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Identification and characterization of HIV-1-specific CD8+ T cell epitopes presented by HLA-A*2601

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Abstract

Since HLA-A*26 is one of the most common alleles in Asia, where approximately 20% of people have this allele, identification of HIV-1-specific epitopes presented by HLA-A*26 is necessary for studies on the immunopathogenesis of AIDS and vaccine development in Asia. As presented herein, we used the reverse immunogenetics approach to identify HIV-1 epitopes presented by HLA-A*2601, one of the major HLA-A*26 subtypes. We selected 24 HLA-A*2601-binding peptides out of 110 HIV-1 peptides by using a HLA-A*2601 stabilization assay. The ability of these HLA-A*2601-binding peptides to induce peptide-specific CD8+ T cells was tested by stimulating PBMCs from HIV-1-infected individuals having HLA-A*2601 with these peptides. Four HLA-A*2601-binding peptides induced peptide-specific CD8 T cells. Analysis using HIV-1 recombinant vaccinia-infected C1R-A*2601 cells indicated that these four peptides were HIV-1 epitopes endogenously presented by HLA-A*2601. Two epitope-specific CD8+ T cells were predominantly detected in HIV-1 infected individuals, suggesting that these epitopes may be useful for vaccine development.
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1. Introduction

In acute and chronic phases of human immunodeficiency virus type-1 (HIV-1) infection, an HIV-1-specific cytotoxic T lymphocyte (CTL) response is effectively induced [1–3]. Several studies have provided direct evidence for high levels of HIV-1-specific CTLs in patients in whom HIV-1 replication is controlled [4,5], suggesting that CTLs may control HIV-1 replication. Therefore, HIV-1 vaccine development and therapy to induce HIV-1-specific CTL might be expected to prevent HIV-1 infection and the development of AIDS.

On the other hand, it is believed that HIV-1 escapes from the host immune system. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific CD8+ T cells [6–11]. A mutation within the viral epitopes recognized by CTL is one of these mechanisms [7]. Therefore, identification and characterization of such epitopes are necessary for studies on vaccine development and immunopathogenesis of AIDS. We previously showed a strategy to determine HIV-1 epitopes by testing whether HIV-1-specific CTLs are induced in PBMCs from HIV-1-seropositive individuals by stimulating the cells with HLA class I-binding HIV-1 peptides [12,13]. Subsequent studies employing this strategy, which is called reverse immunogenetics, identified a large number of HIV-1 epitopes presented by HLA-A*1101, HLA-A*2402, HLA-A*3303 and HLA-B*5101 [14–19].

HLA-A*26 is one of the most common alleles in Asian countries, where approximately 20% of the people have this allele. Although 20 HLA-A*26 subtypes from A*2601 to

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A*2620 have been reported, A*2601, A*2602 and A*2603, are predominantly found in Asian countries including Japan [20,21]. Therefore, identification of HIV-1 epitopes presented by these alleles is required for studies on AIDS pathogenesis and vaccine development in Asia. Since HLA-A*2601 is the most frequently found HLA-A*26 subtype [20,21], we first focused on identification of HIV-1 epitopes presented by this subtype. The strategy of reverse immunogenetics was used to identify HLA-A*2601-restricted epitopes. Peptide-specific $CD8^+$ T cells were measured by counting IFN-γ-producing $CD8^+$ T cells after stimulating PBMCs from HIV-1-infected HLA-A*2601^+ individuals with HLA-A*2601-binding HIV-1 peptides. $CD8^+$ T cell epitopes were finally identified by testing whether peptide-specific $CD8^+$ T cells produced IFN-γ after stimulation with HIV-1 recombinant (r-HIV-1) vaccinia-infected HLA-A*2601^+ cells. We herein describe 4 HLA-A*2601-restricted HIV-1 epitopes identified by using this reverse immunogenetics technique.

2. Materials and methods

2.1. Cells

Cells of C1R and TAP-defective mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2601 (C1R-A*2601) were generated by transfecting the C1R cells with the HLA-A*2601 gene. RMA-S transfectants expressing HLA-A*2601 (RMA-S-A*2601) were previously generated [22]. C1R-A*2601 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A* 2601, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

2.2. Synthetic peptides

Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2601 binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

2.3. HLA-stabilization assay

RMA-S-A*2601 cells express empty HLA-A*2601 on their cell surface when they are cultured at 26 °C. The surface expression of empty HLA-A*2601 rapidly decreases after RMA-S-A*2601 cells are incubated at 37 °C, whereas HLA-A*2601 molecules are stably expressed on the surface of the cells at 37 °C if they bind peptides. Binding of HIV-1 derived peptides to HLA-A*2601 was measured as previously described by using RMA-S-A*2601 cells [22]. Briefly, RMA-S-A*2601 cells were cultured at 26 °C for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [23] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, California, USA). HLA-A*2601-binding peptides were defined as those which at a concentration of 10^{-3} M caused >25% increase in MFI compared with the MFI of control RMA-S-A*2601 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value. Binding peptides were classified into three categories according to their BL_{50}: high binding ($BL_{50} < 10^{-5}$), medium binding ($10^{-5} \leq BL_{50} < 10^{-4}$), and low binding ($BL_{50} \geq 10^{-4}$). High-, medium-, low- and non-binding peptides were ranked as 3, 2, 1 and 0, respectively. The mean binding rank (MBR) of a group of peptides was then calculated. For example, if in a group of 10 peptides, three were high binding, one was medium binding, one was low binding and five non-binding, then the MBR is 12/10 = 1.20. The MBR of each amino acid group and each peptide length were analyzed by the Mann-Whitney *U*-test (Stat View 4.02; Abacus Concepts, Berkeley, CA, USA).

2.4. Patients

Blood samples were collected with informed consent from seven HIV-1 infected patients with HLA-A*2601 (KI-098 with acute HIV-1 clade B infection, and KI-003, KI-134, KI-034, KI-060, KI-123 and KI-125 with chronic HIV-1 clade B infection).

2.5. Detection of IFN-γ-producing $CD8^+$ T cells after stimulation of PBMCs with peptide-pulsed C1R-A*2601 cells

After C1R-A*2601 cells had been incubated for 60 min with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed C1R cells (8×10^4 per well) and cultured PBMC cells (2×10^4 per well) were added to a 96-well round-bottomed plate, which was incubated for 2 h. Subsequently, Brefeldin A (10 μg/ml) was then added and incubation was continued for an additional 4 h. Next the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized with PBS containing 20% newborn calf serum (Summit Biotechnology, Greely, Co.) and 0.1% saponin (permeabilizing buffer) at 4 °C for 10 min. Thereafter the cells were resuspended in permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing

2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by flow cytometry.

2.6. Detection of IFN-γ-producing $CD8^+$ T cells after stimulation with C1R-A*2601 cells infected with recombinant HIV-1 vaccinia

C1R-A*2601 cells were infected with 10 plaque-forming units of recombinant vaccinia virus expressing a given protein (Gag and Pol, Nef, or Env) or WT vaccinia virus per target cells at 37 °C for 1 h, and then cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells in a 96-well round-bottomed plate at 37 °C for 2 h. The activities of the effector cells to produce IFN-γ were tested at an E:T ratio of 1:4. Brefeldin A (10 μg/ml) was added, and then incubation was continued for an additional 4 h. The cells were thereafter stained with anti-CD8 mAb, fixed with 4% paraformaldehyde at 4 °C for 20 min, and incubated at 4 °C for 10 min in the permeabilizing buffer. They were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb. The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by flow cytometry.

2.7. Generation of cytotoxic T lymphocyte (CTL) clones

Gag169–177-specific, Pol604–612-specific, Pol647–656-specific, and Env464–473-specific CTL clones were generated from HIV-1-specific bulk cultured T cells by limiting dilution in U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (about 1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-A*2601 cells pre-pulsed with the corresponding peptide at 1 μM) in RPMI 1640 supplemented with 10% human plasma and 200 U/ml human recombinant IL-2(rIL-2)(Ajinomoto, Tokyo, Japan).

2.8. CTL assay

Cytotoxicity was measured by use of the standard ^{51}Cr release assay. Target cells (5×10^5) were incubated for 60 min with 150 μCi $Na_2{}^{51}CrO_4$ in saline, and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (5×10^3/well) were added to each well of a U-bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark) with the desired amount of the corresponding peptide and were incubated for 1 h at 37 °C. Effector cells were added and the mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% TritonX-100. Percent specific lysis was calculated as follows: percentage specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells.

3. Results

3.1. Identification of HLA-A*2601-binding peptides from HIV-1 peptides carrying HLA-A*2601-binding motif

A previous study revealed that HLA-A*2601-binding peptides have two anchor residues, one at position 2 and the other at the C-terminus [24]. Five (Val, Thr, Ile, Leu and Phe) and 2 (Tyr and Phe) amino acids prevail at position 2 and the C terminus, respectively. Our recent study using an HLA-A*2601 stabilization assay demonstrated that acidic amino acids, Asp and Glu, and a broad range of amino acids with the exception of positively charged ones function as anchors at position 1 and the C-terminus, respectively [22]. Therefore, we chose the sequences of 8-mer to 11-mer containing these anchor residues at position 1, position 2 and the C-terminus from the sequences of Gag, Pol, Nef and Env proteins in the HIV-1 SF2 strain. One hundred-ten peptides matched to these sequences were synthesized. The binding of these synthetic peptides to HLA-A*2601 was then tested by the HLA-stabilization assay using RMA-S-A*2601 cells. A representative result for peptides with high ($BL50 < 10^{-5}$), medium ($10^{-5} \leq BL50 < 10^{-4}$), and low affinity ($BL50 \geq 10^{-4}$) is shown in Fig. 1. Twenty-

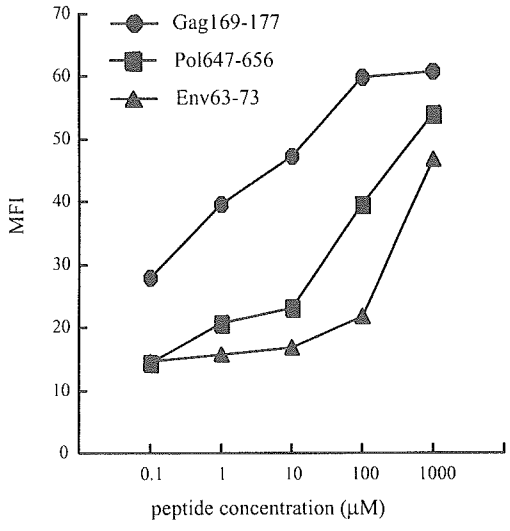

Fig. 1. Binding to HLA-A*2601 of HIV-1 peptides carrying HLA-A*2601 motif. Binding of the peptides carrying A*2601 anchors was measured by a stabilization assay using RMA-S-A*2601 cells. Representative results of binding peptides with high-(Gag169–177), medium-(Pol647–656), and low-(Env63–73) affinity are shown.

Table 1
HLA-A*2601-binding HIV-1 peptides

Sequence	Position	BL50 (M)
EVFRPGGGDM	Env464–473	1.5×10^{-6}
EVIPMFSAL	Gag169–177	2.9×10^{-6}
ETKLGKAGY	Pol604–612	4.1×10^{-6}
ETWEAWWMEY	Pol551–560	6.4×10^{-6}
EVHNVWATHA	Env63–72	1.1×10^{-5}
ELKKIIGQV	Pol872–880	1.9×10^{-5}
ETPGIRYQY	Pol293–301	2.7×10^{-5}
EVNIVTDSQY	Pol647–656	5.2×10^{-5}
ETINEEAAEW	Gag205–214	1.4×10^{-4}
EIYKRWIIL	Gag262–270	1.6×10^{-4}
EILGHRGWEA	Env782–791	2.6×10^{-4}
ETKLGKAGYV	Pol604–613	3.3×10^{-4}
ELYPLTSLRSL	Gag484–494	3.3×10^{-4}
EVVIRSDNF	Env272–280	3.3×10^{-4}
EVYYDPSKDLV	Pol471–481	3.9×10^{-4}
DTTNQKTEL	Pol626–634	4.0×10^{-4}
DVKNWMTETLL	Gag314–324	4.0×10^{-4}
EVNIVTDSQYA	Pol647–657	5.9×10^{-4}
ETGQETAYF	Pol807–815	6.0×10^{-4}
EVHNVWATHAC	Env63–73	$>1.0 \times 10^{-3}$
EICGHKAIGTV	Pol121–131	$>1.0 \times 10^{-3}$
DIISLWDQS	Env106–114	$>1.0 \times 10^{-3}$
EVIPLTEEA	Pol446–454	$>1.0 \times 10^{-3}$
DIVIYQYMDDL	Pol332–342	$>1.0 \times 10^{-3}$

four peptides bound to HLA-A*2601. They included four high-, four medium- and 16 low-affinity peptides (Table 1).

Twenty of seventy-two peptides (27.8%) carrying Glu at P1 bound to HLA-A*2601, whereas only 4 of 38 peptides (10.5%) carrying Asp at P1 bound to this allele (Table 2). This supports a previous study using only 38 peptides, which revealed higher binding ability of peptides carrying Glu at P1 than those carrying Asp at the same position [22]. In addition, peptides carrying Val and Thr at P2 exhibited higher affinity than those carrying Leu and Ile at the same position (Table 2), supporting also the results in a previous study using mutated peptides at position 2 [22].

3.2. Induction of HIV-1 peptide-specific CD8+ T cells from PBMCs of HIV-1-infected individuals with HLA-A*2601

PBMCs from three HIV-1-infected individuals with HLA-A*2601 (KI-003, KI-098 and KI-134) were cultured for 10–14 days after they had been stimulated with cocktails of HLA-A*2601-binding peptides. The cultured cells were then tested for IFN-γ production by $CD8^+$ T cells after stimulation with C1R-A*2601 cells pre-pulsed with the peptide cocktails (Table 3). Cocktail 1 induced a high number of the specific $CD8^+$ T cells in PBMCs from KI-098 and KI-134 and a low number of them in PBMC from KI-003. Cocktail 2 induced a high number of the specific $CD8^+$ T cells in PBMCs from KI-003 and KI-134, whereas cocktails 3, 4 and 5 induced a low number of the specific $CD8^+$ T cells in PBMCs from KI-098, KI-003 and KI-134, respectively. To determine which peptides in the cocktails induced the specific $CD8^+$ T cells, we stimulated the cultured cells with C1R-A*2601 cells pre-pulsed with each peptide contained in the cocktails. Env464–473, Pol604–612, Pol647–656 and Gag169–177 peptides induced the specific $CD8^+$ T cells in 1 (KI-003), 2 (KI-003 and KI-134), 1 (KI-003) and 2 (KI-098 and KI-134) individuals, respectively (Fig. 2).

Table 2
Effect of residues at P1 and P2 on the binding of peptides to HLA-A*2601

Amino acid	NBP[a]/NTP[b]	MBR[c]
Position 1		
E	20/72 (27.78%)	0.44
D	4/38 (10.53%)	0.11
Position 2		
V	10/24 (41.67%)	0.67
T	6/22 (27.28%)	0.55
L	2/37 (5.41%)	0.01
I	5/27 (18.52%)	0.19

[a] Number of binding peptides.
[b] Number of total peptides tested.
[c] Mean binding rank.

3.3. Identification of HIV-1-specific CD8+ T cell epitopes endogenously presented by HLA-A*2601

To clarify whether these peptides are endogenously presented in HIV-1-infected cells, we investigated the ability of these peptide-specific $CD8^+$ T cells to produce IFN-γ after stimulation of these cells with C1R-A*2601 cells infected with r-HIV-1 vaccinia. The cultures containing the four peptide-specific $CD8^+$ T cells significantly produced IFN-γ after stimulation with r-HIV-1 vaccinia-infected cells as

Table 3
Induction of peptide-cocktail-specific $CD8^+$ T cells in cultured cells stimulated with the peptide cocktail

Peptide cocktail	Percentage of IFN-γ-producing cells in $CD8^+$ T cells		
	KI-003	KI-098	KI-134
Cocktail 1 (Gag169–177, Pol551–560, Env464–473, Env63–72)	0.9	55.4	4.7
Cocktail 2 (Pol872–880, Pol293–301, Pol647–656, Pol604–612)	24.5	0	10.3
Cocktail 3 (Gag205–214, Gag262–270, Env782–791)	0	0.8	0.1
Cocktail 4 (Pol604–613, Gag484–494, Env272–280, Pol471–481)	2.5	0	0
Cocktail 5 (Pol626–634, Gag314–324, Pol647–657, Pol807–815)	0	0	0.8
Cocktail 6 (Env63–73, Pol121–131, Env106–114, Pol446–454, Pol332–342)	0	0	0

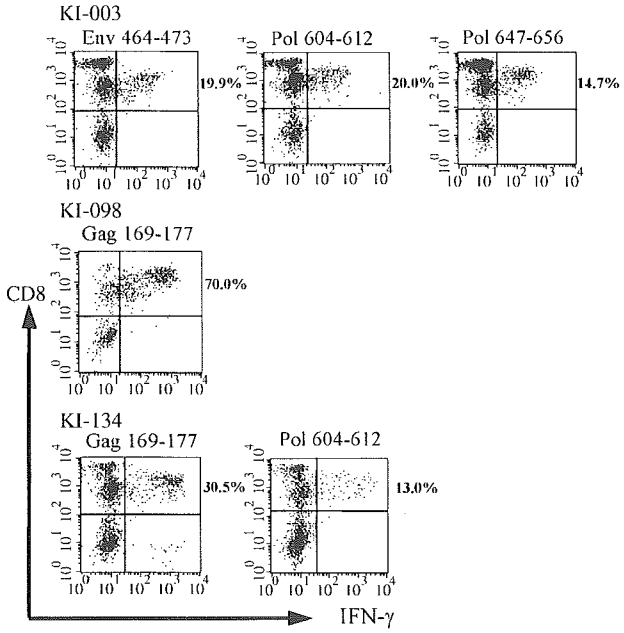

Fig. 2. Induction of HIV-1-specific CD8 T cells from PBMCs of HIV-1-infected individuals with HLA-A*2601. PBMCs from three HIV-1-infected individuals (KI-003, KI-098 and KI-134) were stimulated with cocktails of HLA-A*2601 binding peptides (Table 3), and were then cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2601 cells pre-pulsed for 6 h with the cocktail of HLA-A*2601 binding peptides. IFN-γ-producing $CD8^+$ T cells were measured by using flow cytometry. Cultured cells containing IFN-γ-producing $CD8^+$ T cells from three HIV-1-infected individuals were stimulated with C1R-A*2601 cells pre-pulsed with individual peptides included in the cocktails shown in Table 3. IFN-γ-producing $CD8^+$ T cells were measured by using flow cytometry. Percentage of IFN-γ-producing $CD8^+$ T cells is presented in each figure.

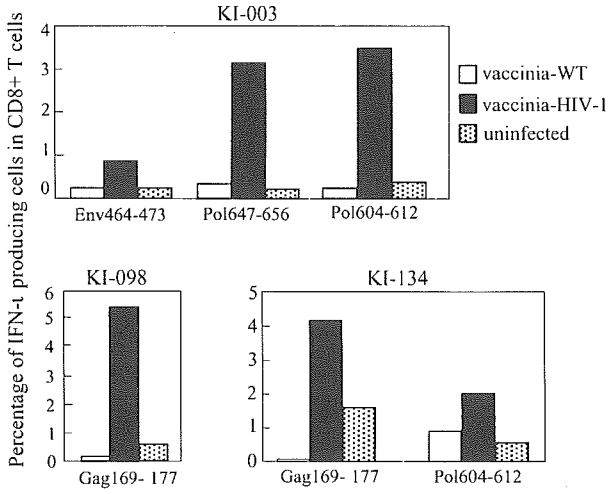

Fig. 3. Recognition of HLA-A*2601-restricted HIV-1 epitopes presented on r-HIV-1 vaccinia-infected cells. Cultured cells containing peptide-specific $CD8^+$ T cells shown, were examined for IFN-γ production after they had been stimulated with C1R-A*2601 cells infected with wild-type vaccinia (vaccinia-WT), those with r-HIV-1-vaccinia-infected C1R-A*2601(vaccinia-HIV-1), or uninfected C1R-A*2601 cells (uninfected).

compared with those stimulated with WT vaccinia-infected cells (Fig. 3). These results indicate that Gag169–177, Pol604–612, Pol647–656 and Env464–473 are endogenously presented in HIV-1-infected cells and recognized as $CD8^+$ T cell epitopes.

To confirm that these $CD8^+$ T cell epitopes are recognized by specific CTLs, we established CTL clones specific for these epitopes. Pol647–656-specific and Env464–473-specific CTL clones were established from patient KI-003, whereas Gag169–177-specific and Pol604–612-specific CTL ones were established from patients KI-098 and KI-125, respectively. These CTL clones effectively killed not only epitope–peptide-pulsed C1R-A*2601 cells but also C1R-A*2601 cells infected with recombinant HIV-1(r-HIV-1)-vaccinia (Fig. 4). These results show that the peptides were epitopes presented by the HLA-A*2601 and indicated that they were recognized as CTL epitopes by the specific CTLs.

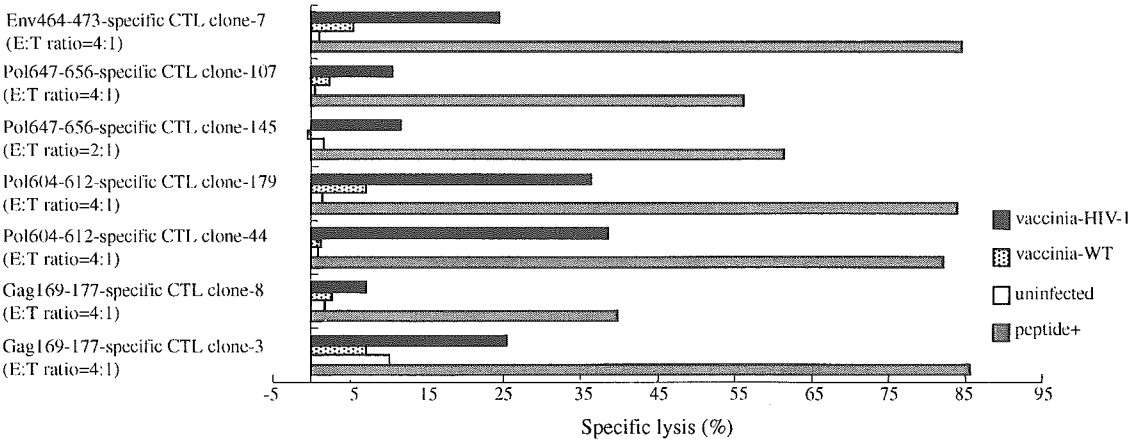

Fig. 4. Cytolytic activity of the HLA-A*2601-restricted CTL clones toward peptide-pulsed or r-HIV-1 vaccinia-infected cells. The activities of HLA-A*2601-restricted CTL clones toward C1R-A*2601 cells pre-pulsed with 1 μM epitope peptides (peptide+) or infected with recombinant vaccinia virus expressing the corresponding proteins, Gag and Pol, or Env (vaccinia-HIV-1), or wild-type vaccinia virus (vaccinia-WT) were tested at an effector-to-target (E:T) ratio of 2:1 or 4:1.

Table 4
Induction of epitope-specific CD8+ T cells in PBMCs from HIV-1-infected individuals

Patients[a]	Viral load[b]	CD4[c]	CD8[c]	Percentage of IFN-γ-producing cells in CD8+ T cells			
				Gag169–177	Pol604–612	Pol647–656	Env464–473
KI-003	3.1×10^3	262	3469	0.1	3.5	3.2	1.1
KI-098	2.2×10^2	981	740	5.3	0.1	0.1	0.2
KI-134	3.7×10^5	422	1545	4.1	0.4	0.7	0.4
KI-123	6.6×10^4	406	1328	2.7	1.2	0.2	0.5
KI-060	8.4×10^3	542	1085	0.5	ND	0.5	0.5
KI-125	2.6×10^4	258	115	4.3	10.6	0.7	0.3
KI-034	2.2×10^4	242	997	4.1	2.9	0.2	0.3

[a] HIV-1-infected individuals with HLA-A*2601.
[b] Copy/ml.
[c] Cell/μl.

*3.4. Gag169–177- and Pol604–612-specific CD8+ T cells are predominantly found in HIV-1-infected individuals with HLA-A*2601*

To clarify whether CD8+ T cells specific for these epitopes were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601, we investigated the induction of the specific CD8+ T cells in PBMCs from 7 HIV-1-infected individuals by stimulating them with these epitope peptides. Gag169–177- and Pol604–612-specific CD8+ T cells (more than 1% of total CD8+ T cells) were found in five and four, respectively, of the seven HIV-1-infected individuals (Table 4). In contrast, Env464–473- and Pol647–656-specific CD8+ T cells were induced in only one of these seven individuals. These results suggest that Gag169–177 and Pol604–612 are predominantly recognized in most HIV-1-infected individuals with HLA-A*2601.

4. Discussion

A previous study that analyzed the sequences of self-peptides eluted from HLA-A*2601 molecules identified the motifs of HLA-A*2601-binding peptides (P2: Val, Thr, Ile, Phe, and Leu, C terminus: Phe and Tyr, Ref. [24]). A subsequent study using an HLA-A*2601 stabilization assay confirmed the anchor residues at position 2 and the C-terminus by using mutated peptides at position 2 and the C-terminus, and further revealed by using 38 peptides that Glu/Asp and non-polar amino acids are preferred at position 1 and the C-terminus, respectively [22]. In the present study using 110 eight- to eleven-mer peptides, we confirmed these anchor residues at positions 1, 2 and the C-terminus. These anchor residues are useful for identification of HLA-A*2601-restricted epitopes including those of viral antigens, tumor antigens, and self-antigens by using reverse immunogenetics.

Although we employed a ^{51}Cr-release cytotoxic assay to identify peptide-specific CD8+ T cells in previous studies employing reverse immunogenetics [15–19], we used the IFN-γ-production assay for the present study. Since the peptide-stimulated, cultured PBMCs contain NK cells, they often show non-specific killing activity toward target cells such as C1R cells, which are sensitive to NK cells. On the other hand, the effect of NK cells is excluded in the IFN-γ-production assay, since peptide-specific CD8+ T cells can be specifically identified by using flow cytometry with anti-CD8 and anti-IFN-γ mAbs. Therefore, this assay is useful for identification of epitope-specific responses by HIV-1-specific CD8+ T cells. On the other hand, it is impossible to show whether these specific CD8+ T cells include cytotoxic T cells by the IFN-γ-production assay. We therefore generated epitope-specific T cell clones and tested whether these CTL clones could kill the target cells. The results showed that these epitopes were indeed recognized by specific CTLs.

The induction of the four epitope-specific CD8+ T cells varied among seven HIV-1-infected individuals carrying HLA-A*2601 (Table 4). Gag169-specific and Pol604-specific CD8+ T cells were found in five of seven and in four of six HIV-1-infected individuals, respectively. In contrast, Pol647-specific and Env464-specific CD8+ T cells were detected only in KI-003. These results suggest that Gag169-specific and Pol604-specific CD8+ T cells were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601. The induction of these HIV-1-specific CD8+ T cells was not correlated with viral load or the number of CD4+ or CD8+ T cells. Epitope mutation may be one factor for the failure to induce some specific CTLs in HIV-1-infected individuals. However, it is difficult to conclude that this would account for all cases where specific CTLs are not induced in HIV-1-infected individuals, because no mutation was detected in some cases [25].

We searched reported HIV-1 sequences of HIV-1 clade A–E (HIV sequence database, Los Alamos, New Mexico, USA) for major variants of these epitopes. The sequences of Gag169–177 (EVIPMFSAL) and Pol604–612 (ETKLGK-AGY) were found in 35 of 36 HIV-1 clade B isolates and in 25 of 33 HIV-1 clade B isolates, respectively (Table 5), indicating that the sequences are relatively conserved in clade B. Since CTLs specific for these epitopes were frequently induced in HIV-1-infected individuals with HLA-A*2601, they may be useful for making a vaccine to induce specific CTLs. These sequences were also conserved in clades A, D and E (Table 5), implying that these sequences are epitopes

Table 5
Variation of HLA-A*2601-restricted epitopes in clades A–E

Epitope	Sequence	Clade A	Clade B	Clade C	Clade D	Clade E
Gag 169-177	EVIPMFSAL	8/11	35/36	1/28	5/5	8/9
	------T--	2/11	1/36	25/28		
	----V----	1/11				
	-I----T--			1/28		
	----I-T--			1/28		
	------P--					1/9
Pol 604-612	ETKLGKAGY	11/11	25/33	7/26	4/5	9/9
	---I-----		1/33	8/26	1/5	
	D--I-----			2/26		
	---VK----		1/33			
	--R------		2/33			
	---K-----		1/33			
	D--S-----		1/33			
	D--------		1/33			
	-----R---		1/33			
	---M-----			2/26		
	-I-M-----			1/26		
	--------C			1/26		
	---K-----			1/26		
	---V-----			1/26		
	-S-I-----			1/26		
	--RI-----			1/26		
	D--K-----			1/26		

in these clades as well. Indeed, our previous study revealed that clade B CTL epitopes, whose sequences were conserved between clades B and E, were recognized as epitopes by CTLs in clade E-infected individuals [26]. That study also revealed that mutants of clade B epitopes, which were predominantly found in clade E, were recognized as CTL epitopes in clade E-infected individuals. The Gag169–177 mutant carrying Thr at position 7 was the consensus sequence in clade C, whereas the Pol604–612 mutant carrying Ile at position 4 was predominantly found in this clade (Table 5). Therefore, these mutants might be recognized as T cell epitopes in clade C-infected individuals. In Asian countries where clades C and E HIV-1 are prevalent in addition to clade B, HLA-A*2601 is one of the commonly found alleles. Identification of HLA-A*2601-restricted HIV-1 epitopes in clades C and E would also be useful for HIV-1 vaccine development in Asia.

In conclusion, we identified 4 HLA-A*2601-resticted $CD8^+$ T cells epitopes by using reverse immunogenetics in the present study. These four epitopes will be useful for studies on the immunopathogenesis of AIDS in HIV-1 clade B-infected individuals. Two epitopes in particular, Gag169–177 and Pol604–612, are promising for the development of an HIV-1 vaccine, since $CD8^+$ T cells specific for these epitopes were predominantly induced in HIV-1-infected individuals bearing HLA-A*2601.

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Patterns of Cytokine Production in Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Human CD8$^+$ T Cells after Stimulation with HIV-1-Infected CD4$^+$ T Cells

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Although human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells can produce various cytokines that suppress HIV-1 replication or modulate anti-HIV-1 immunity, the extent to which HIV-1-specific CD8$^+$ T cells produce cytokines when they recognize HIV-1-infected CD4$^+$ T cells in vivo still remains unclear. We first analyzed the abilities of 10 cytotoxic T-lymphocyte (CTL) clones specific for three HIV-1 epitopes to produce gamma interferon, macrophage inflammatory protein 1β, and tumor necrosis factor alpha after stimulation with epitope peptide-pulsed cells. These CTL clones produced these cytokines in various combinations within the same specificity and among the different specificities, suggesting a functional heterogeneity of HIV-1-specific effector CD8$^+$ T cells in cytokine production. In contrast, the HIV-1-specific CTL clones for the most part produced a single cytokine, without heterogeneity of cytokine production among the clones, after stimulation with HIV-1-infected CD4$^+$ T cells. The loss of heterogeneity in cytokine production may be explained by low surface expression of HLA class I–epitope peptide complexes. Freshly isolated HIV-1-specific CD8$^+$ T cells with an effector/memory or memory phenotype produced much more of the cytokines than the same epitope-specific CTL clones when stimulated with HIV-1-infected CD4$^+$ T cells. Cytokine production from HIV-1-specific memory/effector and memory CD8$^+$ T cells might be a critical event in the eradication of HIV-1 in HIV-1-infected individuals.

Memory and effector CD8$^+$ T cells play an important role in viral eradication through their ability to produce cytokines involved in the suppression of viral replication (6, 10, 15, 26) as well as perforin and granzymes A and B, which are involved in the cytolysis of virus-infected cells (16, 24). The cytokines produced by these cells include gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and chemokines such as RANTES and macrophage inflammatory protein 1β (MIP-1β). IFN-γ increases the surface expression of HLA molecules and can activate macrophages that predominantly synthesize MIP-1β (8, 25). TNF-α induces apoptosis of human immunodeficiency virus type 1 (HIV-1)-infected cells (17). It has been shown that MIP-1β and RANTES can suppress HIV-1 replication in vitro by inhibiting the entry of HIV-1 via CCR5, while IFN-γ induces cellular proteins which suppress viral replication (2, 6).

A previous study showed that HLA-A2-restricted, HCMVpp65$_{495-503}$-specific CD8$^+$ T cells expressed various combinations of three cytokines, IFN-γ, TNF-α, and interleukin 2 (IL-2), after peripheral blood mononuclear cells (PBMC) containing these cells from three individuals had been stimulated with HCMVpp65$_{495-503}$ peptide, suggesting that HCMVpp65$_{495-503}$-specific CD8$^+$ T cells possess functionally heterogeneous cytokine production (18). This functional heterogeneity may be due to the heterogeneous populations in HCMVpp65$_{495-503}$-specific CD8$^+$ T cells. Indeed, these cells for the most part are CD8$^+$ T-cell populations with a memory/effector or effector phenotype (9, 23). Additional studies have revealed the heterogeneity of the production of cytokines and cytolytic effector molecules in human CD8$^+$ T cells (21, 23).

It is well known that virus-specific CD8$^+$ T cells can produce cytokines when they recognize virus-infected cells (4, 13, 14). However, virus-specific CD8$^+$ T cells stimulated with virus-infected cells may not produce cytokines more effectively than those stimulated with cells pulsed with an epitope peptide, because the expression of HLA class I–viral epitope peptide complexes is much lower on virus-infected cells than on peptide-pulsed cells. In particular, HIV-1-specific CD8$^+$ T cells may not produce cytokines effectively when they recognize HIV-1-infected cells, because HLA class I molecules have been actively down-regulated, mostly, although not exclusively, by the Nef protein produced by HIV-1-infected cells (4, 7, 20). An analysis of the cytokine production from HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells is needed to elucidate whether HIV-1-specific CD8$^+$ T cells can recognize HIV-1-infected CD4$^+$ T cells in vivo and to allow the formulation of testable hypotheses on the role of the cytokines from HIV-1-specific CD8$^+$ T cells in the suppression of HIV-1 replication in vivo.

In the present study, we investigated the production patterns of three cytokines, IFN-γ, TNF-α, and MIP-1β, in HIV-1-specific effector CD8$^+$ T-cell clones of the same or different specificities. In addition, we investigated the production of the three cytokines in these cytotoxic T-lymphocyte (CTL) clones and freshly isolated HIV-1-specific CD8$^+$ T cells exposed to

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HIV-1-infected $CD4^+$ T cells whose HLA class I molecules are down-regulated by HIV-1 Nef. The present study elucidates the cytokine production profile of HIV-1-specific $CD8^+$ T cells in response to HIV-1-infected $CD4^+$ T cells.

MATERIALS AND METHODS

CTL clones. Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cell/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human IL-2, 5×10^5 irradiated allogeneic PBMC from a healthy donor, and 1×10^5 irradiated C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells prepulsed with 10^{-6} M of the corresponding peptide, Env77-85 [DPNPQEVVL] [19], Gag28-36 [KYKLKHIVW] [12], or Env830-837 [EVAQ RAYR] [11]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by a standard ^{51}Cr release assay. One Env830-837-specific, HLA-A*3303-restricted CTL clone (clone 1) had been generated previously (11). All CTL clones were cultured in RPMI 1640–10% FCS supplemented with 200 U of recombinant human IL-2/ml and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1 derived peptide.

Antibodies. The peridinin chlorophy protein-conjugated anti-human CD8 monoclonal antibody (MAb) and the ECD-conjugated anti-human CD45RA MAb were purchased from BD Biosciences (San Jose, CA) and the Immunotech Coulter Company (Marseille, France), respectively. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD27, phycoerythrin (PE)-conjugated anti-human IFN-γ, ECD-conjugated anti-human CD28, allophycocyanin (APC)-conjugated anti-human TNF-α, APC-conjugated anti-human IFN-γ, PE Cy7-conjugated anti-human TNF-α, and APC Cy7-conjugated anti-human CD27 MAbs were purchased from PharMingen (San Diego, CA). PE-conjugated anti-human CD28, APC-conjugated anti-human CD8, and FITC-conjugated anti-human MIP-1β MAbs were purchased from DAKO (Glostrup, Denmark). FITC-conjugated anti-HIV-1 p24 MAb KC-57 was purchased from Beckman Coulter (Miami, Fla.). A Cascade Blue-conjugated anti-human CD8 MAb was made by conjugating Cascade Blue (Molecular Probes, Eugene, OR) with the anti-CD8 MAb OKT8.

HLA-peptide tetrameric complexes. HLA class I–peptide tetrameric complexes were synthesized as previously described (3). Briefly, recombinant HLA class I proteins (HLA-A*2402, HLA-A*3303, and HLA-B*3501) and human β_2 microglobulin (β_2m) were produced in *Escherichia coli* cells transformed with the relevant expression plasmids. The heavy chain was modified by deletion of the transmembrane cytosolic tail and COOH-terminal addition of a sequence containing the BirA biotinylation site. Gag28-36, Env830-837, and Env77-85 peptides were used for refolding of the HLA-A*2402, HLA-A*3303, and HLA-B*3501 molecules, respectively. The HLA class I–peptide complexes were refolded in vitro. The 45-kDa complexes were isolated using gel filtration on a Superdex G75 column (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, England). Purified complexes were biotinylated with the BirA enzyme (Avidity, Denver, CO). The biotinylated complexes were purified by using gel filtration first on a Superdex G75 column and then on a MonoQ column (Amersham Pharmacia Biotech UK, Ltd, Buckinghamshire, England). HLA class I–peptide tetrameric complexes (tetramers) were mixed with PE-labeled streptavidin (extravidin-PE; Sigma-Aldrich, St. Louis, MO) at a molar ratio of 4:1.

Identification of HIV-1-specific CTLs by flow cytometry. A total of 0.2×10^6 to 1×10^6 cultured cells were mixed with the tetramers at concentrations of 0.02 to 0.04 mg/ml. After incubation at 37°C for 30 min, the cells were washed twice with RPMI 1640–10% FCS, and then an anti-CD8 MAb was added to the cell suspension. The cells were incubated at 4°C for 30 min, and then the cells were washed twice with phosphate-buffered saline (PBS)–10% FCS. The cells were analyzed using a FACSCalibur with CellQuest software (Becton Dickinson, San Jose, CA). The percentage of tetramer-positive cells was measured in CD8-positive cells.

Phenotype analysis of the CTL clones. Cells were stained for 30 min at 4°C using an FITC-conjugated anti-human CD27 MAb, a PE-conjugated anti-human CD28 MAb, an ECD-conjugated anti-human CD45RA MAb, and an APC-conjugated anti-human CD8 MAb; then they were washed twice in PBS supplemented with 10% NCS. The CD27 CD28 CD45RA phenotype of $CD8^+$ cells was analyzed using FACSCalibur.

Cell surface and intracellular cytokine staining. Specific CTL clones were stimulated with stimulator cells prepulsed with the appropriate HIV-1-derived peptide at each concentration at an effector-to-stimulator ratio of 1:1. Stimulator cells were washed in RPMI 1640–10% FCS before use. Cells were incubated for 6 h at 37°C in 5% CO_2. Brefeldin A (Sigma-Aldrich) at a concentration of 10 μg/ml was added 2 h after stimulation. After a 6-h incubation, the cells were washed in PBS supplemented with 20% NCS. Cell surface staining was performed for 30 min at 4°C using a PerCP-conjugated anti-human CD8 MAb; then cells were washed twice in PBS supplemented with 10% NCS. Freshly isolated $CD8^+$ cells from HIV-1-infected individuals were stained with tetramers after a 6-h incubation, followed by staining with ECD-conjugated anti-human CD28, APC Cy7-conjugated anti-human CD27, and Cascade Blue-conjugated anti-human CD8 MAbs.

After a wash, the cells were fixed with 4% paraformaldehyde for 20 min at 4°C and permeabilized with PBS supplemented with 0.1% saponin containing 20% NCS for 10 min at 4°C. The cells were resuspended in permeabilizing buffer and then were stained with a PE-conjugated anti-human IFN-γ MAb or an APC-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. When the cells were stained with all three MAbs, they were first stained with the PE-conjugated anti-human IFN-γ MAb and the APC-conjugated anti-human TNF-α MAb at room temperature for 20 min and then with the FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C. Freshly isolated $CD8^+$ cells were stained with an APC-conjugated anti-human IFN-γ MAb or a PE Cy7-conjugated anti-human TNF-α MAb at room temperature for 20 min or with an FITC-conjugated anti-human MIP-1β MAb for 30 min at 4°C.

Finally, the cells were washed three times with permeabilizing buffer and were resuspended in 2% paraformaldehyde. The percentages of intracellular IFN-γ-, MIP-1β-, and TNF-α-positive cells among tetramer-positive $CD8^+$ cells were analyzed using FACS Aria (Becton Dickinson, San Jose, CA).

Infection of $CD4^+$ T cells with HIV-1. Cultured $CD4^+$ T cells (purity, >98%) were incubated with an HIV-1 clone, NL-432 (1), or with the chimeric virus NL-432gag^{HXB2} for 4 h at 37°C with intermittent agitation. The cells were then washed once and cultured in RPMI 1640–10% FCS medium supplemented with recombinant human IL-2 (200 U/ml). On the following 2 to 7 days, the cells were harvested to determine the percentage of HIV-1-infected cells by measuring p24 antigen-positive cells using FACSCalibur. When HIV-1 p24-positive cells reached more than 40% of the cultured cells, they were used as stimulator cells.

CTL assay. Cytotoxicity was measured by a standard ^{51}Cr release assay as previously described (19). Target cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and washed three times with RPMI 1640 medium containing 10% NCS. Labeled target cells (2×10^3/well) were added into a 96-well round-bottom microtiter plate (Nunc) with the indicated amount of the corresponding peptide. After a 1-h incubation, effector cells were added and the mixtures were incubated 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 cpm in the supernatant in the wells containing only target cells (cpm spn). Maximum release (cpm 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 counts per minute in the supernatant in the wells containing both the target and effector cells. The activities of the CTL clones on target cells pulsed with peptide were tested at an effector-to-target (E:T) ratio of 2:1.

CTL assay for target cells infected with recombinant vaccinia virus. Recombinant vaccinia virus containing the *env* or the *gag/pol* gene of HIV-1 SF2 was generated as described previously (19). Target cells (C1R-A*2402, C1R-A*3303, and C1R-B*3501 cells) were cultured with 10 PFU of recombinant or wild-type vaccinia virus per target cell overnight. These infected cells (2×10^5) were incubated for 60 min with 100 μCi of $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% NCS. Effector cells were added to the labeled target cells (5×10^3/well), and the mixtures were incubated for 4 h at 37°C. The activities of the CTL clones on target cells infected with recombinant vaccinia virus expressing *env* proteins were tested at an E:T ratio of 2:1.

RESULTS

Production of three cytokines in HIV-1-specific CTL clones. Three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones, three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones, and a further four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones were established from HIV-1-infected individuals. These CTL clones exhibited specific cytolytic activity in both target cells (C1R-A*2402, C1R-A*3303, or

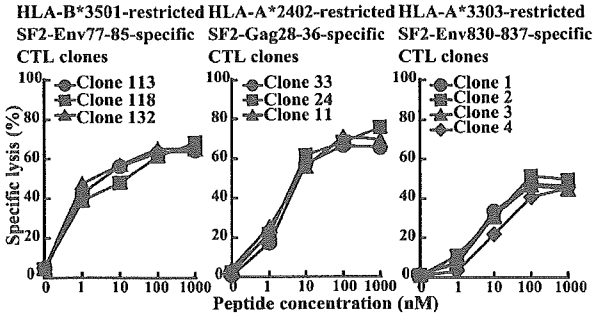

FIG. 1. Cytolytic activities of SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones. Cytolytic activities of three SF2-Env77-85-specific, HLA-B*3501-restricted CTL clones (clones 113, 118, and 132), three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11), and four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) were tested for C1R-B*3501, C1R-A*2402, and C1R-A*3303 cells pulsed with the corresponding peptides (1 to 1,000 nM), respectively. They were tested at an E:T ratio of 2:1.

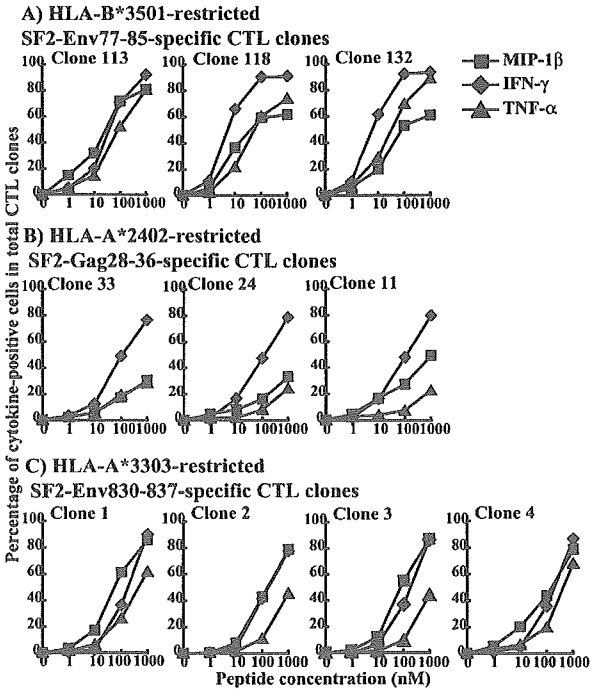

FIG. 2. Intracellular expression of cytokines in SF2-Env77-85-specific, SF2-Gag28-36-specific, and SF2-Env830-837-specific CTL clones responding to epitope peptide-pulsed cells. The 10 CTL clones were incubated for 6 h in the presence of C1R-B*3501, C1R-A*2402, or C1R-A*3303 cells pulsed with specific peptide. Brefeldin A was added 2 h after stimulation, and intracellular staining for IFN-γ, MIP-1β, and TNF-α was carried out using MAbs specific for each cytokine. Cytokine-producing cells were analyzed by flow cytometry. The frequency of each set of cytokine-producing $CD8^+$ cells was measured as the number of cytokine-producing $CD8^+$ cells per total $CD8^+$ cells.

C1R-B*3501 cells) prepulsed with a peptide epitope (Fig. 1) and target cells (C1R-A*2402, C1R-A*3303, or C1R-B*3501 cells) infected with HIV-1 recombinant vaccinia virus (data not shown). The results show no significant difference in cytotoxic activity between CTL clones specific for the same epitopes. We investigated the production of three cytokines, MIP-1β, IFN-γ, and TNF-α, from these HIV-1-specific CTL clones. Intracellular cytokine production by the clones was measured at 6 h after stimulation with the C1R transfectants prepulsed with the epitope peptide. Almost 100% of the cells produced at least one cytokine in all three of the SF2-Env77-85-specific CTL clones, all four of the SF2-Env830-837-specific CTL clones, and all three of the SF2-Gag28-36-specific CTL clones (Fig. 2). Different cytokine production patterns were found among three SF2-Env77-85-specific CTL clones though all of the clones produced IFN-γ more extensively than MIP-1β or TNF-α (Fig. 2A). Different cytokine production patterns were also found for the three SF2-Gag28-36-specific CTL clones (Fig. 2B) and the four SF2-Env830-837-specific CTL clones (Fig. 2C). Clone 11 exhibited much higher production of MIP-1β than of IFN-γ and TNF-α compared with clones 33 and 24. In addition, clones 2 and 3 exhibited much lower production of TNF-α than of IFN-γ and MIP-1β, while the difference in production between these cytokines was at a minimum for clones 1 and 4. These results were confirmed by performing the experiments twice. Thus, the results reveal heterogeneity in the production of these cytokines between CTL clones of the same as well as different specificities. To exclude contamination by T cells that are not epitope-specific CTLs, we stained the CTL clones with an epitope-specific HLA class I tetramer. More than 97% of the cells in nine CTL clones bound the epitope-specific tetramer, while only 92% of the cells bound to the specific tetramer in one (clone 33) of the SF2-Gag28-36-specific CTL clones (Table 1). However, this difference does not seem to be significant for the functions, since the three SF2-Gag28-36-specific CTL clones exhibited the same cytotoxic activity and IFN-γ production.

Since the T-cell clones used in the present study possess cytolytic activity (Fig. 1), they are thought to be mature effector $CD8^+$ T cells. To analyze the relation between the maturation stage of the CTL clone and cytokine production, we stained these CTL clones with anti-CD27, anti-CD28, and anti-CD45RA MAbs. Nine CTL clones were mostly of the $CD27^-$ $CD28^-$ $CD45RA^-$ type, while one (clone 2) of the SF2-Env830-837-specific CTL clones exhibited both $CD27^-$ $CD28^-$ $CD45RA^-$ and $CD27^+$ $CD28^-$ $CD45RA^-$ phenotypes (Table 1). Clone 2, stimulated with SF2-Env830-837 peptide, exhibited a much smaller number of TNF-α-producing cells than of IFN-γ-producing or MIP-1β-producing cells, but this is not a characteristic restricted to clone 2.

Coproduction of three cytokines by HIV-specific CTL clones stimulated with peptide-pulsed cells. The heterogeneity of the CTL clones shown in Fig. 2 suggests that the CTL clones are actually composed of cell populations which produce different combinations of the cytokines. To clarify this, we investigated the coproduction of these three cytokines in the CTL clones by simultaneously staining multiple intracellular cytokines with anti-IFN-γ, anti-MIP-1β, and anti-TNF-α MAbs. The results of the multiple staining of the three SF2-Env77-85-specific CTL clones are shown in Fig. 3A. The SF2-Env77-85-specific CTL clones stimulated with 1,000 nM of specific peptide revealed different patterns of cytokine production. Clone 113 included a high number of cells producing all three of the cytokines ($IFN\text{-}\gamma^+$ $TNF\text{-}\alpha^+$ $MIP\text{-}1\beta^+$) (72.0%). In contrast,

TABLE 1. CD27 and CD28 expression in HIV-1-specific CTL clones

CTL clone	% Tetramer-positive cells	% of total $CD8^+$ $CD45RA^-$ cells with the following phenotype:			
		$CD27^+$ $CD28^+$	$CD27^+$ $CD28^-$	$CD27^-$ $CD28^-$	$CD27^-$ $CD28^+$
Env77-85					
Clone 113	98.2	0.8	1.2	84.8	13.2
Clone 118	99.7	0.2	13.6	86.0	0.2
Clone 132	95.1	0.3	6.3	91.5	1.9
Gag28-36					
Clone 33	91.7	0.0	1.5	97.8	0.7
Clone 24	97.0	0.0	17.2	81.6	1.2
Clone 11	96.0	0.0	19.2	80.7	0.1
Env830-837					
Clone 1	99.5	0.0	13.0	86.0	1.0
Clone 2	99.7	0.0	46.1	53.9	0.0
Clone 3	99.7	0.1	8.9	89.8	1.2
Clone 4	99.2	0.0	0.2	99.8	0.0

clone 118 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (58.1%) and IFN-γ^+ TNF-α^+ cells (30.6%), while clone 132 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (54.7%), IFN-γ^+ TNF-α^+ cells (17.8%), and IFN-γ^+ cells (13.1%). This difference between these CTL clones became even more apparent when the CTL clones were stimulated with 10 nM of specific peptide. Clone 113 included MIP-1β^+ cells (16.1%) and IFN-γ^+ MIP-1β^+ cells (7.3%). In contrast, clone 118 included IFN-γ^+ cells (29.1%) and IFN-γ^+ TNF-α^+ cells (17.0%), while clone 132 included IFN-γ^+ cells (27.5%) and IFN-γ^+ MIP-1β^+ cells (20.6%). This difference was consistent over two different experiments performed on different days.

A similar analysis was performed on the four SF2-Env830-837-specific and the three SF2-Gag28-36-specific CTL clones. The seven CTL clones were stimulated with 1,000 nM of specific peptide (Fig. 3B and C). Among SF2-Gag28-36-specific CTL clones, clones 33 and 24 showed similar patterns of cytokine production. They included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (12 to 14%), IFN-γ^+ TNF-α^+ cells (11 to 13%), and IFN-γ^+ MIP-1β^+ cells (14 to 18%). In contrast, clone 11 included IFN-γ^+ TNF-α^+ cells (5%) and IFN-γ^+ MIP-1β^+ cells (34%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. The major populations of cytokine-producing cells were as follows: for clone 11, IFN-γ^+ cells (24.5%), IFN-γ^+ MIP-1β^+cells (15.4%), and MIP-1β^+cells (7.7%); for clone 33, IFN-γ^+ cells (28.0%), IFN-γ^+ MIP-1β^+cells (9.9%), and IFN-γ^+ TNF-α^+ cells (8.0%); and for clone 24, IFN-γ^+ cells (27.2%), IFN-γ^+ MIP-1β^+cells (9.1%), and IFN-γ^+ TNF-α^+ cells (7.6%). Among SF2-Env830-837-specific CTL clones, clones 1 and 4 showed similar patterns of cytokine production. These included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (50 to 60%), IFN-γ^+ TNF-α^+ cells (13 to 17%), and IFN-γ^+ MIP-1β^+ cells (12 to 14%). In contrast, clones 2 and 3 included IFN-γ^+ TNF-α^+ MIP-1β^+ cells (40 to 42%) and IFN-γ^+ MIP-1β^+ cells (35 to 37%). The difference became more complex when the clones were stimulated with a lower concentration (100 nM) of the peptide. At this concentration the major populations of cytokine-producing cells were as follows: for clone 1, MIP-1β^+ cells (17.6%) and IFN-γ^+ MIP-1β^+cells (10.9%); for clone 2, IFN-γ^+ MIP-1β^+ cells (19.1%), IFN-γ^+ cells (15.7%), and MIP-1β^+ cells (12.4%); for clone 3, MIP-1β^+ cells (20.8%), IFN-γ^+ MIP-1β^+ cells (20.6%), and IFN-γ^+ cells (10.9%); and for clone 4, IFN-γ^+ cells (15.1%), IFN-γ^+ MIP-1β^+ cells (12.0%), and IFN-γ^+ TNF-α^+ MIP-1β^+ cells (10.1%). These differences were also confirmed by two different experiments performed on different days.

Cells expressing all three cytokines were most frequently found in the CTL clones stimulated with the stimulator cells prepulsed with 1,000 nM peptide, while cells expressing either two or all three of the cytokines were not found in the CTL clones stimulated with the stimulator cells prepulsed with 1 nM peptide (Fig. 4). The CTL clones stimulated with stimulator cells prepulsed with 1 nM peptide predominantly produced a single cytokine. These results indicate that CTL clones produce multiple cytokines when they are stimulated with cells prepulsed with a high concentration of an HLA–epitope peptide complex but produce a single cytokine when they are stimulated with cells prepulsed with a low concentration. This implies that HIV-1-specific CTL clones produce a single cytokine when they are stimulated with HIV-1-infected cells because a small number of HIV-1 CTL epitope peptides is presented in HIV-1-infected cells.

Coproduction of three cytokines by HIV-1-specific CTL clones stimulated with HIV-1-infected $CD4^+$ T cells. Nef-mediated down-regulation of HLA class I critically affects the ability of HIV-1-specific CTLs to kill HIV-1-infected cells; this could be the result of a lowered frequency of cytokine-producing cells after stimulation with HIV-1-infected $CD4^+$ T cells. Indeed, our previous study showed that the lower frequency of HIV-1-specific CTL clones produced fewer cytokines by stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells than by stimulation with Nef^- HIV-1-infected $CD4^+$ T cells (22). We investigated the coproduction of the three cytokines by HIV-1-specific CTL clones after stimulation with Nef^+ HIV-1-infected $CD4^+$T cells. Approximately 7 to 9% of the cells in five clones produced cytokines after stimulation with Nef^+ HIV-1-infected $CD4^+$ T cells (Fig. 5). Most of these cytokine-secreting cells produced only a single cytokine but were able to produce any one of the three. Thus, the results indicate that after stimulation with HIV-1-infected $CD4^+$T cells, HIV-1-specific CTL clones can produce various kinds of

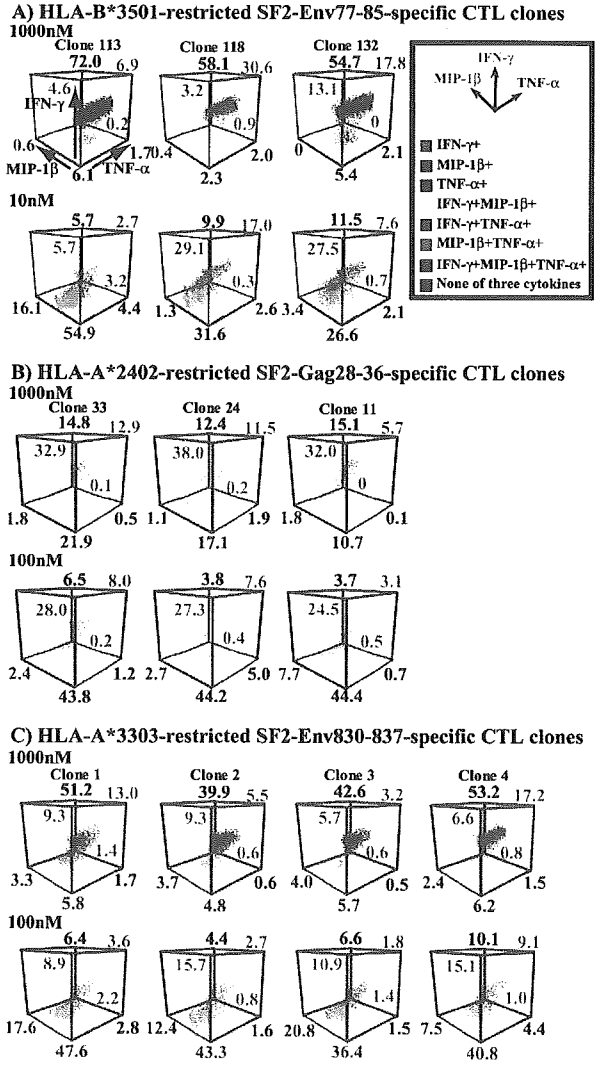

FIG. 3. Coexpression of three cytokines in HIV-1-specific CTL clones responding to epitope peptide-pulsed cells. (A) Env77-85-specific CTL clones. Intracellular cytokine production of three SF2-Env77-85-specific CTL clones was measured 6 h after stimulation with C1R-B*3501 cells prepulsed with the corresponding peptides (1,000 nM and 10 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. (B) Gag28-36-specific CTL clones. Intracellular cytokine production of three SF2-Gag28-36-specific, HLA-A*2402-restricted CTL clones (clones 33, 24, and 11) was tested 6 h after stimulation with C1R-A*2402 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). (C) Env830-837-specific CTL clones. Intracellular cytokine production of four SF2-Env830-837-specific, HLA-A*3303-restricted CTL clones (clones 1, 2, 3, and 4) was tested 6 h after stimulation with C1R-A*3303 cells prepulsed with the corresponding peptides (1,000 nM and 100 nM). Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. Fluorescence-activated cell sorter data were analyzed with Paint-A-Gate^{PRO} (BD Biosciences). The frequencies of cells expressing these cytokines are shown as percentages of the total number of cells. Cells expressing these cytokines are shown in a 3-dimensional presentation as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

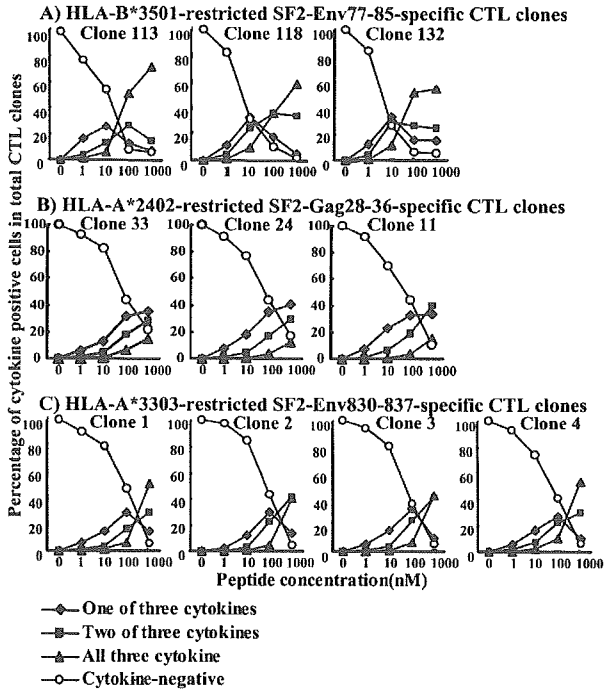

FIG. 4. Kinetics of multiple cytokine expression in the HIV-1-specific CTL clones corresponding to epitope peptide stimulation. The frequency of CTL clones expressing a single cytokine, two cytokines, or all three cytokines was measured 6 h after stimulation with cells prepulsed with the corresponding peptide at different concentrations as follows: the frequency of cells expressing a single cytokine was the sum of the frequency of cells expressing IFN-γ only, MIP-1β only, and TNF-α only in the total CD8$^+$ cells, while the frequency of cells expressing two cytokines was the sum of cells expressing IFN-γ and MIP-1β, IFN-γ and TNF-α, and MIP-1β and TNF-α in total CD8$^+$ cells.

cytokines but that each CTL clone is able to produce only a single cytokine. Heterogeneity in the production of these cytokines among CTL clones of the same specificity is barely noticeable.

Cytokine production of freshly isolated HIV-1-specific CD8$^+$ T cells stimulated with HIV-1-infected CD4$^+$ T cells. The cytokine production results for HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells suggest the possibility that when HIV-1-specific CD8$^+$ T cells recognize HIV-1-infected CD4$^+$ T cells in HIV-1-infected individuals, they produce various combinations of cytokines but each of them produces only a single cytokine. To clarify the accuracy of this hypothetical picture, we performed ex vivo analysis of the cytokine production of HIV-1 Gag28-36-specific CD8$^+$ T cells after stimulation with Nef$^+$ HIV-1-infected CD4$^+$ T cells (Fig. 6). We isolated CD8$^+$ T cells from fresh PBMC of two HIV-1-infected individuals (KI-144 and KI-158). Analysis using a Gag28-36-specific HLA-A*2402 tetramer as well as anti-CD27 and anti-CD28 MAbs showed that approximately 0.2 to 0.3% of CD8$^+$ T cells were Gag28-36-specific CD8$^+$ T cells and that the Gag28-36-specific CD8$^+$ T cells predominantly have either a CD27$^+$ CD28$^-$ memory/effector phenotype or a CD27$^+$ CD28$^+$ memory phenotype (Fig. 6). We then stimulated the CD8$^+$ T cells with NL-432-infected CD4$^+$ T cells. Approximately

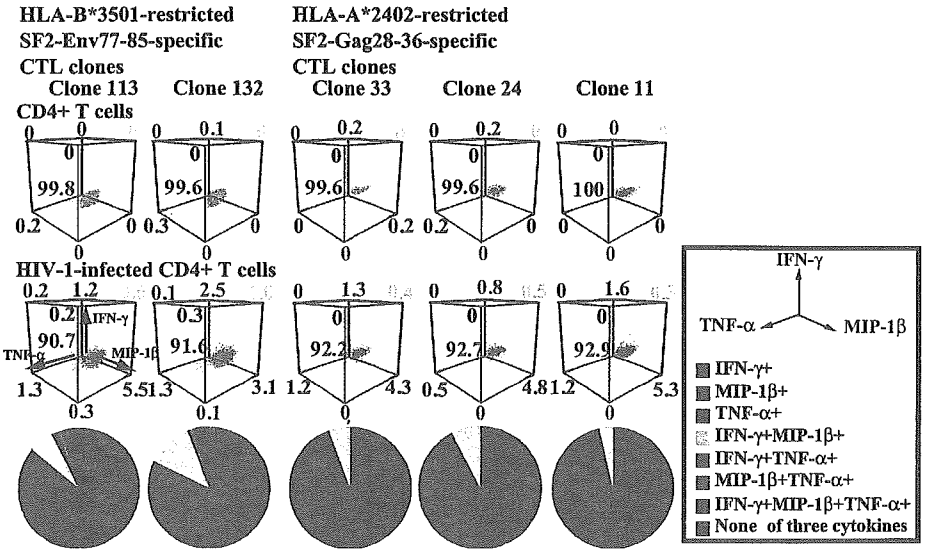

FIG. 5. Coexpression of all three cytokines in HIV-1-specific CTL clones responding to HIV-1-infected CD4$^+$ T cells. Intracellular cytokine production of two SF2-Env77-85-specific CTL clones and three Gag28-36-specific CTL clones was measured 6 h after stimulation with CD4$^+$ T cells infected with the Nef$^+$ HIV-1 clone NL-432 or NL-432gag^{HXB2}. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The frequency of cells expressing these cytokines is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown as a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

30 and 10% of Gag28-36-specific CD8$^+$ T cells produced at least one cytokine in PBMC from two different HIV-1-infected individuals (Fig. 6). The frequency of cytokine-producing cells was much higher in freshly isolated Gag28-36-specific CD8$^+$ T cells than in Gag28-36-specific CTL clones. KI-144 cells predominantly produced only MIP-1β or both MIP-1β and IFN-γ, while KI-158 cells predominantly produced only MIP-1β or both MIP-1β and TNF-α. This is in contrast to the finding that Gag28-36-specific CTL clones produced a single cytokine: either MIP-1β, IFN-γ, or TNF-α.

DISCUSSION

In the present study, we employed three kinds of HIV-1-specific CTL clones of the same specificity from the same individual. These CTL clones mostly exhibited an effector phenotype (CD27$^-$ CD28$^-$ CD45RA$^-$) and strong cytolytic activity. The HIV-1-specific CTL clones exhibited functional heterogeneity in the production of three cytokines in clones of the same specificity as well as of different specificities, indicating that HIV-1-specific effector CD8$^+$ T cells, even when of the same specificity, display functional heterogeneity in cytokine production. The mechanism for this heterogeneity of function, however, is still unclear. One possibility is that these CTL clones carry different T-cell receptors (TCR), with the difference in receptor signaling triggering the functional heterogeneity, although it is not understood how different activation of TCR influences cytokine production. The fact that cells expressing different cytokine production patterns do in any event exist in each clone tends to exclude this hypothesis but does support the idea that the cytokine production pattern is determined by various combinations of interacting factors, such as certain specific characteristics of the T cells, including the usage and expression level of TCR, the expression level of accessory molecules, and the relative activation status of the T cells.

Cytokine production by HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells reflects the response of HIV-1-specific CTLs in vivo much better than cytokine production by such clones stimulated with peptide-pulsed cells. The analysis of HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells revealed that only 7 to 9% of cells in each CTL clone produce one of the cytokines IFN-γ, TNF-α, and MIP-1β, suggesting limited cytokine production by HIV-1-specific effector CD8$^+$ T cells carrying the CD27$^-$ CD28$^-$ phenotype in vivo. This limited cytokine production may be explained by either or both of two factors: (i) the amount of HLA class I–virus peptide complexes on virus-infected cells is much smaller than that on peptide-pulsed cells; (ii) HLA class I–HIV-1 peptide complexes are down-regulated on the surfaces of HIV-1-infected cells. Previous studies have demonstrated that HIV-1-specific CTL clones may partially suppress HIV-1 replication, although these cells hardly kill HIV-1-infected CD4$^+$ T cells because of the Nef-mediated down-regulation of the HLA-A and -B molecules (20). The partial suppression may be explained by the limited cytokine production of HIV-1-specific CTL clones stimulated with HIV-1-infected CD4$^+$ T cells. Thus, it is hypothesized that cytokines produced by HIV-1-specific CD8$^+$ T cells play an important role in the suppression of HIV-1-replication.

Heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities was not found when the CTL clones were stimulated with HIV-1-infected CD4$^+$ T cells. This may be explained by the fact that

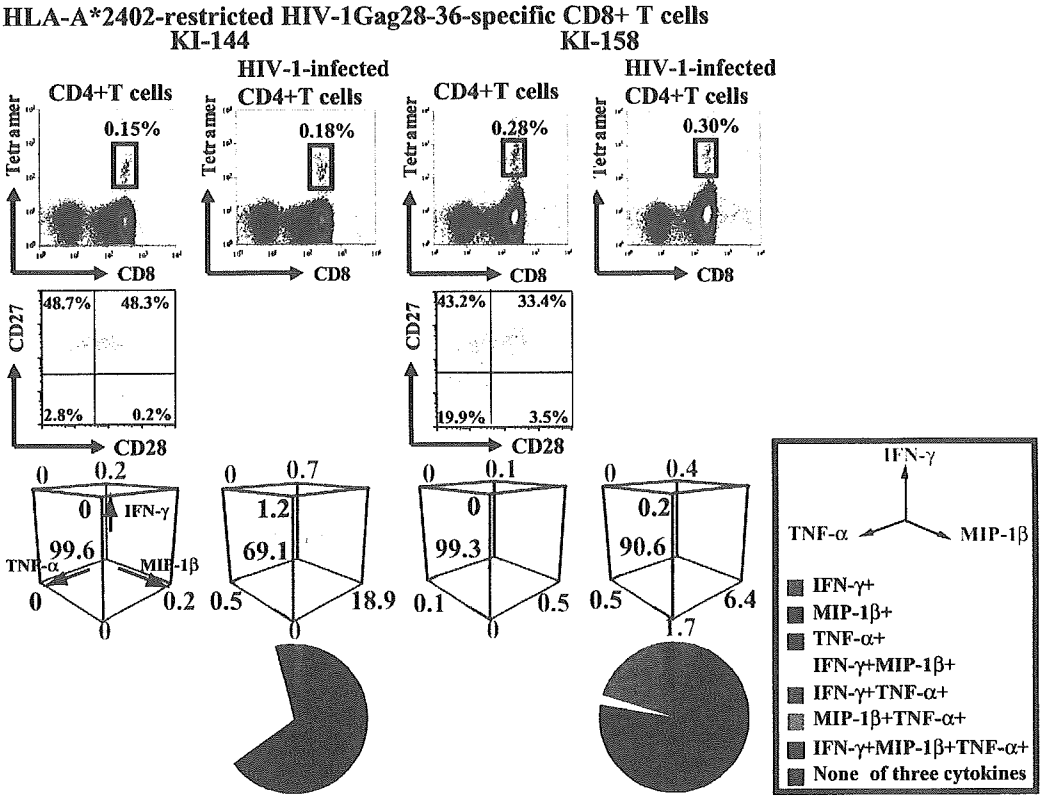

FIG. 6. Coexpression of three cytokines in freshly isolated $HIV_{Gag28-36}$-specific $CD8^+$ T cells responding to HIV-1-infected $CD4^+$ T cells. Intracellular cytokine production of Gag28-36-specific $CD8^+$ T cells was measured 6 h after stimulation with $CD4^+$ T cells infected with the Nef^+ HIV-1 clone NL-$432gag^{HXB2}$. Intracellular staining for the three cytokines IFN-γ, MIP-1β, and TNF-α was simultaneously carried out using the three MAbs specific for these cytokines, and then the expression of these cytokines was analyzed by flow cytometry. The $CD8^+$ tetramer-positive cells are gated to determine both the frequency of cells expressing these cytokines and the CD27 and CD28 expression. The frequency is shown as a percentage of the total number of cells in a 3-dimensional presentation. The frequency of cells expressing these cytokines among total cytokine-producing cells is also shown in a pie chart. Cells expressing these cytokines are shown as follows: none of the three cytokines (gray), IFN-γ only (red), MIP-1β only (green), TNF-α only (blue), IFN-γ and MIP-1β (yellow), IFN-γ and TNF-α (violet), MIP-1β and TNF-α (cyan), and all three cytokines (black).

heterogeneity in cytokine production between the CTL clones was not found when they were stimulated with cells pulsed with lower concentrations of HIV-1 peptides. Therefore, it is still unclear whether the heterogeneity in cytokine production between CTL clones of the same specificity as well as of different specificities influences disease progression.

A previous study revealed that HLA-A2-restricted, $HCMVpp65_{495-503}$-specific $CD8^+$ T cells are able to produce various combinations of IFN-γ, TNF-α, and IL-2 after PBMC from healthy individuals are stimulated with $HCMVpp65_{495-503}$ peptide, suggesting that the virus-specific $CD8^+$ T cells possess a functional heterogeneity of cytokine production in vivo (18). Since the HCMV-specific $CD8^+$ T cells are heterogeneous with regard to the surface markers CD45RO, CD45RA, CD27, CD28, CD57, and CD62L (18, 21), it is thought that they include various population types, ranging from memory to effector T cells. Therefore, the heterogeneity at maturation of $CD8^+$ T cells may reflect the capacity for functional heterogeneity in cytokine production. Our recent study revealed that $CD8^+$ T cells with effector phenotypes ($CD27^-$ $CD28^-$ $CD45RA^{+/-}$) or memory/effector phenotypes ($CD27^{low}$ $CD28^-$ $CD45RA^{+/-}$) can produce IFN-γ after stimulation of $CD8^+$ T cells with an anti-CD3 MAb (23), indicating that effector and memory/effector $CD8^+$ T cells have a more pronounced ability to produce IFN-γ than memory $CD8^+$ T cells. Thus, the heterogeneity in maturation or differentiation of $CD8^+$ T cells reflects the functional heterogeneity in cytokine production capacity seen in vivo.

Freshly isolated Gag28-36-specific $CD8^+$ T cells produced more cytokines than CTL clones with the same specificity. Since these Gag28-36-specific $CD8^+$ T cells carried either a $CD27^+$ $CD28^-$ memory/effector or a $CD27^+$ $CD28^+$ memory phenotype, it is likely that they are less mature than the CTL clones. These results suggest that HIV-1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype can produce more cytokines than those with an effector phenotype. Approximately 20 to 30% of cytokine-producing $CD8^+$ T cells produced two or three cytokines, whereas most CTL clones of the same specificity produced a single cytokine. This indicates the ability of HIV-1-specific memory and memory/effector $CD8^+$ T cells to produce multiple cytokines when they recognize HIV-1-infected cells. Various differentiation ranges of HIV-1-specific $CD8^+$ T cells, from memory to effector, are found in PBMC from HIV-1-infected individuals (5, 22). HIV-

1-specific $CD8^+$ T cells with a $CD27^+$ $CD28^-$ or $CD27^+$ $CD28^+$ phenotype may play both a direct and an indirect role in the suppression of HIV-1 replication in vivo via cytokines secreted from these $CD8^+$ T cells. Approximately 30 and 10% of Gag28-36-specific $CD8^+$ T cells produced cytokines in KI-144 and KI-158, respectively. This difference might be explained by the fact that approximately 20% of Gag28-36-specific $CD8^+$ T cells from KI-158 carried effector phenotype $CD27^-$ $CD28^-$, whereas no Gag28-36-specific $CD8^+$ T cells from KI-144 carried it.

In summary, HIV-1-specific CTL clones for the most part produced a single cytokine and did not exhibit heterogeneity of cytokine production among clones after stimulation with HIV-1-infected $CD4^+$ T cells, although they exhibited multiple cytokine production and functional heterogeneity of cytokine production between clones after stimulation with HIV-1 peptide-pulsed cells. Freshly isolated HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype produced much greater amounts of the cytokines than CTL clones with the same epitope specificity after stimulation with HIV-1-infected $CD4^+$ T cells. HIV-1-specific $CD8^+$ T cells with an effector/memory or memory phenotype might directly or indirectly play a crucial role in the eradication of HIV-1 via the cytokines secreted from these T cells in HIV-1-infected individuals.

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Susceptibility of Mink (*Mustera vision*)-Derived Cells to Replication by Human Immunodeficiency Virus Type 1

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In vivo studies for understanding viral transmission and replication, host immune responses, and pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection would greatly benefit from the establishment of a small-animal model. In this study, we explored the potential of American mink (*Mustera vison*) as a susceptible host. We found that primary cells and cell lines derived from this species efficiently supported *trans*-activation of the HIV-1 long terminal repeat by Tat. Accordingly, the cysteine residue at position 261, which has been shown to be important for interaction of the human cyclin T1 with the HIV-1 regulatory protein Tat, is conserved in the mink homologue. No species-specific defect in Rev function could be detected in mink cells. In addition, primary splenocytes, fibroblasts, and the Mv.1.Lu cell line from American mink supported early as well as late HIV-1 gene expression following infection with vesicular stomatitis G protein-pseudotyped HIV-1 viruses, at levels comparable to those seen with permissive human cells. Furthermore, the mink Mv.1.Lu cell line stably expressing human CD4 and CCR5 receptors supported a spreading HIV-1 infection with few, if any, deficiencies compared to findings in human cell lines. This indicates the potential of HIV-1 to replicate in these cells once the blockade at the stage of virus entry has been removed. These results clearly show that cells from American mink generally pose no functional intracellular block to HIV-1 replication, and collectively they raise the possibility that this animal species could be engineered to support HIV-1 infection, providing a useful small-animal model for evaluating de novo infection by HIV-1.

Human immunodeficiency virus type 1 (HIV-1) replicates efficiently only in humans and certain nonhuman primates such as chimpanzees. Cellular entry has been considered to be a major restriction of HIV-1 replication in cells from nonhuman species. The identification of roles played by chemokine receptors as entry coreceptors with human CD4 (reviewed in references 3, 17, and 26) was seen to offer possibilities for overcoming species-specific restrictions to HIV-1 replication in rodent cells, leading to the development of a transgenic rodent model. However, primary lymphocytes from mice transgenic for human CD4 and either the human CCR5 or human CXCR4 coreceptor, while largely able to overcome the entry block, exhibited little or no sign of productive infection (6, 30). Recently, primary cells, especially macrophages and microglia from rats transgenic for human CD4 and CCR5 were found to support HIV-1 replication at levels higher than those described for comparable transgenic mouse models, but in vivo replication of HIV-1 in this host appeared to be limited (15).

Multiple intracellular steps at which the HIV-1 replication cycle is blocked have been noted, especially in cells from rodent species. The inability of the HIV-1-encoded *trans*-activator Tat to activate viral RNA transcription from the long terminal repeat (LTR) is one major restriction at the postintegration phase in rodent cells (13). This intracellular restriction could be partially overcome by the introduction of human cyclin T1 (CycT1) (4, 10, 11, 19, 36), indicating that human CycT1 is essential for Tat-mediated transcription. CycT1 is a component of the positive-transcription-elongation factor-β transcription factor complex (23, 37), which associates with the cyclin-dependent kinase CDK9. Human CycT1, in association with CDK9, interacts with HIV-1 Tat to form a heterodimer with high affinity for the *trans*-activation response element stem-loop at the 5′ ends of all nascent viral transcripts. This complex promotes hyperphosphorylation of the carboxy-terminal repeat domain of RNA polymerase II, causing increased transcriptional processivity (11). Human and murine forms of CycT1 are 90% identical at the amino acid level; a single amino acid change from cysteine to tyrosine at position 261 of murine CycT1 prevents it from interacting with Tat (4, 11, 19). Although expression of human CycT1 in mouse NIH 3T3 cells coexpressing human CD4 and an appropriate coreceptor allows HIV-1 to proceed through entry, reverse transcription, integration, and proviral gene expression, it is not sufficient to reconstitute the full replication cycle (5, 11, 25).

Reduced levels of unspliced genomic RNA synthesis as well as structural gene expression of HIV-1 have also been noted in rodent cells. However, the issue of whether HIV-1 Rev, which is known to associate with the cellular export factor CRM1, in cells from rodent species is functional or nonfunctional has been controversial (22, 34). More recent studies suggest a relative, rather than an absolute, limitation in the function of this regulatory protein in rodent cells (5, 25). Lastly, there are blocks to the late, posttranslational phase of viral replication, including Gag polyprotein processing, virion assembly, and release, that result in failure of the virus to spread. Although these blocks could be partially circumvented by human-mouse heterokaryon fusions, the underlying mechanism has yet to be

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clarified (5, 18, 24, 25). This assembly defect of HIV-1 in murine cells further complicates the development of a permissive small-animal model of HIV-1 disease.

Nevertheless, the potential usefulness of a small-animal model of HIV-1 infection and disease warrants further effort directed at an assessment of the quantitative as well as qualitative limitations and blocks in the viral replication cycle in animals that may serve as hosts. Among the small-animal species studied, we previously observed efficient proviral gene expression and virion assembly and release in certain cell lines from American mink (*Mustera vision*) stably transduced with HIV-1 proviruses (18). The indication that mink-derived cells are permissive for postintegration steps in the HIV-1 replication cycle prompted us to extend these provocative findings with established cell lines to primary cultures, with the intent of further exploring mink as a potential small laboratory animal model for HIV infection.

MATERIALS AND METHODS

Primary cells and cell lines. Primary fibroblast and splenocytes were prepared from kidney and spleen, respectively, which had been removed aseptically from euthanized *M. vision* animals at 6 months of age. Primary fibroblasts from *M. vision* kidney were prepared by filtering tissue pieces through a nylon mesh screen (Falcon cell strainer; 70-μl pore size; Becton Dickinson) after treatment with 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 30 min at room temperature. After centrifugation, cells were washed with Ca^{2+}- and Mg^{2+}-free PBS and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) and antibiotics (stabilized penicillin-streptomycin solution; Sigma). Single-cell suspensions of splenocytes were prepared by pushing tissue pieces through a nylon mesh screen and purified on Lympholyte-M CL5030 (Cedarlane Laboratories). Activation of mink splenocytes was achieved by an initial overnight stimulation with 1 μg of concanavalin A (ConA) (Wako, Osaka, Japan) per ml and subsequent culturing in RPMI 1640 (Sigma) containing 15% FCS, human recombinant interleukin 2 (IL-2) (40 IU/ml) (Genzyme), 5×10^{-5} M 2-mercaptoethanol (GIBCO-BRL), nonessential amino acids (GIBCO-BRL), 1 mM sodium pyruvate (GIBCO-BRL), minimum essential medium vitamin solution (GIBCO-BRL), and antibiotics. Human peripheral blood mononuclear cells (PBMC) from healthy donors were prepared by using Ficoll-Paque (Ficoll-Paque PLUS; Amersham Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation and then cultured in RPMI1640 containing 10% FCS and 40 IU of human recombinant IL-2 per ml after activation with 3 μg of phytohemagglutinin (PHA) P (Sigma) per ml for 2 days. Primary mouse splenocytes were prepared from spleens removed aseptically from euthanized female C57BL/6J mice by passage through a nylon mesh screen and purified on Lympholyte-MCL5030. Cells were cultured in RPMI 1640 containing 10% FCS and 4 ng of recombinant murine IL-2 (R&D Systems, Inc.,) per ml, followed by activation with 1 μg of ConA per ml. Mink Mv.1.Lu (NBL-7) cells (a mink-fibroblast-like cell line derived from the lung of a normal *M. vision* embryo or fetus with no detectable reverse transcriptase activity) was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained in DMEM supplemented with 10% FCS and antibiotics. 293T, HeLa, HOS, and NIH 3T3 cells were maintained in DMEM with 10% FCS and antibiotics. NIH 3T3 cells stably expressing human CycT1 (18) were maintained in DMEM supplemented with 10% FCS, antibiotics, and 200 μg of G418 (Geneticin; GIBCO-BRL) per ml. The $CD4^+$ human osteosarcoma GHOST cell line derivative that stably expresses human CCR5, GHOST-hi5 (27), was obtained through the AIDS Research and Reference Reagent Program and was maintained in DMEM supplemented with 10% FCS, 200 μg of G418 per ml, 100 μg of hygromycin (Sigma) per ml, 1 μg of puromycin (Sigma) per ml, and antibiotics.

To generate mink cells expressing human CD4, parental mink Mv.1.Lu cells were transfected with pMOSCD4 (33) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and selected in culture medium containing 1 mg of G418 per ml. To introduce human CCR5, Mv.1.Lu cells expressing human CD4 (Mv.1.Lu-CD4 cells) were transfected with pBMGCCR5 (33) and selected in medium with 700 μg of hygromycin per ml. Clones with surface expression of either human CD4 (Mv.1.Lu-CD4) or both human CD4 and CCR5 (Mv.1.Lu-CD4-CCR5) were selected with phycoerythrin-conjugated CD4 monoclonal antibody (eBioscience) and fluorescein isothiocyanate-conjugated anti-CCR5 monoclonal antibody 2D7 (PharMingen) by flow cytometry (FACSCalibur; Becton Dickinson) after limiting dilution.

HIV-1 molecular clones, envelope expression vectors, and generation of pseudotyped and HIV-1 viral stocks. The pNL4-3 Luc E^-R^- reporter plasmid (7) was obtained through the AIDS Research and Reference Reagent Program. The infectious HIV-1 molecular clones R7/3/SF162, R7/3/SF162P3, and R7/3/SF33 were constructed as described previously (21). A vesicular stomatitis virus G (VSV-G)-expressing plasmid (pVSV-G) was purchased from Clontech. For expression of HIV-1 SF162, SF162P3, SF33, and IIIB gp160, each *env* gene was subcloned into the β-actin-based expression vector pCAGGS (28). Single-round replication-competent luciferase reporter virus stocks were produced in 293T cells transfected with an equal amount of pNL4-3 Luc E^-R^- and pVSV-G or various HIV-1 envelope expression vectors by using Lipofectamine 2000. Culture supernatants were harvested at 48 h posttransfection, passed through 0.45-μm-pore-size filters, and frozen in aliquots at −80°C. The p24 contents of the viruses were determined with enzyme-linked immunosorbent assay kits (Cellular Products Inc.), as were those of standards provided by the manufacturer. To generate the replication-competent VSV-G-pseudotyped virus, 293T cells were cotransfected with an equal amount of the R7/3/SF162P3 proviral plasmid and pVSV-G. VSV-G is incorporated into the HIV-1 virions during production and mediates entry into cells from a broad range of vertebrate animals. HIV-1 virus stocks were produced in 293T cells transfected with respective proviral DNA clones. Culture supernatants were harvested, quantitated for p24 content, and frozen as described above.

Viral entry assay. Target cells were infected for 3 h with 15-ng equivalents of luciferase reporter viruses and cultured for 48 h. Infected cells were lysed with 100 μl of cell lysing buffer (Luc PGC-50, PicaGene; Wako), and 20 μl of each lysate was assayed for photon emission after the addition of 100 μl of luciferase assay substrate (Wako) with a luminometer (Lumat B9506; Bertold). The protein concentration of each sample was determined with the Bio-Rad protein assay.

Infectivity assay. Target cells were exposed to HIV-1 SF162P3/(VSV-G) replication-competent pseudotypes or HIV-1 R7/3/SF162, R7/3/SF162P3, and R7/3/SF33 proviruses for 3 h at 37°C. After being washed three times with PBS, cells were treated with trypsin (0.025%)–EDTA (0.27 mM) (Sigma) for 3 min at 37°C and then washed three times with complete medium. The p24 antigen in the medium was assayed immediately after the cell washing (day 0), and that in the culture supernatants was assayed periodically. Background levels, taken to be those on day 0, were subtracted from the amount of p24.

Transient transfection and luciferase assay. To determine the transcriptional activity of the HIV-1 LTR, cells (5×10^5) were plated onto 60-mm-diameter plates. Transient transfections were done with 2 μg of a luciferase reporter plasmid, 1 μg of pAct-β-gal plasmid, and 2 μg of pBC12/CMV/hCycT1 (human CycT1 under the control of the cytomegalovirus promoter) (4) or pcDNA3.1, using Lipofectamine 2000. Cells were harvested at 48 h posttransfection. Lysates were prepared from a portion of the transfected cells by using cell lysing buffer (Luc PGC-50) and assayed for luciferase activities. Another portion was used to prepare cell lysates for β-galactosidase (β-Gal) measurements to ensure comparable efficiency of transfection. β-Gal activity was measured by standard colorimetric methods with β-Gal detection kits (Invitrogen).

cDNA sequencing of mink CycT1. Total RNA was prepared from mink Mv.1.Lu cell by using the TRIzol reagent (Invitrogen), and first-strand cDNA was generated with SuperScriptII (Invitrogen) according to the manufacturer's instructions, using oligo(dT) as a primer. The 5′ and 3′ halves of cDNA encoding the entire open reading frame of mink CycT1 were amplified by using primer sets (5′-ATGGAGGGAGAGAGGAAGAAC-3′–5′-ATGAGAAAGGAGAT TCTGGGC-3′ and 5′-CAATGTGAAGTCACAATATGC-3′–5′-TTTACTTAG GAAGGGGTGGAAG-3′) designed based on the sequence of human CycT1 (GenBank accession number AF048730). *Taq* polymerase-amplified PCR products were cloned into a vector by using pCR2.1-TOPO TA cloning (Invitrogen), and a TA clone of each half was obtained and sequenced.

RNase protection assays. A 262-bp fragment (nucleotide 78 to 340 relative to the site of transcription) was PCR amplified from the R7/3/162 proviral plasmid by using the primer sets 5′-GCTTGCCTTGAGTGCTTCAAG-3′–5′-CCCATC TCTCTCCTTCTAGCCTCC-3′) and inserted into pCR2.1 TOPO TA vector containing the T7 promoter to provide a template for the synthesis of an antisense RNA probe. This plasmid was linearized with *Hin*dIII, and the antisense RNA probe spanning the HIV-1 major 5′ splice donor was generated by in vitro transcription with T7 polymerase in the presence of [α-^{32}P]dCTP (Amersham) by using RNA transcription kits (Stratagene), heated to 85°C, and used as a probe in hybridization. Ten micrograms of total RNA, extracted from HIV-1 R7/3/162P3(VSV-G)-infected cell lines with the TRIzol reagent, was hybridized to the antisense RNA probe overnight and digested with an RNase A-RNase T_1 mixture (RNase cocktail; Ambion). Protected fragments that corresponded to

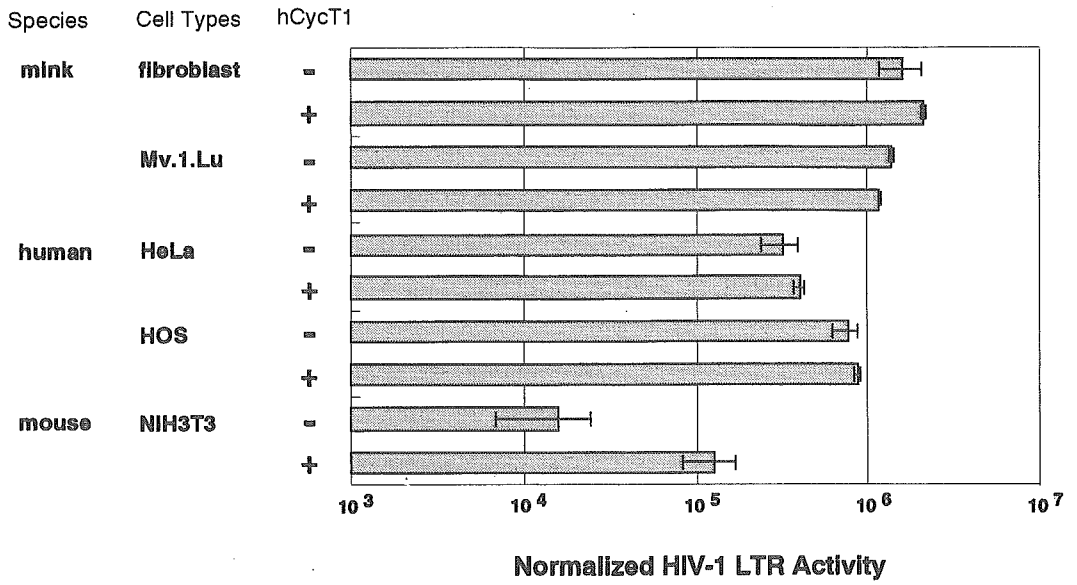

FIG. 1. The HIV-1 LTR exhibits significant activity in mink cells. Mink fibroblasts and Mv.1.Lu, human HeLa, HOS, and mouse NIH 3T3 cells were transfected with 2 μg of pNL4-3 Luc E^-R^-, 1 μg of pAct-β-gal, and 2 μg of pBC12/CMV/hCycT1 (expression plasmid for human CycT1) or 2 μg of cytomegalovirus immediate-early promoter-based vector pcDNA3.1 (empty vector). Luciferase and β-Gal activities in cell lysates were determined 48 h after transfection, and the firefly luciferase activity was normalized to the β-Gal activity measured by standard colorimetric methods. Values are arithmetic means ± standard deviations from duplicate transfections. Results are representative of those from three independent experiments.

spliced and unspliced HIV-1 RNA were visualized by autoradiography after separation on a 5.0% denaturing acrylamide gel.

CD4 down-regulation assay. Transfection to assess CD4 down-regulation by Nef was performed with Mv.1.Lu-CD4-CCR5 and GHOST-hi5 cells. Cells (7×10^5) plated onto 60-mm-diameter plates were transfected by using Lipofectamine 2000 with 3 μg of the pRcCMV-CD8-SF2Nef expression construct (2). As a control, a chimeric CD8 fusion protein with Nef in the antisense orientation (pRcCMV-CD8-antisense SF2Nef) was used. At approximately 40 h posttransfection, cells were harvested and stained with a mixture of phycoerythrin-conjugated anti-CD4 (eBioscience) and fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibodies (PharMingen). Surface CD8 expression was used as a marker for Nef-expressing cells. CD4 down-regulation was determined by comparing the percentage of CD4 expression on CD8-positive cells transfected with CD8/Nef expression plasmid to that on cells transfected with CD8/antisense Nef by using a FACSCalibur.

$TCID_{50}$ determination. The infectious titer (50% tissue culture infective dose [$TCID_{50}$]) of HIV-1 in cell culture supernatants was determined by end point limiting dilution on PHA–IL-2-activated human PBMC from HIV-1-seronegative donors 5 days after inoculation.

RESULTS

HIV-1 LTR activity is robust in mink cells and cannot be enhanced by human CycT1. To characterize the efficiency of *trans*-activation and transcript elongation from the HIV-1 LTR in mink cells, the proviral plasmid pNL4-3 Luc E^-R^- was introduced by transfection, and luciferase activities in cellular lysates were quantified 48 h later. Both adherent primary mink fibroblasts and the Mv.1.Lu cell line from American mink (*M. vison*) were used, and HIV-1 LTR activity was normalized for variability in transfection efficiency by cotransfection of an LTR-independent β-Gal reporter construct (14). Transfections of human HeLa and HOS cells were included as positive controls, while mouse NIH 3T3 cells served as a negative control.

HIV-1 LTR activity that was 2 log units higher than that found in mouse NIH 3T3 cells and comparable to that of the human HeLa and HOS cells was observed in primary mink fibroblasts and Mv.1.Lu cells (Fig. 1). The high-level transcriptional activity observed in mink fibroblasts and Mv.1.Lu cells could not be further enhanced by cotransfection of an expression plasmid encoding human CycT1. In contrast, the luciferase signal in NIH 3T3 cells was significantly augmented in the presence of human CycT1. A single amino acid change at residue 261 from cysteine to tyrosine in murine CycT1 has been shown to be the major determinant in restriction of Tat-mediated HIV-1 LTR *trans*-activation in NIH 3T3 cells (4, 10, 11, 19, 36). The ability to *trans*-activate the HIV-1 LTR in mink cells suggests that the mink homologue of CycT1 is capable of forming functional complexes with HIV-1 Tat. To confirm this at the genetic level, the gene encoding CycT1 was isolated from cDNA of mink Mv.1.Lu cells, and the sequence was compared to those of human and murine CycT1s (GenBank accession numbers AF048730 and AF095640, respectively). Alignment of the predicted amino acid sequences showed that the CycT1s from human and mink have 726 amino acid residues, while mouse CycT1 has 724 amino acids (Fig. 2). There are 47 amino acid substitutions between human and mink CycT1, many of which are also found in mouse CycT1. Importantly, however, the cysteine residue at position 261 that is critical for productive interaction of human CycT1-Tat to the *trans*-activation response element is absent in mouse CycT1 (4, 11) but is conserved in mink CycT1. The identity of residue 261 in mink CycT1 was further confirmed by preparing another set of RNA samples from mink primary fibroblasts and amplifying the region around residue 261 (data not shown). Collectively, these results suggest that the ability to support robust HIV-1 LTR activity is a property unique to *M. vision* among small-animal species.

Efficient spliced and unspliced HIV-1 mRNA syntheses in