Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 23 (2005) 3783–3790
Vaccine
www.elsevier.com/locate/vaccine

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

Manami Satoh[a], Yuji Takamiya[b], Shinichi Oka[c], Katsushi Tokunaga[b], Masafumi Takiguchi[a,*]

[a] *Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[b] *Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[c] *AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan*

Received 3 August 2004; received in revised form 8 February 2005; accepted 17 February 2005
Available online 17 March 2005

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.

Keywords: HIV-1; CTL; HLA-A*2601

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

* Corresponding author. Tel.: +81 96 3736529; fax: +81 96 3736532.
E-mail address: masafumi@kaiju.medic.kumamoto-u.ac.jp (M. Takiguchi).

0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.02.022

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-

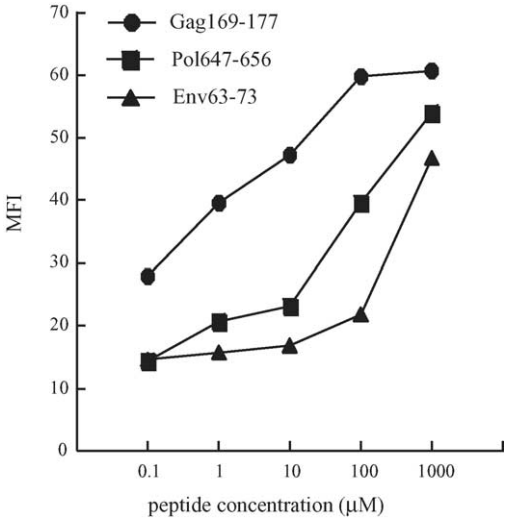

Fig. 1. Binding to HLA-A*2601 of HIV-1 peptides carrying HLA-A*2601 motif. Binding of the peptides carrying A*2601 anchors was measured by a stabilization assay using RMA-S-A*2601 cells. Representative results of binding peptides with high-(Gag169–177), medium-(Pol647–656), and low-(Env63–73) affinity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

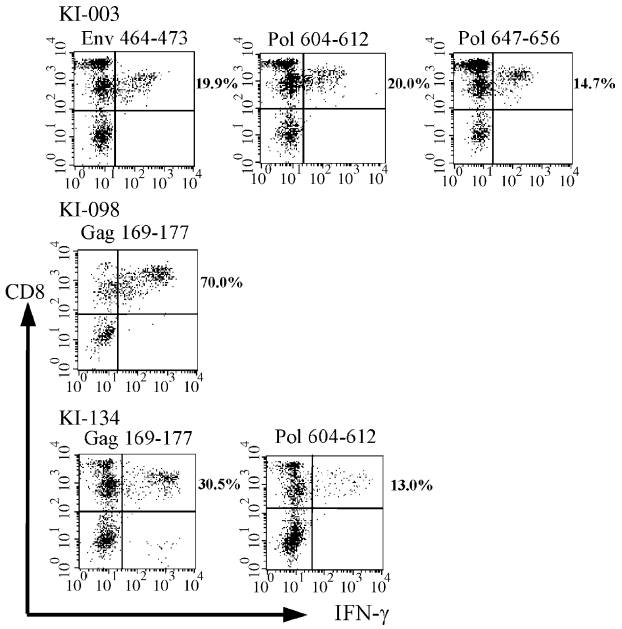

Fig. 2. Induction of HIV-1-specific CD8 T cells from PBMCs of HIV-1-infected individuals with HLA-A*2601. PBMCs from three HIV-1-infected individuals (KI-003, KI-098 and KI-134) were stimulated with cocktails of HLA-A*2601 binding peptides (Table 3), and were then cultured for 10–14 days. The cultured 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.

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.

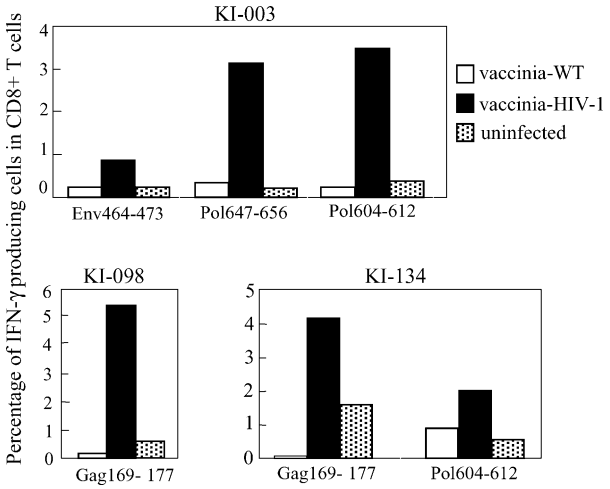

Fig. 3. Recognition of HLA-A*2601-restricted HIV-1 epitopes presented on r-HIV-1 vaccinia-infected cells. Cultured cells containing peptide-specific $CD8^+$ T cells shown, were examined for IFN-γ production after they had been stimulated with C1R-A*2601 cells infected with wild-type vaccinia (vaccinia-WT),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

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.

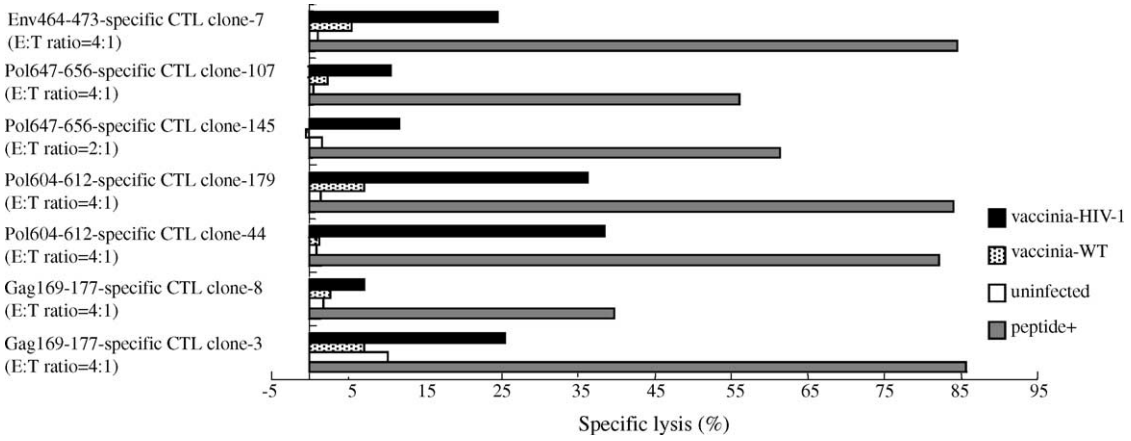

Fig. 4. Cytolytic activity of the HLA-A*2601-restricted CTL clones toward peptide-pulsed or r-HIV-1 vaccinia-infected cells. The activities of HLA-A*2601-restricted CTL clones toward C1R-A*2601 cells pre-pulsed with 1 μM epitope peptides (peptide+) or infected with recombinant vaccinia virus expressing 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 (ETKLGKAGY) 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.

Acknowledgements

The authors thank Dr. S. Ferrone for the gift of mAB TP25.99 and Sachiko Sakai for secretarial assistance. This research was supported by a grant-in aid for scientific research from the Ministry of Health, Labour and Welfare, the government of Japan, by a grant from the Japan Health Science Foundation, and by a grant from the Organization for Pharmaceutical Safety and Research.

References

[1] Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994;684:4650–5.
[2] Pantaleo G, Demarest JF, Soudeyns H, Graziosi C, Denis F, Adelsberger JW, et al. Major expansion of $CD8^+$ T cells with a predominant Vb usage during the primary immune response to HIV. Nature 1994;370:463–7.

[3] Moss PA, Rowland-Jones SL, Frodsham PM, McAdam S, Giangrande P, McMichael AJ, et al. Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors. Proc Natl Acad Sci USA 1995;92:5773–7.
[4] Rowland-Jones S, Sutton J, Ariyoshi K, Dong T, Gotch F, McAdam S, et al. HIV-1 specific cytotoxic T cells in HIV-exposed but uninfected Gambian women. Nat Med 1995;1:59–64.
[5] Langlade-Demoyen P, Ngo-Giang-Huong N, Ferchal F, Oksenhendler E. Human immunodeficiency virus (HIV) Nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. J Clin Invest 1994;93:1293–7.
[6] Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat Med 1997;3:205–11.
[7] Goulder PJ, Philips RE, Colbert RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T-lympnocyte response associated with progression to AIDS. Nat Med 1997;3:212–7.
[8] Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature 1998;391:397–401.
[9] Xu XN, Laffert B, Screaton GR, Kraft M, Wolf D, Kolanus W, et al. Induction of Fas Ligand expression by HIV involves the interaction of Nef with the T cell receptor z chain. J Exp Med 1999;189:1489–96.
[10] Muller YM, De Rosa SC, Hutton JA, Witek J, Roederer M, Altman JD, et al. Increased CD95/Fas-induced apoptosis of HIV-specific $CD8^+$ T cells. Immunity 2001;15:871–82.
[11] Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory HIV-1 specific CD8 T lymphocytes. Nature 2001;410:106–11.
[12] Shiga H, Shioda T, Tomiyama H, Takamiya Y, Oka S, Kimura S, et al. Identification of multiple HIV-1 cytotoxic T cell epitopes presented by human leukocyte antigen B35 molecules. AIDS 1996;10:1075–83.
[13] Tomiyama H, Miwa K, Shiga H, Ikeda-Moore Y, Oka S, Iwamoto A, et al. Evidence of presentation of multiple HIV-1 cytotoxic T lymphocyte epitopes by HLA-B*3501 molecules that are associated with the accelerated progression of AIDS. J Immunol 1997;158:5026–34.
[14] Threlkeld SC, Wentworth PA, Kalams SA, Wilkes BM, Ruhl DJ, Keogh E, et al. Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily: implications for vaccine development. J Immunol 1997;159:1648–57.
[15] Fukada K, Chujoh Y, Tomiyama H, Miwa K, Kaneko Y, Oka S. HLA-A*1101-restricted cytotoxic T lymphocyte recognition of HIV-1 Pol protein. AIDS 1999;13:1413.
[16] Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242–52.
[17] Hossain MS, Tomiyama H, Inagawa T, Sriwanthana B, Oka S, Takiguchi M. HLA-A*3303-restricted cytotoxic T lymphocyte recognition for novel epitopes derived from the highly variable region of the HIV-1 Env protein. AIDS 2001;15:2199–208.
[18] Hossain MS, Tomiyama H, Inagawa T, Ida S, Oka S, Takiguchi M. Identification and characterization of HLA-A*3303-restricted, HIV type 1 Pol- and Gag-derived cytotoxic T cell epitopes. AIDS Res Hum Retrovir 2003;19:503–10.
[19] Tomiyama H, Sakaguchi T, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification of multiple HIV-1 CTL epitopes presented by HLA-B*5101 molecules. Hum Immunol 1999;60:177–86.
[20] Imanishi T, Akaza T, Kimura A, Tokinaka K, Gojibori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, editors. HLA1991. Oxford: Oxford Scientific Publication; 1992. p. 1065–220.
[21] Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S, et al. Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. Immunogenetics 1997;46:199–205.
[22] Yamada N, Ishikawa Y, Dumrese T, Tokunaga K, Juji T, Nagatani T, et al. Role of anchor residues in peptides binding to three HLA-A26 molecules. Tissue Antigens 1999;54:325–32.
[23] Tanabe M, Sikimata M, Ferrone S, Takiguchi M, Structural. functional analysis of monomorphic determinants recognized by monoclonal antibodies reacting with the HLA class I a_3 domain. J Immunol 1992;148:3202–9.
[24] Dumrses T, Stevanovic S, Seefer FH, Yamada N, Ishikawa Y, Tokunaga K, et al. HLA-A26 subtype A pockets accommodate acidic N-termini of ligands. Immunogenetics 1998;48:350–3.
[25] Cardinaud S, Moris A, Fevrier M, Rohrlich PS, Weiss L, Langlade-Demoyen P, et al. Identification of cryptic MHC I-restricted epitopes endoded by HIV-1 alternative reading frames. J Exp Med 2004;199:1053–63.
[26] Fukada K, Tomiyama H, Wasi C, Matsuda T, Kusagawa S, Sato H, et al. Cytotoxic T cell recognition of HIV-1 cross-clade and clade-specific epitopes in HIV-1-infected Thai and Japanese patients. AIDS 2002;16:701–11.