HIV-1 Variation Diminishes CD4 T Lymphocyte Recognition

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Summary

Effective long-term antiviral immunity requires specific cytotoxic T lymphocytes and $CD4^+$ T lymphocyte help. Failure of these helper responses can be a principle cause of viral persistence. We sought evidence that variation in HIV-1 $CD4^+$ T helper epitopes might contribute to this phenomenon. To determine this, we assayed fresh peripheral blood mononuclear cells from 43 asymptomatic HIV-1^+ patients for proliferative responses to HIV-1 antigens. 12 (28%) showed a positive response, and we went on to map dominant epitopes in two individuals, to p24 Gag restricted by human histocompatibility leukocyte antigen (HLA)-DR1 and to p17 Gag restricted by HLA-DRB52c. Nine naturally occurring variants of the p24 Gag epitope were found in the proviral DNA of the individual in whom this response was detected. All variants bound to HLA-DR1, but three of these peptides failed to stimulate a $CD4^+$ T lymphocyte line which recognized the index sequence. Antigenic variation was also detected in the p17 Gag epitope; a dominant viral variant present in the patient was well recognized by a specific $CD4^+$ T lymphocyte line, whereas several natural mutants were not. Importantly, variants detected at both epitopes also failed to stimulate fresh uncultured cells while index peptide stimulated successfully. These results demonstrate that variant antigens arise in HIV-1^+ patients which fail to stimulate the T cell antigen receptor of HLA class II–restricted lymphocytes, although the peptide epitopes are capable of being presented on the cell surface. In HIV-1 infection, naturally occurring HLA class II–restricted altered peptide ligands that fail to stimulate the circulating T lymphocyte repertoire may curtail helper responses at sites where variant viruses predominate.

Key words: human immunodeficiency virus • CD4 • immune escape • altered peptide ligands

Loss and malfunction of $CD4^+$ lymphocytes is one of the hallmarks of HIV-1 infection (1). Before severe $CD4^+$ lymphocyte depletion occurs, defective proliferation of these cells to recall antigens is common (2, 3). Few studies have detected specific proliferative responses to HIV-1–derived antigens in HIV^+ individuals. Recently, Rosenberg et al. (4) detected p24 Gag–specific helper responses in two HIV-1^+ individuals with long-term nonprogressive disease. They also found that the use of potent highly active antiretroviral therapy restored p24 Gag proliferative responses.

Antigenic variation in HIV-1 HLA class I–restricted epitopes has been shown to cause loss of T cell recognition (5–8). Some of these variant antigens failed to bind to the HLA class I molecule (9, 10). Other epitope variants failed to trigger the full T cell signaling cascade (11) despite partial engagement of the TCR. Studies carried out on HLA class II–restricted T cell responses in HIV-1 seronegative individuals showed that CD4 responses were also influenced by antigenic variation (12), but further work on Th cells has been frustrated by the difficulty of detecting such responses in HIV-1^+ individuals at any stage of infection. We detected responses in asymptomatic HIV-1^+ patients who had not received any antiretroviral therapy, many of whom had long-term disease. We were also able to localize a dominant $CD4^+$ lymphocyte response to HIV-1 Gag peptides in two individuals. Proviral DNA sequencing revealed substantial sequence variation within both these peptide epitopes. In general, epitope variation did not diminish binding to class II molecules, although several of the variants detected failed to stimulate proliferation of both fresh PBMCs and cultured T cell lines.

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Materials and Methods

Patient Details. 43 HIV-1$^+$ patients were screened for CD4$^+$ lymphocyte proliferative responses. The duration of infection varied from 10 to <1 yr; all the patients were asymptomatic.

Peptides and Recombinant Antigens. Peptides were obtained from the Medical Research Council AIDS Directed Programme and included 22 20-mers overlapping by 10 amino acids (aa)[1] covering all of p24 Gag (aa 133–363 of HIV-1 SF2) and 13 15-mers covering all of p17 Gag overlapping by 5 aa (aa 1–134 of HIV-1SF2). Synthetic peptides representing the variant viral sequences detected in the blood of the patients were manufactured by standard f-moc chemistry using an automated synthesizer (model SMP2 350; Zinsser Analytic GmbH, Frankfurt, Germany). All peptides were analyzed by HPLC and found to be >90% pure. They were dissolved in RPMI and stored at −20°C in aliquots.

Proliferation Assays of Fresh PBMCs. PBMCs were separated from whole blood by centrifugation on a density gradient (Lymphoprep; Nycomed Amersham plc, Little Chalfont, Bucks, UK) and were washed and cultured in RPMI 1640 containing glutamine, penicillin/streptomycin, and 10% heat-inactivated human A–positive serum. PBMCs were cultured with or without antigens in medium at 2×10^5 cells/well in round-bottomed 96-well plates in a total volume of 200 μl. Triplicate wells were set up for each antigen, which included PHA and tetanus toxoid (2 μg/ml), and the pooled peptides covering all of p24 or p17 at 1 and 0.2 μM concentrations. After 72 h, cultures were pulsed with 1 μCi of [^{3}H]thymidine per well for 16 h before harvesting and counting in a flat-bed scintillation counter (Wallac, Turku, Finland). Results are expressed either as cpm or stimulation indices (SI = cpm in the presence of antigen/cpm without antigen). An SI >2.5 was regarded as positive. As variant peptides were identified by sequencing, some of these were tested in fresh proliferation assays.

Isolation of T Cell Lines. PBMCs were separated from whole blood, and the CD8$^+$ lymphocytes were removed using Dynabeads (Dynal A.S., Oslo, Norway). The CD8-depleted cells were then grown in culture medium at 4×10^6 in 1 ml for 6 d in the presence of 10 μg of the relevant pooled peptides. Lymphoblasts were separated on Percoll on day 7 and then cultured in medium containing 10% IL-2 (Lymphocult-T; Biotest AG, Frankfurt, Germany). Lines were expanded using an alternating cycle of restimulation with peptide pool plus irradiated (30 Gy) autologous PBMCs as APCs followed by culture in IL-2. The epitopes were mapped using individual peptides, and the phenotype of the cells was determined by FACScan® (Becton Dickinson, San Jose, CA) analysis. Once the individual peptide epitopes had been identified, the lines were maintained using the relevant peptide at 0.1 μg/ml as the antigenic stimulus.

Proliferation assays on the T cell lines were performed as described for fresh PBMCs using 5×10^4 T cells plus 10^5 irradiated (30 Gy) autologous PBMCs as APCs in a final 200 μl volume.

Identification of Restricting HLA Class II Molecules. To identify the restricting HLA class II isotype of the T cell lines, antigen presentation by autologous APCs was assayed after addition of mAb against HLA-DR (L243), -DQ (Genox 3.53), or -DP (B7.21) at the beginning of the culture time. To identify the restricting allele, HLA-sharing or mismatched PBMCs from healthy donors were used as APCs. Mouse cells (L cells) transfected with HLA-DRB52c (obtained from Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) were used as APCs to identify T cells restricted by this allele using uninfected L cells as control. In brief, 5×10^4 T cells were cocultured with mouse L cells (5×10^4/well) plus 1 μM of the relevant peptide.

HLA Typing. HLA typing was carried out using sequence-specific primer PCR as described previously (13).

Detection of Secreted IL-2. Identical cultures were set up in a separate 96-well plate for proliferation assays and for detection of secreted IL-2. Additionally, 20 μl of anti–IL-2R antibody was added to each well to prevent the use of secreted IL-2 by the cells. 100 μl of supernatant was collected from the T cell cultures after 7 d and frozen at −20°C until ready to assay. The IL-2–dependent cell line CTLL was used to assay for IL-2 production. 8×10^3 CTLL cells per well were grown in RPMI plus 20% FCS in the presence of doubling dilutions of cell culture supernatants or in known concentrations of IL-2 standard. After 24 h of culture, the plate was labeled with [^{3}H]thymidine for 16 h and then harvested. The amount of IL-2 produced was assessed according to the standard curve obtained using known amounts of IL-2.

Antagonism Assay. Antagonism assays were carried out according to the method of De Magistris et al. (14). APCs (autologous or HLA class II–matched PBLs) were pulsed with a suboptimal dose of the relevant index peptide for 2 h at 37°C and then washed twice. They were then pulsed for an additional 2 h with varying concentrations of potential antagonist peptides, washed, and irradiated. 10^5 of these APCs were added to wells containing 2.5×10^4 T cells along with the relevant controls and cultured for 72 h before labeling with [^{3}H]thymidine and harvesting.

Sequencing of Proviral DNA. DNA was purified from patients' PBMCs after 24–48 h of culture with PHA and extracted using proteinase K. PCR primers were as follows: for p24-1, external 5′ primer 5′-GTAAGAAAAAAGCACAGCAAGC-3′, external 3′ primer 5′-TTTCTCCTACTGGGATAGGTGG-3′, internal 5′ primer 5′-GCTAGAATTCCCAGCAAGCAGCAGCTGACA-3′, and internal 3′ primer 5′-CTAGATCTAGATGGATTATTTGTCATCCATCC-3′; for p17-3, external 5′ primer 5′-ACTAGCGGAGGCTAG-3′, external 3′ primer 5′-GTTCTAGGTGATATGG-3′, internal 5′ primer 5′-GCTAGAATTCCATGGGTGCGAGAGCGT-3′, and internal 3′ primer 5′-CAGTTCTAGATCTAGTATAATTTTGGCTGACC-3′. At any one time point, at least 20 M13-HIV clones were sequenced to identify the viral variants present (8).

Peptide Binding Studies. These were carried out according to the method of Davenport et al. (15) using a competitive binding assay. This determines the ability of a peptide to compete with biotinylated invariant chain (Ii) for binding to affinity-purified MHC class II molecules. The amount of biotinylated Ii bound was quantitated using an avidin–horseradish peroxidase conjugate system. The results were expressed as IC_{50}, the concentration of peptide necessary to reduce the binding of Ii to 50% of the noninhibited value.

Results

Screening

43 HIV-1 seropositive patients were screened for proliferative responses to the mitogen PHA, the recall antigen tetanus toxoid, and HIV-1 antigens consisting of pooled peptides covering Gag p24 and p17 (Table 1). 19 individuals (44%) showed a response to PHA only, 12 (28%) a response to PHA and tetanus toxoid only, and the remaining 12 (28%) a response to PHA, tetanus toxoid, and one or

[1]*Abbreviations used in this paper:* aa, amino acid(s); Ii, invariant chain; SI, stimulation index.

Table 1. *Summary of Patient Clinical Data and Proliferative Responses*

Proliferative responses to:	PHA only	PHA and tetanus toxoid only	PHA, tetanus toxoid, and HIV antigens	Total
No. of patients	19 (44%)	12 (28%)	12 (28%)	43
Mean CD4 counts (cells/μl)	351 ± 169	350 ± 153	497 ± 148	398 ± 167
Length of time of infection (yr)	<1–10	<1–8	<1–4	<1–10
Mean proliferation (SI) to:				
PHA	49 ± 53	78 ± 118	138 ± 170	82 ± 118
Tetanus toxoid	<1	45 ± 117	31 ± 32	21 ± 65
HIV antigens	<1	<1	15 ± 18	5 ± 11

Results are expressed as SI = mean cpm of PBMCs plus antigen/mean cpm of PBMCs alone. *HIV antigens*, pools of overlapping peptides covering all of Gag p24 or Gag p17.

both of the HIV-1 antigens. All cultures in which a proliferative response to HIV-1 peptides was detected also showed an increase in IL-2 production. T cell lines from two patients, 744 and 024, were then used to map and characterize the natural epitopes that evoked these HIV-1 proliferative responses.

When the epitopes had been mapped and their natural variants identified, proliferation assays were performed on fresh PBMCs from the two patients to assess the capacity of HLA-bound altered peptides to stimulate the prevailing T cell specificities.

Patient 744

Epitope Mapping. Patient 744 is a male Caucasian homosexual who acquired HIV-1 in 1993. His initial CD4 count was 580 cells/μl on day 0 of the study and had declined to 280 by day 323. His HLA type is A24/74; B35/44; DR1/12, DR52. A proliferation assay performed on fresh PBMCs from this patient showed a response to PHA, tetanus toxoid, and pooled p24 peptides but not to p17. An 8-wk-old T cell line showed that a single dominant epitope, p24-1, representing aa 1–20 of p24 Gag (PIVQNLQGQMVHQAISPRTL) was recognized (Fig. 1 *A*). FACScan® analysis revealed this line to be 97% $CD4^+$, 0% $CD8^+$. After antigen stimulation, the T cell line produced IL-2, consistent with a Th1 phenotype (data not shown). HLA-matched and mismatched PBMCs used as APCs, and blocking studies using mAbs to HLA-DR, -DQ, and -DP, revealed the HLA class II–restricting molecule to be HLA-DR1 (Fig. 1 *B*). p24-1 was the sole antigen recognized repeatedly from Gag in four assays performed on blood samples taken over a 1- and an 18-mo period. Assays of fresh PBMCs confirmed the response to p24-1 (Table 2).

Epitope Variation. Nine variants of the p24-1 epitope in 744 HIV-1 proviral DNA were detected and synthesized (Fig. 2). Other shorter (11-mer) peptides were also made to determine the requirement for binding to HLA-DR1. These peptides were also tested for their affinity for HLA-DR1.

Peptide Binding. Peptide binding is shown in Table 3. Truncated peptide studies revealed that loss of the NH_2-terminal aa PIVQ (p24-1/13) abolished binding. Loss of

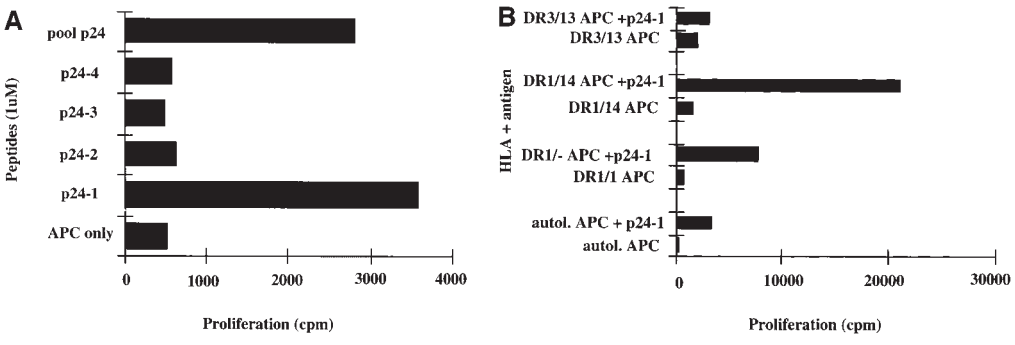

Figure 1. (*A*) Identification of HIV p24 Gag epitope recognized by T cell line 744. Proliferation assays were performed by culturing 5×10^4 T cells with 10^5 irradiated (30 Gy) autologous PBMCs as APCs with or without 1 μM peptide for 3 d. Peptides used to verify the specificity of the Gag response were as follows: p24-1, aa 133–153, PIVQNLQGQMVHQAISPRTL; p24-2, aa 143–163, VHQAISPRTLMAWVKVVEEK; p24-3, aa 153–173, MAWVKVVEEKAFSPEVIPMF; and p24-4, aa 163–183, AFSPEVIPMFSALSEGATQP. Cells were labeled with 1 μCi [^{3}H]thymidine per well for 16 h, harvested, and counted on a beta counter. *APC only*, cpm obtained for T cells and APCs only. (*B*) Identification of the MHC haplotype that restricts presentation of antigen to T cell line 744. HLA class II–restricting elements were determined by proliferation assays using HLA-matched and mismatched PBMCs from healthy donors as APCs in the presence or absence of 1 μM peptide p24-1. HLA type of patient 744 (*autol.APC*) was A24/74;B35/44;DR1/12, DR52. Donor DR1/− was homozygous for DR1.

Table 2. *Proliferative Responses of Fresh PBMCs from Patients 744 and 024 over Time*

Patient 744				
Day of bleeding	Day 0	Day 56	Day 178	Day 498
PHA	44	12	385	nt
Tetanus toxoid	40	nt	6	nt
Gag p24 peptide pool	10	3.3	3.2	19
p24-1	nt	3.2	5.6	11
p24-1/16 variant	nt	nt	nt	1.1
Patient 024				
Day of bleeding	Day 0	Day 29	Day 271	
PHA	239	126	400	
Tetanus toxoid		98	nt	
Gag p17 peptide pool	63	25	18	
p17-3	24	21	25	
p17-3/1	nt	nt	1	
p17-3/2	nt	nt	1.1	
p17-3/3	nt	nt	3.7	
p17-3/4	nt	nt	1.3	

Results are expressed as SI = mean cpm of PBMCs + antigen/mean cpm of PBMCs alone. SI >2.5 was regarded as a positive response. *nt*, not tested.

the COOH-terminal aa sequence AISPRTL (P24-1/11) did not interfere with binding. These results imply that the core sequence VQNLQGQMV (p24-1/12) is sufficient to mimic binding (but not necessarily antigenicity) of the natural epitope. Subsequent studies showed the importance of the NH_2-terminal aa PI for TCR recognition of this epitope.

Two other peptide sequences, p24-1/18 (HIV-1 consensus H) and 20 (HIV-1; our unpublished results), bound well to HLA-DR1. Of the nine natural variants from patient 744, all bound well. Although variant p24-1/19 bound less well to HLA-DR1, this peptide stimulated T cells, so this difference was not functionally significant in this assay system.

CD4 T Lymphocyte Responses to p24-1 Peptide Variants. Of five truncated peptides tested, only p24-1/11 stimulated a line from patient 744 (Fig. 3). Since both p24-1/11 and 12 bound well in the assay, this result implies that NH_2-terminal proline and isoleucine were required for T cell recognition. The peptides p24-1/13, 14, and 15 failed to bind and failed to stimulate the T cell line.

Natural Variants. In assays of fresh PBMCs, the most common variant, p24-1/16, failed to stimulate proliferation (Table 2), although this sequence bound well (Table 3), whereas the index peptide p24-1 elicited a significant response. This result was confirmed when p24-1/16 and other variants were tested against a T cell line from this patient. Three peptides, p24-1/21, 23, and 25, elicited an SI <1 and yet had satisfactory binding (Table 3). Thus, loss of recognition was likely to be caused by loss of TCR engagement or signaling, rather than failure of presentation.

To investigate whether these variant peptides could antagonize the CD4 helper response to virus, we identified two peptides (p24-1/16 and p24-1/25; see Table 3) in which there was no T cell recognition but in which the aa changes were conservative as potential antagonists (14). The p24-1/16 viral variant comprised 100% of the virus in this patient on day 0 and was a common variant subsequently (Fig. 2). Despite several attempts to demonstrate an antagonist effect with peptides (p24-1/16 and p24-1/25) on the response to wild-type p24-1, no inhibition was seen.

Patient 024

Epitope Mapping. Patient 024 is a male Caucasian HIV-1^+ homosexual. His CD4 count was 780 on day 1 of the study and 800 on day 335. His HLA type is A3/30; B13/37; DR15/13.02; DR51/52; DQ6. PBMCs from this patient showed a strong proliferative response to p17 pooled

EPITOPE 403	P CCT	I ATA	V GTG	Q CAG	N AAC	L CTC	Q CAG	G GGG	Q CAA	M ATG	V GTA	H CAT	Q CAG	A GCC	I ATA	S TCA	P CCT	R AGA	T ACT	L TTA	462		
Peptide																						Frequency (%)	Days after initial sample
p24-1/16	P	I	V	Q	N	I ATC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		100	Day 0
p24-1	P	I	V	Q	N	L	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		9	Day 182
p24-1/16	P	I	V	Q	N	I ATC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		55	
p24-1/21	P	I	A GCG	Q	N	I ATC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		9	
p24-1/23	P	M ATG	V	Q	N	I ATC	R CGG	G	Q	M	V	H	Q	A	I	S	P	R	T	L		9	
p24-1/24	P	I	V	Q	N	I ATC	Q	G	Q	V GTG	V	H	Q	A	I	S	P	R	T	L		9	
p24-1/25	P	I	V	E GAG	N	I ATC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		9	
p24-1/16	P	I	V	Q	N	I ATC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		31	Day 323
p24-1/17	P	I	V	Q	N	Q CAG	Q	G	Q	M	V	H	Q	P CCC	I	S	P	R	T	L		13	
p24-1/19	P	I	V	Q	N	L	Q	G	Q	M	V	H	Q	P CCC	I	S	P	R	T	L		44	
p24-1/29	F TTT	I	V	Q	N	A GCC	Q	G	Q	M	V	H	Q	A	I	S	P	R	T	L		6	
p24-1/30	P	I	V	E GAG	N	I ATC	Q	G	Q	I ATA	I ATA	H	Q	A	I	S	P	R	T	L		6	

Figure 2. Naturally occurring variants of p24-1 Gag from patient 744. Proviral DNA was purified from PBMCs of patient 744 collected at intervals. The region of interest was amplified by nested PCR and sequenced. At least 20 clones were studied to identify any variants.

Table 3. *The Binding Affinity (IC_{50}) of p24-1 Variant Peptides to HLA-DR1*

Peptide no.	Peptide	Binding results	
		IC_{50} av.*	IC_{50}/index‡
Truncated peptides			
p24-1/11	PIVQNLQGQMV	0.07	1.75
p24-1/12	VQNLQGQMVHQ	0.08	2
p24-1/13	NLQGQMVHQAI	8.74	475
p24-1/14	QGQMVHQAISP	11.77	600
p24-1/15	QMVHQAISPRTL	6.62	325
p24-1/26	VQNLQGQMV	12.79	450
p24-1/27	PGVQNLQGQMVHQ	0.09	2.25
p24-1/28	PIGQNLQGQMVHQ	0.64	16
Ii	LPKPPKPVSKMRMATPLLMQ	0.04	1
HA	PKYVKQNTLKLAT (306–318)	0.14	3.5
Natural database variants§			
p24-1/18	PIVQNAQGQMVHQAISPRTL	0.04	1
p24-1/20	PIVQNLRGQMVHQAISPRTL	0.91	22.75
Natural 744 variants			
p24-1	PIVQNLQGQMVHQAISPRTL	0.04	1
p24-1/16	PIVQNIQGQMVHQAISPRTL	0.1	2.5
p24-1/17	PIVQNQQGQMVHQPISPRTL	0.1	2.5
p24-1/19	PIVQNLQGQMVHQPISPRTL	1.19	29.75
p24-1/21	PIAQNIQGQMVHQAISPRTL	0.16	4
p24-1/23	PMVQNIRGQMVHQAISPRTL	0.01	0.25
p24-1/24	PIVQNIQGQVVHQAISPRTL	0.05	1.25
p24-1/25	PIVENIQGQMVHQAISPRTL	0.02	0.5
p24-1/29	FIVQNAQGQMVHQAISPRTL	0.32	8
p24-1/30	PIVENIQGQIIHQAISPRTL	0.44	11

IC_{50}, concentration of peptide required to bind 50% of Ii. *Ii*, invariant chain CLIP peptide. *HA*, influenza hemagglutinin peptide.
*Average of triplicate binding assays.
‡IC_{50} of variant peptides divided by value for index p24-1.
§Reference 28.

peptides and to peptide p17-3, aa 21–35 of p17 (LRPGGKKKYKLHIV; data not shown). This peptide sequence contains a known cytotoxic T cell epitope, usually restricted by HLA-A3 (16) or -B8 (8). Since this patient is HLA-$A3^+$, we ensured that only CD4 lymphocyte proliferation was measured by depleting $CD8^+$ lymphocytes before assay or culture. Proliferation assays performed using total PBMCs, CD8-depleted lymphocytes, and $CD8^+$ lymphocytes revealed that the response measured was confined to the CD8-depleted population (data not shown). T cell lines grown out from fresh PBMCs were shown to be $CD4^+$ by FACScan® analysis. This cell line had a Th1 phenotype, since IL-2 was produced on stimulation with antigen. Using HLA-matched and mismatched PBMCs as APCs, the HLA restriction molecule was initially identified as DR13.02. Peptide binding studies using purified DR13.02 showed that p17-3 binds very poorly if at all to this allele ($IC_{50} > 100$ μM). Since DR52c is invariably associated with DR13.02, we used mouse L cells transfected with HLA-DR52c to present peptide p17-3 and confirmed that this was the restricting allele. Although presentation of p17-3 by the L cells was not optimal, probably because of a lack of accessory molecules, there was a fivefold increase in cpm over that obtained with the control untransfected L cells, confirming that DR52c was the class II restricting molecule for p17-3.

Sequencing of proviral DNA from this patient over 12 mo showed considerable variation (Fig. 4). There are four commonly identified naturally occurring variants of HIV-1 SF2 p17-3 in the database:

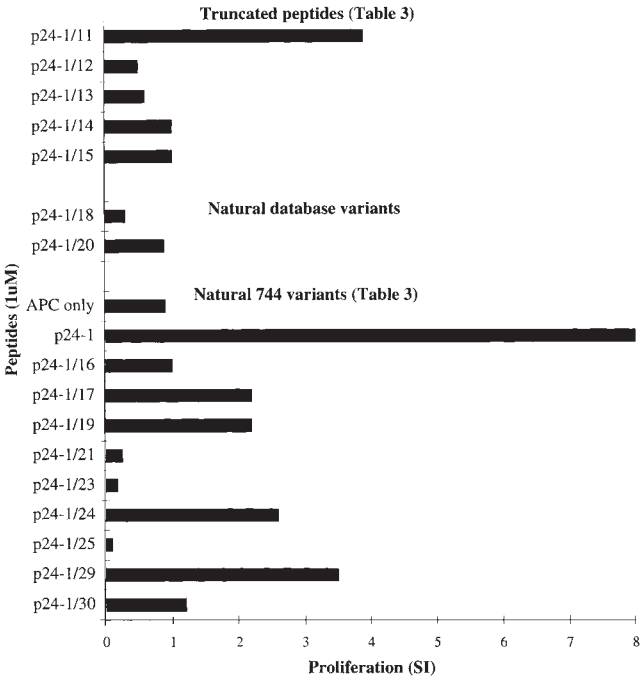

Figure 3. Proliferation of T cell line 744 to variant peptides of p24-1 Gag. Recognition of 16 variant peptides (see Table 3) of p24-1 Gag by T cell line 744 was assayed by proliferation, as described for Fig. 1 *A*. *APC only*, background for T cells and APCs alone, i.e., SI = 1.

p17-3	aa 20–35 of p17 Gag	LRPGGKKKYKLKHIV
p17-3/1	HIV-1 RF	LRPRGKKRYKLKHIV
p17-3/2	HIV-1 CD41	LRPGGKKQYRLKHIV
p17-3/3	HIV-1 MAN	LRPGGKKKYQLKHIV
p17-3/4	HIV-1 NDK	LRPGGKKKYALKHLI

Two of these, index p17-3 and p17-3/3, occurred in this individual. p17-3/3, which was the most common variant detected in patient 024 on days 0 and 335, was recognized well by the T cell line derived from PBMCs initiated on day 71, and substantially better than peptide p17-3, which had been used to grow out the line. The natural database variants p17-3/1 and p17-3/2 failed to stimulate this line, whereas p17-3/4 was a strong proliferative agonist (Fig. 5). The proliferative response of fresh PBMCs on day 271 revealed a marked difference in the capacity of p17-3 epitope variants to elicit proliferation (Table 2). The index sequence, p17-3, which may have been the predominant antigen at that time (Fig. 4), produced a clear positive response. The common variant p17-3/3 induced a much weaker response. This result and the fresh responses to variants in patient 744 imply that circulating HLA class II–restricted T lymphocytes do not bear specificities for every variant antigen present in the infecting virus population.

Discussion

When systematically sought, HIV-1–specific proliferative responses were present in 28% of our asymptomatic patients. We mapped the HIV-1–specific Th response in two individuals to epitopes within HIV-1 Gag. We defined the HLA class II restriction molecules and determined the antigenic variation within these epitopes. This work has revealed that natural antigenic variation in an HLA-DR1–restricted epitope does not lead to loss of HLA class II binding, but some variants detected failed to stimulate T cell proliferative responses in fresh uncultured cells and in a T cell line. Similar results were also demonstrated in an HLA-DRB52c–restricted p17 Gag epitope variant. These findings imply that CD4 T lymphocyte help may be diminished when these cells fail to be stimulated by naturally occurring HIV-1 antigenic variation.

There is evidence that HIV variants can escape from $CD8^+$ CTL responses (5–10, 17). This escape can arise through impairment of epitope binding to the presenting HLA class I molecule (10, 17) or by alterations in residues in contact with the TCR (5). Can $CD4^+$ T cell responses provide analogous pressure for the generation of escape mutants? Siliciano et al. (12) demonstrated that HIV genomic heterogeneity had a profound effect on recognition by a gp120-specific $CD4^+$ line derived from uninfected donors, suggesting that natural escape in seropositive people might occur. In that study, variant peptides induced three different types of proliferative response. One peptide

EPITOPE 21	L	R	P	G	G	K	K	K	Y	K	L	K	H	I	V	35		
	TTA	AGG	CCA	GGG	GGA	AAG	AAA	AAA	TAT	AAA	TTA	AAA	CAT	ATA	GTA			
Peptide																	Frequency (%)	Days after initial sample
p17-3/3	L	R	P	G	G	K	K	K	Y	Q CAA	L	K	H	I	V		95	Day 0
p17-3/5	L	R	P	G	G	K	K	K	Y	Q CAA	L	R AGA	H	I	V		5	
p17-3	L	R	P	G	G	K	K	K	Y	K	L	K	H	I	V		90	Day 245
p17-3/6	L	R	P	G	G	K	K	K	Y	K	L	K	R CGA	V GTA	V		5	
p17-3/7	L	R	P	G	G	K	K	K	Y	K	S TCA	K	H	I	V		5	
p17-3/3	L	R	P	G	G	K	K	K	Y	Q CAA	L	K	H	I	V		80	Day 335
p17-3/8	L	R	P	G	G	K	K	K	Y	R CGG	L	K	H	I	V		10	
p17-3/8	L	R	P	G	G	K	K	K	Y	R CGA	L	K	H	I	V		10	

Figure 4. Naturally occurring variants of p17-3 Gag in patient 024. Viral DNA was purified from PBMCs of patient 024 at the time intervals stated. The region of p17-3 was amplified by PCR, cloned, and sequenced. At least 20 clones were sequenced to identify any variants.

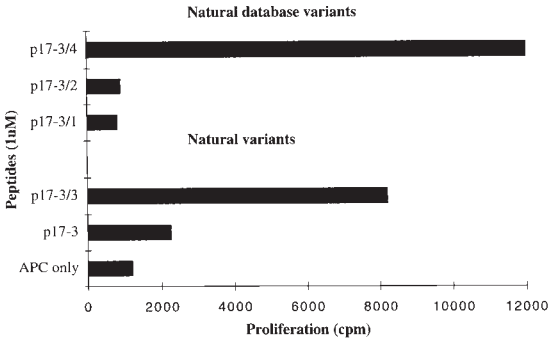

Figure 5. Proliferation of T cell line 024 to variants of p17-3 Gag. Recognition of peptides by T cell line 024 was assessed by proliferation assays as described for Fig. 1 *A*. Sequences of the p17 Gag variant peptides are as follows: p17-3, LRPGGKKKYKLKHIV; p17-3/1, LRPRGKKRYKLKHIV; p17-3/2, LRPGGKKQYRLKHIV; p17-3/3, LRPGGKKKYQLKHIV; and p17-3/4, LRPGGKKKYALKHLI.

induced greater proliferation, but the majority showed a significant reduction in stimulation or no stimulation at all. Here we show that single aa changes within HLA class II–restricted epitopes can alter recognition by $CD4^+$ T cells as gauged by diminished proliferative responses. These mutant epitopes could confer a survival advantage on viruses in an environment where the loss of T cell help impaired HIV-specific CTL activity against infected cells.

In patients 744 and 024, fresh PBMCs responded to the mapped peptides (Table 2), confirming the presence of cells with these specificities in circulating T lymphocytes. In patient 744, the viral variant p24-1/16 dominated in vivo (Fig. 2; 100% on day 0, 55% on day 182) and it bound adequately to HLA-DR1. However, it was not recognized by fresh PBMCs from this patient tested on day 498. Although results obtained with T cell lines suggest important differences in the capacity of these cells to recognize variants, the failure of recognition by fresh uncultured cells is likely to more closely reflect events in vivo.

Since $CD4^+$ lymphocytes failed to proliferate despite adequate binding of the altered peptides to the restricting HLA-DR1 molecule, the mutation must have impaired TCR recognition in some way. HLA-DR1 was the first HLA class II allele to be crystallized (18, 19), and there appear to be five pockets in the peptide binding groove. Studies using mutant HLA-DR1 molecules have revealed that changes in the contact residues of pockets 1, 4, and 7 can greatly alter binding. Hammer et al. (20) proposed a motif for peptides binding to HLA-DR1 using a phage display library. This identifies two potential anchor positions, at positions 1 and 4, and an enrichment for the small aa residues at 6. Peptide p24-1 and its variants all bound well to HLA-DR1, with affinities comparable to the influenza hemagglutinin peptide. Our results confirm that HLA-DR1 will tolerate peptide variation since binding in one pocket can be compensated for elsewhere, stabilizing the interaction. Analysis of the crystal structure of HLA-DR1 complexed with influenza hemagglutinin revealed that ~35% of the peptide remains solvent accessible in the peptide–MHC complex. Thus, if p24-1 binds in a similar manner to HLA-DR1, the alteration of any of its exposed peptide residues would directly alter the surface of the peptide–MHC complex that interacts with the TCR.

Evavold and Allen (21) have described a Th cell specific for hemoglobin in which the alteration of a single aa in the immunogenic peptide induced only cytokine secretion and not proliferation. This shows that the TCR response to ligand is not an all-or-nothing phenomena and that "partial signaling" can be triggered by an altered peptide ligand such as those described here. Purbhoo et al. (11) showed that altered HIV-1 peptide ligands failed to induce phosphorylation of pp36 in CTLs. Madrenas et al. (22) and Sloan-Lancaster et al. (23) have also shown that altered peptide ligands can induce partial signaling in $CD4^+$ lymphocytes.

$CD4^+$ T cells play a central role in virtually all immune responses by releasing cytokines that enhance the activity of immune effector cells such as $CD8^+$ T and B cells (24). Ossendorp et al. (25) have shown that it is possible to confer protective immunity to virus-induced tumors by vaccination with a specific viral Th epitope. The $CD4^+$ cells induced acted synergistically with $CD8^+$ cytotoxic T cells to eradicate the tumor. The use of Th epitopes in vaccines can greatly enhance their efficiency in inducing CTL responses and maintaining CTL memory, as has been shown for lymphocytic choriomeningitis virus (26). Lack of Th responses through engagement with weak agonist peptides could impair CTL activity in HIV-1 infection (27).

The vast capacity for mutation of HIV-1 has often been alleged to be a basis for immune escape and viral persistence in this infection. Experimental evidence for this contention has been accumulating in studies of HIV-1–specific CTL-mediated immunity.

There has been much more uncertainty as to why $CD4^+$ lymphocyte responses are impaired. In this study we defined the HIV-1–specific CD4 lymphocyte response by mapping the peptide epitope and determining the HLA class II restriction in infected individuals. We also sequenced viral variants present in the same patients. This enabled us to determine the effect on immune recognition of natural viral variation. Most peptide variants were capable of binding to class II and hence could be presented at the cell surface, but they often failed to stimulate T cell proliferation in both fresh preparations and cell lines. These findings raise important questions regarding the integrity of T lymphocyte help even during the early phases of HIV-1 infection. It is plausible that within lymph nodes or at other sites there are T lymphocytes with specficities capable of recognizing a greater range of variant antigens than are found circulating in the blood. However, some of the altered peptides we have identified are likely to be weak agonists, incapable of driving out proliferative responses. Dominant variants such as p24-1/16, which always failed to elicit proliferation in fresh cells and cell lines, could diminish the provision of necessary cytokine-mediated help in vivo. Modern antiretroviral therapy that restored CD4 lymphocyte function might have the additional advantage of diminishing the prevalence of such variants by switching off viral replication.

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