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Novel, Cross-Restricted, Conserved, and Immunodominant Cytotoxic T Lymphocyte Epitopes in Slow Progressors in HIV Type 1 Infection

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ABSTRACT

HIV-specific cytotoxic T lymphocytes (CTLs) play an important role in the immune response to HIV infection. Long-term nonprogressors (LTNPs) or slow progressors (SPs) in HIV infection may make qualitatively different CTL responses compared to those generated by seropositive individuals who progress to disease at a faster rate. The class I molecule HLA-B*57 has been identified as one restriction element overrepresented in SP groups studied, and, together with the closely related molecule HLA-B*58, occurs commonly in ethnic groups where HIV is most prevalent. In this study, we have identified five new HLA-B*57-restricted CTL epitopes recognized by SP donors, one of which is also HLA-B*5801 restricted. These HLA-B*57-restricted responses represent the dominant HIV-specific CTL response in each of the SP donors tested. These and other such epitopes may be an important component in future vaccine design.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS-specific cytotoxic T lymphocytes (CTLs) are believed to play an important role in the immune response to HIV infection.¹ The appearance of HIV-specific CTLs in the blood of individuals at or prior to seroconversion closely corresponds to the disappearance of viremia,² and high levels of CTLs have been associated with the maintenance of the disease-free phase of infection.³ However, the great majority of HIV-infected individuals generate a strong CTL response to the virus during the asymptomatic phase of infection, and it is not possible to distinguish on the basis of CTL precursor frequency at this stage between the group who will progress to disease more rapidly, and those who will not progress or who will progress more slowly (slow progressors: SPs).⁴

One hypothesis, to explain this paradox, would be that there is a qualitative difference between the CTL responses shown

by SP donors and the progressors. We therefore identified a group of SP donors, and focused on those HLA class I molecules overrepresented in this group compared to a control group of progressors, initially in order to identify and define CTL responses made through these restriction elements.

One such restriction element of potential interest is HLA-B*57, since this and the closely related class I molecule HLA-B*58 are extremely common in African populations. For example, one of these two class I molecules is present in 43% of Zimbabwean⁵ and 40% of Ugandan populations.^{5a} In addition, HLA-B*57 is the single class I molecule that has been identified as occurring more frequently than expected in a preliminary study of the well-studied Amsterdam long-term nonprogressor (LTNP) cohort⁶ and also is a strong marker in a second LTNP study.⁷ Convincing HLA associations are exceedingly difficult to obtain,⁸ principally because of the large sample sizes required, the very large number of comparisons made, and also

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because of inaccuracies in 12–37% of serological class I typing, compared to methods that employ sequence-specific oligonucleotide primers now available^{9,10} (and M. Bunce, personal communication). The study by Kaslow *et al.*⁷ is the most thorough to date, and HLA-B*57 was second to HLA-B*27 as the marker most closely associated with nonprogression in both cohorts studied.

Those CTL epitopes restricted by one or both of the HLA class I molecules HLA-B*57 or -B*58, and that are also associated with long-term nonprogression in HIV infection, might therefore be important ultimately in vaccine development. A previous study of CTL epitopes within the Nef protein¹¹ noted that one HLA-B*57-restricted epitope was equally well presented by HLA-B*58 (not subtyped).¹² The possibility that the five novel HLA-B57-restricted epitopes identified here in SP donors are also “cross-restricted” by HLA-B*58 is explored. One of the five appears to be equally presented by HLA-B*5801 targets to HLA-B*57 effector cells, and is shown to be recognized also by HLA-B*5801 effectors on HLA-B*57 targets.

This study is designed to identify and define the CTL responses made by HLA-B*57- and HLA-B*58-positive donors as an initial component of a long-term longitudinal analysis in order to determine correlates of progression and nonprogression in HIV infection.

MATERIALS AND METHODS

Long-term slow progressor and progressor donors

Criteria for inclusion into the slow progressor (SP) study group were as follows: HIV infected for 7 years or more, asymptomatic, CD4 count in the normal range (above 400 cells/ μ l), and no history of antiretroviral therapy. Criteria for inclusion into the progressor group were as follows: HIV infected less than 5 years, CD4 count less than 400 cells/ μ l.

HLA class I tissue typing

HLA class I tissue typing was performed by polymerase chain reaction (PCR), using sequence-specific primers.^{13,14}

Identification of novel HLA-B57-restricted epitopes and putative B5801 cross-restricted epitopes

Seropositive donors with HLA-B*57 and HLA-B*58 were identified. Autologous B lymphoblastoid cell lines (BCLs) were infected for 16 hr with recombinant vaccinia viruses expressing a panel of HIV-1 proteins. Overlapping 20-mer peptides within the protein recognized were then tested for recognition. Shorter peptides within the 20-mer recognized were then synthesized, including 8- to 12-mers fitting the motif previously described for HLA-B*5801.¹⁵ HLA restriction and cross-restriction was then established using BCLs matched through single HLA class I molecules.

Having identified three novel HLA-B*57 epitopes that fitted the HLA-B*5801 motif, further putative epitopes consistent with this motif (alanine, valine, serine, or threonine at position 2; tryptophan or phenylalanine at the C-terminal position) were identified from the Gag, Pol, and Nef amino acid sequences¹⁶ and synthesized.

Generation of cytotoxic T lymphocyte cultures

Peripheral blood mononuclear cells (PBMCs) were separated from blood by Ficoll-Hypaque density gradient centrifugation. CTL “bulk” cultures were generated according to the protocol set out by Nixon *et al.*¹⁷ Briefly, one-fifth of PBMCs were phytohemagglutinin (PHA) activated and washed twice after 24 hr before being added back to the other four-fifths. Cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with antibiotics and 10% heat-inactivated fetal calf serum (FCS) (Globepharm, Esher, UK). After 7 days, 10% Lymphocult T (Biotest, Solihull, UK) was added to the medium. Peptide-specific lines were generated using irradiated autologous peptide-pulsed Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines as weekly feeders.

Vaccinia viruses

Vaccinia viruses used to infect targets were recombinants encoding gp120, Gag, Pol, Nef, and Tat of the HIV-1 BRU isolate, with the control vaccinia being a recombinant encoding the influenza protein PB2. The vaccinia constructs were supplied by the Medical Research Council AIDS Directed Programme (MRC ADP).

Cytotoxic T lymphocyte assays

Target cells (autologous or HLA class I-matched BCLs) were labeled with radioactive chromium and peptide pulsed for 1 hr prior to the assay. A total of 5×10^3 targets was used per well. Percent lysis was calculated according to the formula: $100 \times (E - M)/(T - M)$, where E is the chromium release from the supernatant of wells containing targets and effectors, M is the release from wells containing targets and medium only, and T is the release from wells containing targets and 5% Triton X. Background lysis (M/T) was less than 30% in all assays. Assays were harvested at 4–6 hr. Specific lysis was calculated by subtracting the percent lysis of targets pulsed with test peptide or infected with test vaccinia from the percent lysis of targets pulsed with no peptide or infected with control vaccinia, respectively.

Peptides

Overlapping peptides 20 amino acids in length were supplied by the MRC ADP. Shorter peptides 8–12 amino acids in length were synthesized on an automated peptide synthesizer (Advanced Chem Tech, Comberton, UK) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The purity of peptides was established by high-performance liquid chromatography (HPLC); they were then weighed and dissolved in RPMI. Peptides that did not dissolve easily in RPMI were initially dissolved in dimethyl sulfoxide (DMSO) to a maximum concentration of 1% DMSO at a peptide concentration of 100 μ M.

RESULTS

Ethnicity of donors within slow progressor and progressor groups

Thirty of the 33 donors within the SP group were Caucasian; the remaining 3 were Nigerian, Zimbabwean, and Thai. All 29 donors within the progressor group were Caucasian.

*Frequency of HLA-B*57 in slow progressor and progressor groups*

HLA-B*57 was present in 9 of the 30 Caucasians in the SP group studied (HLA-B*57-positive donors are listed in Table 1). Compared with the frequency of this class I molecule in the 2 of 29 Caucasian donors in the progressor group, HLA-B*57 is overrepresented in the SP group (odds ratio 5.79; 95% confidence interval [CI] 26.23–1.28; $p < 0.013$, using $2 \times 2 \chi^2$, not correcting for multiple comparisons). Not included in this comparison were the tissue types of the three non-Caucasians within the SP group, two of whom were HLA-B*57 positive, and one of whom was HLA-B*5801 positive (donor 19M). For comparison, the frequency of HLA-B*57 in the general population of British Caucasians is 11%.⁵

*Definition of a novel HLA-B*5801-restricted epitope, TSTLQEIQGW*

Bulk CTLs from donor 19M, a Zimbabwean, recognized an epitope in p24^{gag} within the 20-mer GSDIAGTTSTLQEIQGWMTN (Fig. 1a). The optimal peptide had the sequence TSTLQEIQGW (HIV-1 LAI Gag amino acid residues 250–260) (Fig. 1b) and was well recognized at concentrations of 10 nM by a peptide-specific line (Fig. 1c). The HLA restriction of the response was established as HLA-B*5801, but peptide-pulsed HLA-B*57 targets were also recognized by CTLs from this donor (Fig. 1d).

*Identification of an HLA-B*57-restricted response to the peptide TSTLQEIQGW by two HLA-B*57-positive slow progressors*

Cytotoxic T lymphocytes from four HLA-B*57-positive SP donors recognized this peptide TSTLQEIQGW bound to HLA-B*57-matched targets (Table 2; and CTLs from donor 422, not shown). This was the dominant response identified in donor 422

(not shown) and one of several strong responses identified donor 051. A peptide-specific line from donor 051 recognized the peptide equally well when presented by HLA-B*57 targets and HLA-B*5801 targets; but did not recognize the peptide on HLA-B*5802 targets (Fig. 2a).

Identification of four further HLA-B57-restricted epitopes in slow progressors

Bulk culture from donor 032 made a dominant response to Gag-vaccinia-infected autologous targets (Fig. 3a), and the 20-mer peptide within Gag best seen by these cells was p24.2, sequence VHQAISPRTLNAWVKVVEEK, HIV-1 LAI Gag amino acid sequence residues 143–162 (not shown). Using the HLA-B*5801 motif of serine, threonine, or alanine at position 2 and tryptophan or phenylalanine at position 9¹⁷ (Table 3), the predicted epitope sequence ISPRTLNAW (residues 147–155) was synthesized and tested, and was well recognized (Fig. 3b). Four other donors have made responses to this epitope (Table 2; and donor 949, not shown); in Nigerian donor 949 this was the dominant response (not shown).

The dominant Gag response seen in bulk CTLs from donor 998 was to the 20-mer NAWVKVVEEKAFSPEVIPMF (residues 153–172; not shown). Using the motif for HLA-B*5801, the peptides FSPEVIPMF and KAFSPEVIPMF (residues 162–172) were tested, and only the latter was well recognized (Figs. 3c and 2b). This epitope was also well seen by CTLs from other donors (Table 2).

The panel of 19 putative HLA-B*57-restricted peptide epitopes, which was derived using the peptide motif for HLA-B*5801,¹⁷ was tested for recognition by bulk CTLs from several donors. The Nef peptide GPGVRYPLTFGWY (residues 130–143) was strongly recognized by CTLs from four donors, and there was recognition of another Gag peptide, QASQDVKNW (residues 309–317), by CTLs from two donors (Table 2).

TABLE 1. ETHNICITY, HLA CLASS I A AND B TYPES, LATEST CD4 COUNT, AND DURATION/DATE OF INFECTION OF HLA-B57 AND HLA-B5801 DONORS STUDIED^a

Donor/ ethnic group	HLA class I A*/ B* tissue type	CD4 count (cells/ μ l)	Duration/date of infection (years/year infected)
Slow progressors			
031 (Caucasian)	A*01/*02 B*51/*57	410	13/1983
032 (Caucasian)	A*01/*32 B*44/*57	1190	13/1983
051 (Caucasian)	A*01/*24 B*44/*57	620	13/1983
058 (Caucasian)	A*28/*29 B*51/*57	520	11/1983
422 (Caucasian)	A*24/*31 B*27/*57	1040	9/1987
998 (Caucasian)	A*01/*01 B*52/*57	400	7/1989
220 (Caucasian)	A*02/*02 B*35/*57	480	10/1986
824 (Caucasian)	A*01/*02 B*44/*57	580	12/1984
1M (Caucasian)	A*01/*02 B*07/*57	850	8/1988
19M (Zimbabwean)	A*11/*30 ₀₂ B*52/*5801	490	7/1989
20M (Thai)	A*02/*33 B*61/*57	520	10/1987
949 (Nigerian)	A*23/*33 B*45/*57	480	7/1989
Progressors			
196 (Caucasian)	A*03/*03 B*35/*57	320	5/1991
5M (Caucasian)	A*02/*03 B*07/*57	210	6/1990

^aDonor 058 died in 1994 from HIV-unrelated causes, and tissue typing of this donor was performed using standard serological methods.

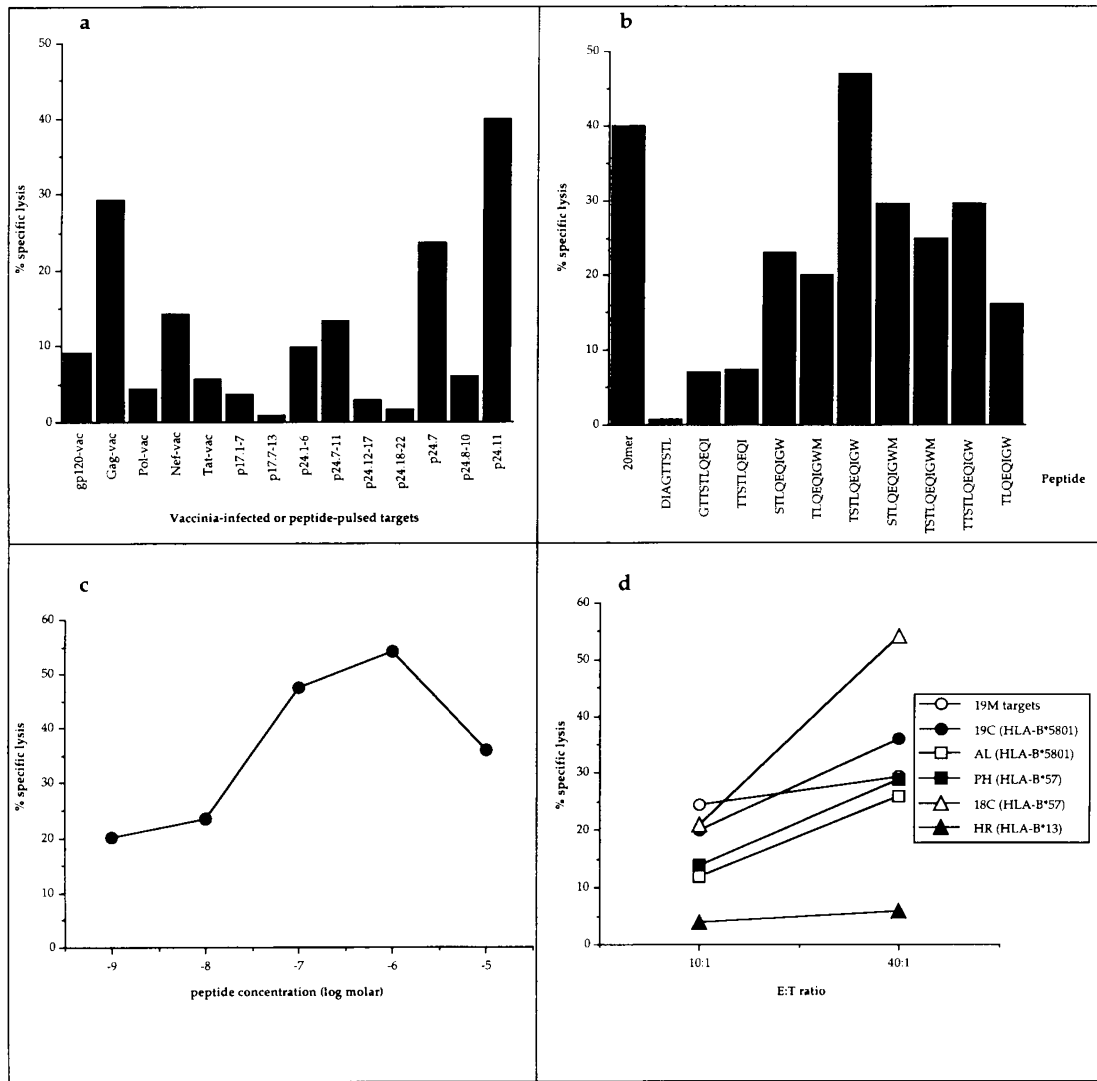


FIG. 1. (a) Recognition of Gag-vaccinia-infected and peptide-pulsed autologous targets by bulk CTLs from donor 19M (HLA class I type: A*11/30.02 B*52/5801). Effector:target (*E:T*) ratio, 40:1; p17.1-13, overlapping 15-mers spanning p17^{gag} protein; p24.1-22, overlapping 20-mers spanning p24^{gag} protein; p24.1-6, pool of six 20-mers p24.1-p24.6 inclusive; other pools designated likewise. The 20-mers recognized: p24.7 (sequence GHQAAMQMLKETINEEAAEW, HIV LAI positions 193-212) and p24.11 (GSDIAGTTSTLQEQIGWMTN, positions 233-252). Lysis of vaccinia-influenza PB2 control subtracted (4.1%) and lysis of targets pulsed with no peptide (1.2%) subtracted to calculate percent specific lysis. (b) Optimization of epitope within p24.11, sequence GSDIAGTTSTLQEQIGWMTN. The 8-12-mers were assayed at 1 μ M concentrations, the 20-mer assayed at 10 μ M. Lysis of targets pulsed with no peptide (3.2%) subtracted. (c) Titration of optimal epitope, TSTLQEQIGW. Effectors, peptide-specific line; *E:T* 12:1. Lysis of targets pulsed with no peptide (1.4%) subtracted. (d) Presentation of TSTLQEQIGW on HLA-B57 and HLA-B5801 targets to HLA-B5801 effectors: 19M (HLA-A*11/30.02 B*52/5801). Bulk cultured lymphocytes, *E:T* shown. Other HLA class I A and B types: 19C, A*11/30.02 B*8/5801; AL, A*3/24 B*18/5801; PH, A*28/29 B*51/57; 18C, A*1/3 B*7/57; HR, A*30.01/- B*13/-.

TABLE 2. HLA-B*57-RESTRICTED CTL RESPONSES OF FOUR DONORS TESTED FOR RECOGNITION OF FIVE NOVEL EPITOPES DESCRIBED AND ONE PREVIOUSLY DESCRIBED^a

Donor	Percent lysis of targets peptide pulsed with:						
	No peptide	ISPRTLNAW	KAFSPEVIPMF	TSTLQEQIGW	QASQDVKNW	GPGVRYPLTFWCY	HTQGYFPDW
032	2	43	24	20	-6	0	11
051	12	29	30	50	54	67	26
998	12	25	34	29	25	53	10
824	7	56	55	6	7	44	8
1M	-2	2	28	0	2	38	1

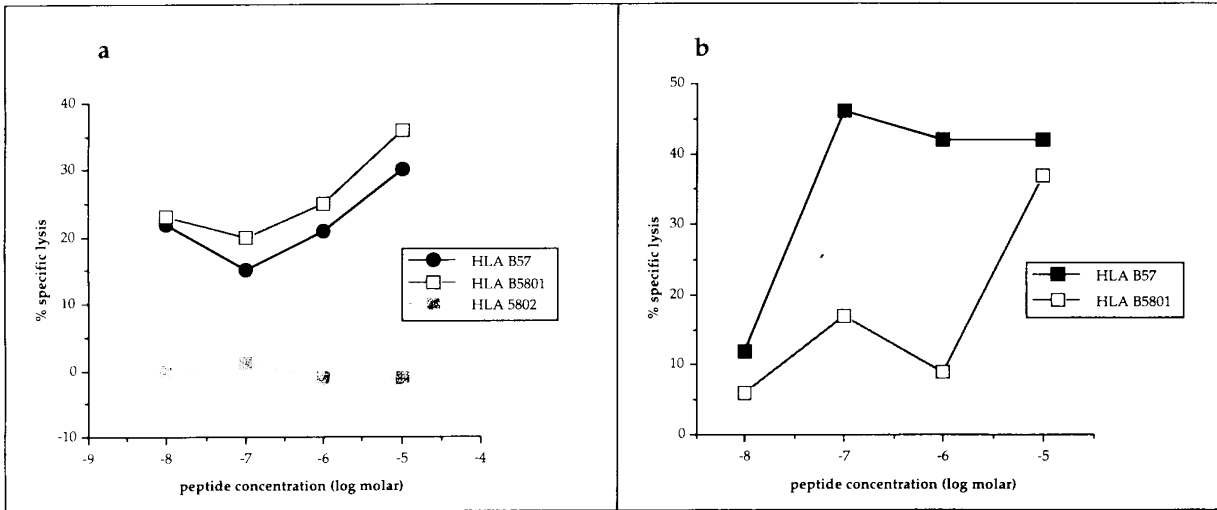


FIG. 2. (a) Presentation of TSTLQEIQGW by HLA-B*57, HLA-B*5801, and HLA-B*5802 targets to HLA-B*57-positive effectors: 051 (HLA-A*1/24 B*44/57). Peptide-specific line, *E:T* 5:1. Lysis of targets pulsed with no peptide (3.2%) subtracted. (b) Presentation of KAFSPEVIPMF by HLA-B*57 and HLA-B*5801 targets. Effectors: 998 (HLA-A*1/- B*52/57), peptide-specific line, *E:T* 5:1. Lysis of targets pulsed with no peptide (6%) subtracted.

These four epitopes (ISPRTLNAW, KAFSPEVIPMF, QASQDVKNW, and GPGVRYPLTFGWY) were shown to be HLA-B*57 restricted but not HLA-B*5801 cross-restricted (data not shown), except at high peptide concentrations (Fig. 2b). ISPRTLNAW and KAFSPEVIPMF are found within 20- to 22-mers (respectively, VHQAISPRTLNAWVKVVEEKAF and NAWVKVVEEKAFSPEVIPMFSA) that have previously been shown to be HLA-B*57 restricted.¹⁸

*Immunodominant cytotoxic T lymphocyte responses in HLA-B*57- and HLA-B*5801-positive donors*

The dominant HIV-specific CTL response in a donor was defined by the peptide best recognized by "bulk" cultured lymphocytes on peptide-pulsed autologous targets. The three HLA-B*57-restricted responses identified in donor 824 were the dominant responses observed. The only response identified that was

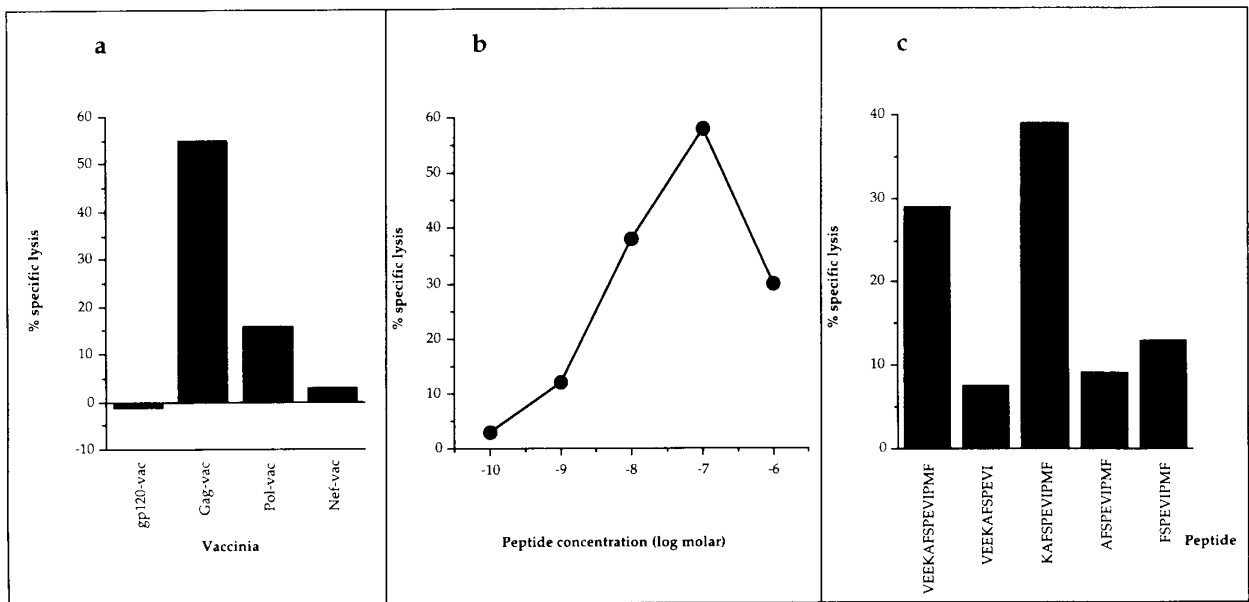


FIG. 3. (a) Recognition of vaccinia-infected autologous targets by bulk lymphocyte culture from donor 032 (HLA-A*1/32 B*44/57). *E:T* ratio 50:1. Lysis of targets infected with control vaccinia (PB2-vac; 5.0%) subtracted. (b) Titration of peptide ISPRTLNAW. Effectors: peptide-specific line from donor 032, *E:T* 10:1. Lysis of targets pulsed with no peptide (1.8%) subtracted. (c) Optimization of epitope KAFSPEVIPMF within 15-mer VVEEKAFSPEVIPMF. Effectors: peptide-specific line from donor 998, *E:T* 8:1. Lysis of targets pulsed with no peptide (6.1%) subtracted. Peptide concentrations all 1 μ M.

TABLE 3. PEPTIDE MOTIF FOR HLA-B*5801^a
AND CONSISTENCY OF NOVEL HLA-B*57-RESTRICTED
EPITOPES WITH THIS MOTIF^b

HLA B5801 motif ^c	
Position 2	Position 9
S	W
T	F
A	

HIV-1 HLA B57- and B5801-restricted epitopes ^d	
Sequence	Epitope
YHTQGYFPDWQ	(Nef 115–125) ^e
GPGVRYPLTFGW <u>WCY</u>	(Nef 130–143)
ISPRTLNA <u>W</u>	(Gag 147–155)
KAFSPEVIPMF	(Gag 162–172)
TSTLQEQIGW	(Gag 240–249)
QASQDVKNW	(Gag 309–317)

^aSee Ref. 15.

^bPreviously identified cross-restricted epitope YHTQGYFPDWQ^{11,12} and currently identified optimized epitopes shown. Predicted anchor residues underlined.

^cSee Ref. 17.

^dAll except HTQGYFPDW described in this article.

^eSee Ref. 11.

directed through non-HLA-B*57 class I molecules was a relatively weak response to the HLA-A*2-restricted epitope ILKEPVHGV¹ (Fig. 4). In all seven HLA-B*57 or HLA-B*5801 donors in the SP group tested, the dominant CTL response was made through the HLA-B*57 or HLA-B*5801 molecule. In the single HLA-B*57-positive donor (196) in the progressor group whose bulk CTLs were tested, there was no recognition of Gag- or Pol-expressing HLA-B*57-matched targets, and the only peptide recognized by CTLs from this donor was the HLA-B*35-restricted epitope, VPLRPMY¹ (not shown).

DISCUSSION

A candidate HIV vaccine would ideally incorporate epitopes that dominate CTL responses in SP donors. Importantly, these epitopes would also need to be restricted by HLA class I molecules commonly found in ethnic groups where the prevalence of HIV infection is high.

It is easy to identify which HLA class I molecules occur frequently in areas of high HIV prevalence, but exceedingly difficult to demonstrate a significantly high frequency of any particular MHC class I in a group of LTNP donors because the *p* value must be multiplied by the number of possible MHC class I types and subtypes, of which there are well over 100. In this case, we have examined a single class I molecule identified in different studies^{6,7} as being more frequent than expected in an SP group. Although the number of slow progressors and progressors in each group is fairly small, the observation that HLA-B*57 is found at higher than expected frequencies in these three

independent studies is highly suggestive of an association of HLA-B*57 with slow progression in HIV-1 infection.

Following the description of a "cross-restricted" HLA-B*57-restricted CTL epitope within Nef that was equally presented by HLA-B*58 targets,¹² and the identification of HLA-B*57 as a class I molecule overrepresented in two SP groups,^{6,7} we sought new HLA-B*57-restricted responses in a group of SP donors and tested for cross-restriction in these responses. We have identified five new HLA-B*57-restricted epitopes in SP donors. One of these new epitopes is cross-restrictive, and indeed a dominant CTL response to the cross-restricted epitope TSTLQEQIGW was identified in both HLA-B*57 donors and an HLA-B*5801 donor.

The only difference in residues contributing to the binding of peptides between HLA-B*57 and HLA-B*5801 is at position 45, within the B pocket, where a methionine in HLA-B*57 is replaced by a threonine in HLA-B*58.¹⁹ This is a fairly minor change, so it is not surprising that the anchor residues of peptides binding to these two molecules appear to be the same. Elution of peptides from C1R-B*5801 molecules revealed that these anchors are serine, threonine, or alanine at position 2, which would bind into the B pocket; and tryptophan or phenylalanine at the C-terminal positions 9–11.¹⁵ All the peptide epitopes newly described here fit this motif, as does the cross-restricted epitope previously described¹² (Table 1).

The anchor residues for peptides binding to HLA-B*5802 may not be the same as for HLA-B*57 and HLA-B*5801, as there are differences in the critical residues involved,^{19,20} and effectors did not see HLA-B*5802 targets pulsed with the pep-

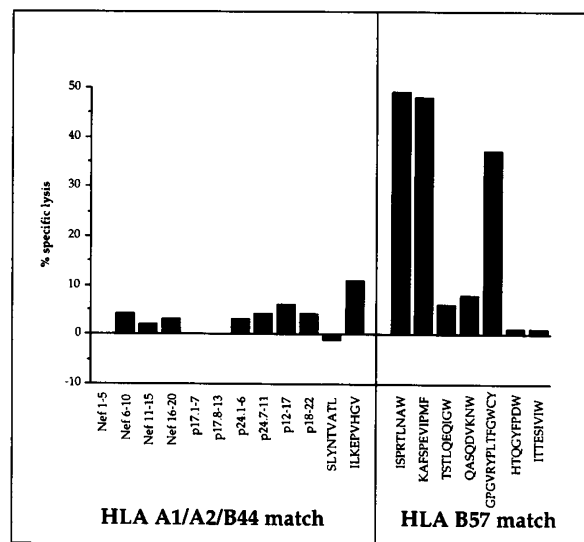


FIG. 4. Immunodominance of HLA-B*57-restricted responses in donor 824 (HLA class I type: A*1/2 B*8/44). Effectors: bulk cultured lymphocytes; *E:T* 50:1. Targets: HLA-A*1/2 B*44-matched BCL targets pulsed with pools of 20-mers spanning the Nef and Gag proteins and two described¹ HLA-A*2-restricted epitopes, SLYNTVATL and ILKEPVHGV. HLA-B*57-matched targets (HLA class I type: A*24/31 B*27/57) pulsed with HLA-B*57-defined epitopes or putative epitopes. Lysis of unpulsed targets subtracted (4.0% HLA-A*1/2 B*44-matched targets; 7.0% HLA-B*57-matched targets).

tide TSTLQEIQGW, when similarly pulsed HLA-B*57 and HLA-B*5801 targets were seen. The probable explanation for this is the presence of a tryptophan residue at position 97 in HLA-B*5802, in place of an arginine at this position in HLA-B*5801. This could affect binding in the F pocket as well as the interaction with contact residues in the peptide.^{19,20}

It is striking that the CTL responses of the donors are all dominated by HLA-B*57- or HLA-B*5801-restricted responses (for example, see Figs. 1a and 4). The reason for this is not known. However, it may provide an additional clue to the explanation for HIV-infected individuals with HLA-B*57 being overrepresented in the SP group.

Finally, it is notable that the four epitopes described here within Gag all are strongly conserved. In HIV-1 amino acid sequences listed in the Los Alamos database¹⁶ and in a study of 70 HIV-1 international isolates,²¹ there are no variants within 32 B clade strains listed for KAFSPEVIPMF, and one variant only for TSTLQEIQGW (alanine for glycine at position 9) and the ISPRTLNAW epitope (leucine for isoleucine in position 1). QASQDVKNW is similarly conserved, but with relatively common changes at position 5 of glutamate for aspartate. The GPGVRYPLTFGWY epitope within Nef is more variable, conservative changes at positions 4 (isoleucine or threonine for valine) and 6 (phenylalanine or tryptophan for tyrosine) being seen in database sequences.

Conservation within database epitope sequences may mean that escape mutation in these regions is constrained without damaging the viability of the virus. This may in turn relate to slow progression in HIV infection, as CTL responses directed toward such regions may be more long-lasting. The conservation of these HLA-B*57-restricted epitope sequences supports the evidence that they may represent qualitatively important components of the immune response in slow progressor individuals in HIV infection.

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