer binding was not due to the surface density of the TCR/CD3 complex. Consistent with this, when the tetramer$^+$ cells were divided into two subsets in the ex vivo flow cytometric analysis, the Vδ1$^+$ cells were mostly found in the tetramer^{high} subset, whereas the Vα12$^+$ cells were in the tetramer^{low} subset (Fig. 4*A*). These observations are interesting, because the extent of T cell activation is known to be sensitive to subtle differences in the duration of TCR-ligand interactions (12, 13, 17).

Because large temperature effects on TCR-pMHC interactions have been noted (12, 25, 26), we first examined the effect of reaction temperature on binding activity toward the tetramer. Pol peptide-stimulated lymphocytes were stained with various concentrations of the tetramer at 4 or 37°C, and the mean fluorescence intensity (MFI) of both subsets was determined by flow cytometry. At either temperature, the Vδ1$^+$CD8$^+$tetramer$^+$ cells showed ~3-fold higher MFI for the tetramer than the corresponding Vα12$^+$ cells (Fig. 4*B*), indicating that both T cell subsets could bind the tetramer with sufficient sensitivity and specificity.

We next examined the kinetic interactions of the tetramer with the Vδ1$^+$ or Vα12$^+$ subset at 4°C, because the level of tetramer binding and its half-life were previously shown to correlate with monomer affinity and off-rate, respectively (13, 27). Although the association of the tetramer with the two subsets was not significantly different (Fig. 4*C*), the dissociation of the tetramer from Vδ1$^+$ cells substantially delayed compared with that from Vα12$^+$ cells (Fig. 4*D*). The half-lives of the tetramer dissociation from the Vδ1$^+$ and Vα12$^+$ subsets were calculated to be 30.0 ± 1.6 and 8.1 ± 0.4 min, respectively. These results suggest that the >3-fold longer half-life of the Vδ1$^+$ subset for interaction with the tetramer than that of the Vα12$^+$ subset was a cause of the impaired antiviral effector functions of the Vδ1$^+$ subset.

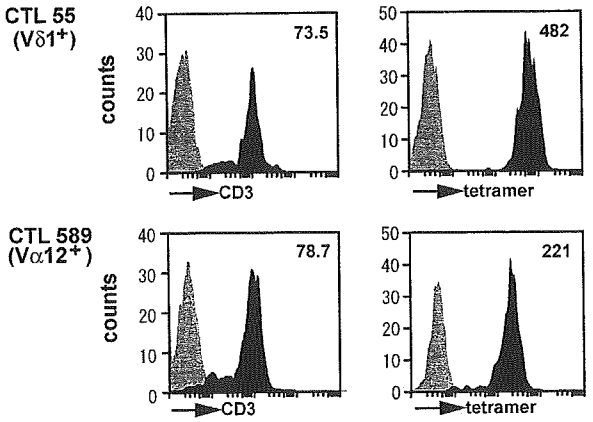

FIGURE 3. Flow cytometric analysis of CTL clones. Two CTL clones, CTL 55 and 589, selected from the Vδ1$^+$ and Vα12$^+$ CD8 T cell subsets, respectively, were stained (■) or not stained (▨) with anti-CD3 mAb or the tetramer at 4°C, and analyzed by flow cytometry. MFI values are shown in the *upper right* corners.

Impaired responsiveness of CD8 T cells to HIV-infected cells solely caused by TCR

To clarify whether the high affinity interaction with the tetramer and the impaired responsiveness to HIV-infected cells observed in the Vδ1$^+$tetramer$^+$CD8 T cell subset were caused by the ligand recognition property of the TCR, we transduced human primary CD8 T cells with both TCR genes separately.

Both α and β TCR genes for CTL 55 (Vδ1.1/Vβ13.3) and 589 (Vα12.1/Vβ5.6) were cloned into a pGC-based retrovirus vector with the gene encoding murine CD24 or GFP downstream of IRES for bicistronic expression of α or β TCR genes, respectively (Fig. 5*A*). Human primary CD8 T cells prepared from an HIV-negative donor carrying *HLA-B**3501 were sequentially transduced with α and β TCR genes and analyzed by flow cytometry. As shown in Fig. 5*B*, tetramer$^+$CD8$^+$ subsets reached 15 and 34% of the total lymphocytes for 55 TCR and 589 TCR-transduced cells, respectively, whereas the tetramer$^+$CD8$^+$ subset remained 0.05% of the total lymphocyte population in the case of mock-transduced cells. As measured by the MFI values for the tetramer, the tetramer$^+$CD8$^+$ fraction (gated cells in Fig. 5*B*) of 55 TCR-transduced cells appeared to have 2.8-fold greater binding activity toward the tetramer than that of the 589 TCR-transduced ones. In contrast, both tetramer$^+$CD8$^+$ fractions showed comparable MFI values for CD24 and GFP (within 1.5-fold; Fig. 5, *B* and *C*), which should reflect the expression levels of α and β TCR genes, respectively. These data indicate that the difference in tetramer binding activity observed between the Vδ1$^+$ and Vα12$^+$tetramer$^+$CD8 T cells was solely due to their TCR.

The tetramer$^+$CD8$^+$ fractions of the TCR-transduced cells were then sorted and tested for their cytotoxic activity toward cells either pulsed with the Pol peptide or infected with HIV-1. Both 55 and 589 TCR-transduced cells showed substantial cytotoxic activity toward peptide-loaded cells (Fig. 6*A*), and the activities were as potent as those of their parental CTL clones (cf., Figs. 1*A* and 6*A*). Also, both transduced cells showed modest differences in cytotoxic activities at low concentrations of the Pol peptide (Fig. 6*A*), consistent with the observations made on parental CTL clones (Fig. 1*A*). In contrast, the 55 TCR-transduced cells did not show cytotoxic activity toward HIV-infected cells, whereas 589 TCR-transduced ones killed cells infected with HIV-1 (Fig. 6*B*), again in agreement with the observations made on the parental CTL clones (Fig. 1*C*). These data strongly support our conclusion that the different cytotoxic activities toward HIV-infected cells observed in