JOURNAL OF VIROLOGY, Mar. 2005, p. 3653–3663 Vol. 79, No. 6
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.6.3653–3663.2005

Immunologic Pressure within Class I-Restricted Cognate Human Immunodeficiency Virus Epitopes during Highly Active Antiretroviral Therapy

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Received 24 June 2004/Accepted 22 October 2004

Cytotoxic T lymphocytes (CTL) and highly active antiretroviral therapy (HAART) are known to exert strong evolutionary pressures on the virus population during human immunodeficiency virus (HIV) infection. However, it is not known whether CTL responses continue to substantially affect viral evolution during treatment. To study the effect of immunologic pressure on viral sequences during HAART, we identified 10 targeted HIV-specific CD8$^+$-T-cell epitopes in five treatment-naïve patients, sequenced each epitope in plasma-derived viruses, and then identified evidence of immunologic pressure at these epitopes by comparing the frequency of viral variants in plasma to the frequency of the CD8$^+$-T-cell response for each variant identified. For one of the five patients, evidence of viral evolution was found during therapy. The sequence of the CTL-targeted epitope changed from an apparent escape variant prior to the initiation of therapy, to the sequence that is best recognized by the CTL response after the initiation of therapy, and then finally to a new escape variant during continued therapy. These data show that CTL-mediated pressure can continue to affect viral evolution after the initiation of HAART, even when treatment drives the viral load below detectable levels, and suggest that antiretroviral therapy may preferentially inhibit those virus variants that escape the CTL response.

In the two decades since AIDS was first described (14, 30), it has become clear that human immunodeficiency virus (HIV)-specific CD8$^+$ T lymphocytes play an important role in the control of HIV replication. A temporal correlation exists between the emergence of virus-specific CD8$^+$ T cells and the initial control of viremia during primary infection (5, 26, 37). A rapid expansion of clonal subsets containing HIV-specific cytotoxic T lymphocytes (CTL) also occurs during this time (38). More recently, structured treatment interruption studies with patients who were treated soon after infection showed that the control of viremia is associated with an increase in the strength and breadth of virus-specific CTL responses (44, 54). Viral escape from immunologic containment after mutations affecting the amino acid sequence of an immunodominant epitope (15, 23, 29) and viral escape in response to infusions of large numbers of monospecific cytotoxic T lymphocytes (25) also suggest that HIV-specific CD8$^+$ T cells contribute to viral control during chronic infection. In simian immunodeficiency virus (SIV)-infected rhesus macaques, the depletion of CD8$^+$ T cells results in higher viral loads and a more rapid disease progression (21, 48). Other rhesus macaque studies have demonstrated predictable patterns of T-cell immune escape through amino acid changes within cognate CD8$^+$ T-cell epitopes (12) and a correlation between vaccine-elicited CD8$^+$ T-cell gamma interferon (IFN-γ) production and the control of viremia after simian-human immunodeficiency virus challenge (4).

Highly active antiretroviral therapy (HAART) has revolutionized the treatment of AIDS. By lowering the viral load as much as 5 log, HAART has increased longevity and allowed the restoration of immune competency in HIV-infected subjects (10, 11, 32, 52). Despite the potency of these drugs, viral replication continues at low levels during antiretroviral therapy (13, 18, 19, 42, 45). The decrease in viral load caused by antiretroviral therapy also results in a rapid decrease in, but not elimination of, HIV-specific CD8$^+$ T cells (2, 9, 34-36).

Viral quasispecies diversity and evolution are hallmarks of HIV infection. Most evolution occurs in response to antiviral pressures exerted by the immune response or by specific antiretroviral agents. Both the level of viral replication and the degree of antiviral pressure affect the rate of viral quasispecies evolution. In general, it is believed that HAART exerts more antiviral pressure upon HIV than that exerted by the T-cell immune response. In fact, antiretroviral therapy exerts such potent antiviral pressure that antiretroviral resistance mutations evolve even when viral replication is extremely low. It is also known that during periods of high viral replication, when CD8$^+$ T cells are likely the dominant antiviral pressure upon the quasispecies, the CD8$^+$ T-cell pressure is sufficient to drive escape within cognate epitopes (6, 41). What remains unclear is whether CD8$^+$ T-cell pressure becomes insignificant compared to the antiviral pressure of HAART during treatment. Does this new pressure upon the quasispecies alter the balance of viruses that do and do not escape CD8$^+$ T-cell recognition, and is there sufficient residual pressure exerted by CD8$^+$ T cells to drive the further evolution of escape variants within cognate epitopes even when there is minimal viral replication?

To assess whether HIV-specific T cells continue to exert evolutionary pressure on the virus during HAART, we as-

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TABLE 1. Primers used for amplification of viral sequences

Primers	Forward sequence	Reverse sequence
P17 primary	AGAGCGTCAGTATTAAGCGGG	CCCATGCATTTAAAGTTCTAGGTG
P17 secondary	GGGAAAATTGGATAAATGGG	CTAGGTGATATGGCCTGATGTAC
P24 primary	AGATTGCATCCAGTGCATGC	CCACATTTCCAACAGCCCTT
P24 secondary	GGGAAGTGACATAGCAGGAACTACT	ACATGCTGTCATCATTTCTTCTAGTGTA
Pol primary	TTTGTACAGAAATGGAAAAGGAAGG	AGGCTGTACTGTCCATTTATCAGGAT
Pol secondary	CTCAAGACTTCTGGGAAGTTCAATT	CAGATCCTACATACAAATCATCCATGTAT
Nef primary	GGTGGGAGCAGCATCTCG	TGCAGCTCTCGGGCCA
Nef secondary	CCTGGGAAAACATGGAGCAA	GTTGTTCTCTCCTTCATTGGCC
Env primary	GAGGATTGTGGAACTTCTGGGAC	CCCTGGTGTGTAGTTCTGCCA
Env secondary	GGAGTCAGGAACTAAAGAATAGTGCTG	TGCTTCTAGCCAGGCACAATC

sessed viral sequence evolution within cognate epitopes and epitope-specific $CD8^+$ IFN-γ production both before and during the first 20 weeks of HAART for five previously treatment-naïve subjects.

MATERIALS AND METHODS

Subjects. Five HIV-infected, antiretroviral treatment-naïve subjects with initial $CD4^+$ T-cell counts of >200 cells/μl were recruited for this study. All subjects achieved viral loads of <400 copies/ml upon initiation of HAART and maintained this level throughout the course of the study. HLA typing was performed on all five subjects by a PCR sequence-specific primer methodology (8). Subjects signed informed consent forms approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas.

Antibodies. Unconjugated mouse anti-human CD28, unconjugated mouse anti-human CD49d, fluorescein isothiocyanate-conjugated mouse anti-human IFN-γ, phycoerythrin-conjugated mouse anti-human CD3, peridinin chlorophyll protein-conjugated mouse anti-human CD8, and allophycocyanin-conjugated mouse anti-human CD69 monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS) (San Jose, Calif.).

Cell preparation. Peripheral blood mononuclear cells (PBMC) were prepared by standard Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). Both fresh and frozen PBMC were used for intracellular IFN-γ staining. PBMC were frozen in heat-inactivated fetal calf serum containing 10% dimethyl sulfoxide in a Forma CryoMed cell freezer (Marietta, Ohio). The cells were stored at −140°C.

Peptides. Peptides were synthesized by Bio-Synthesis Inc. (Lewisville, Tex.) and were >70% pure by high-performance liquid chromatography. The final concentration of individual peptides in $CD8^+$ T-cell intracellular cytokine staining (ICS) assays was 2 μg/ml unless otherwise noted. Screening and peptide preparation were performed as described previously (9).

Cell stimulation. Cell stimulation was performed as described previously (24). One million PBMC in 1 ml of R-10 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin G/ml, 100 μg of streptomycin sulfate/ml, and 1.7 mM sodium glutamate) were incubated with 1 μg each of costimulatory anti-CD28 and -CD49d monoclonal antibodies and 2 μg of each peptide. Cells incubated with costimulatory antibodies only were included in every experiment to control for the spontaneous production of cytokines and the activation of cells prior to the addition of peptides. Cultures were incubated at 37°C in a 5% CO_2 incubator for 1 h, followed by an additional 5-h incubation in the presence of brefeldin A (10 μg/ml; Sigma, St. Louis, Mo.). PBMC that were used to compare the response to autologous peptides, determined by viral sequencing prior to the initiation of therapy, to the response to optimized peptides were prepared within 16 weeks of the initiation of HAART.

Immunofluorescence staining. Peptide-stimulated and control cultures were washed twice in cold Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide (fluorescence-activated cell sorting [FACS] buffer) and then stained with directly conjugated anti-human CD3 and anti-human CD8 antibodies for 30 min on ice. After a further wash, the cells were resuspended in 750 μl of a solution containing 50 μl of enzyme-grade Tween (Sigma) in 100 ml of 2× FACS-Lyse solution (BDIS) for 10 min in the dark at room temperature. Permeabilized cells were immediately washed twice with cold FACS buffer and spun down at 600 × *g* for 8 min. The cell pellet was resuspended in a minimal volume and stained with directly conjugated anti-IFN-γ and anti-CD69 antibodies for 30 min at 4°C. After a final wash, the cells were resuspended in Dulbecco's phosphate-buffered saline containing 1% paraformaldehyde (Electron Microscopy Systems, Fort Washington, Pa.) and stored at 4°C until use.

Flow cytometric analysis. Six-parameter flow cytometric analysis was performed with a FACSCalibur flow cytometer (BDIS). Between 50,000 and 130,000 events were acquired, gated on small lymphocytes, and assessed for CD3, CD8, CD69, and IFN-γ expression. The results were analyzed with either PAINT-A-GATE Plus (BDIS) or FlowJo (Tree Star Software, Ashland, Oreg.) software.

Autologous virus sequencing. HIV was concentrated from 8 to 11 ml of plasma by centrifugation at 28,000 × *g* for 1 h. The supernatant was removed, and the viral pellet was extracted by use of a QIAmp viral RNA extraction kit (QIAGEN, Valencia, Calif.). First-strand cDNA synthesis was performed with the outer reverse primers shown in Table 1 by use of a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, Calif.). A two-step nested PCR amplification of the first-strand cDNA was performed by the use of Platinum *Taq* polymerase Hi-Fidelity kits (Invitrogen). The cycling parameters were as follows: activation at 94°C for 2 min followed by 30 cycles of 15 s of denaturation at 94°C, 30 s of annealing at 54°C, and 30 s of extension at 68°C for both primary and secondary amplification. Amplified DNAs were purified by gel electrophoresis, extracted by use of a QIAquick gel extraction kit (QIAGEN), and ligated into the p-GEM T-Easy vector system (Promega, Madison, Wis.). Plasmids were transformed into competent DH5α *Escherichia coli* cells (Invitrogen). Individual clones were picked by α-complementation and then amplified by the use of Platinum *Taq* polymerase Hi-Fidelity kits (Invitrogen) and M13 primers. Colony PCRs used a 2-min activation step at 94°C followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 57°C, and 3 min of extension at 72°C. Excess primers were degraded by the incubation of 4 μl of reaction product with 2 U each of exonuclease I and shrimp alkaline phosphatase at 37°C for 1 h. Heating to 72°C for 15 min terminated these reactions. The reaction products were sequenced on an automated DNA sequencer (Applied Biosystems, Foster City, Calif.) by use of an M13 forward or reverse primer.

At least 11 individual plasma-derived clones were sequenced at each region of interest for each time point. The nucleotide sequence of each clone was compared to those of all other clones isolated from the peripheral blood for that day and subject. Each difference from the consensus sequence for that clone was manually checked to ensure correct identification of the base sequence. Any base sequence that was not identified at least twice within a specific epitope was considered to be the result of polymerase infidelity and was disallowed, as previously suggested (50). All PCR amplifications were performed with procedural safeguards, including the division of all reagents into aliquots and the physical separation of sample processing and post-PCR handling steps (27). Control amplifications were performed by using first-strand DNA reaction mixtures to which no viral RNA was added. These reactions were carried through the secondary PCR amplification step to ensure that there was no carryover contamination.

Primers. Primers for *gag*, *pol*, *nef*, and *env* sequence amplification and first-strand cDNA synthesis were designed based on the consensus B clade sequence (51) and were synthesized by Sigma Genosys (Woodlands, Tex.) (Table 1). The nucleotide-encoded regions sequenced with each primer set were as follows: p17, Gag 4-149; p24, Gag 232-348; Pol, Pol 240-345; Env, Env 773-824 and Nef 1-60; and Nef, Nef 36-161.

HLA-B51 stabilization studies. HLA-B51 stabilization experiments were performed by a modification of the method of Hansen and Myers (17), using T2 (174 × CE.T2) cells (46) obtained from the American Type Culture Collection (Manassas, Va.). T2 cells were grown to log phase in R-10 and then washed three times with RPMI 1640 supplemented with 100 U of penicillin G/ml, 100 μg of streptomycin sulfate/ml, and 1.7 mM sodium glutamate. Each incubation con-

TABLE 2. HLA types, optimized epitopes used to screen for $CD8^+$ T-cell responses, and frequencies of epitope sequences found prior to the initiation of therapy based on viral RNA sequencing

Subject	HLA type	Optimized epitope sequence	Epitope restriction	Initial $CD8^+$-T-cell response (%)	Plasma viral sequence[a] (no. of clones with sequence/ total no. of clones)
A	A2, A2, B44, B70	$SLYNTVATL_{(p17\ 77–85)}$	A2	0.62	--------- (20/20)
B	A3, A31, B18, B39	$YPLTFGWCY_{(nef\ 135–143)}$	B7, B18, B35, B49, B53	0.58	--------F (11/11)
F	A1, A3, B8, B35	$IRLRPGGKK_{(p17\ 19–27)}$	B27[b]	0.65	--------- (3/15)
					-------R- (12/15)
		$EIYKRWII_{(p24\ 128–135)}$	B8	2.35	D------- (12/17)
					-V------ (5/17)
		$YPLTFGWCY_{(nef\ 135–143)}$	B7, B18, B35, B49, B53	1.84	--------F (15/15)
G	A2, A3, B7, B44	$RLRPGGKKKY_{(p17\ 20–29)}$	A3, A30, B42, Bw62	0.62[a]	---------- (18/18)
		$QVPLRPMTYK_{(nef\ 73–82)}$	A3	0.37[a]	---------- (18/18)
R	A2, A31, B51, B58w4	$SLYNTVATL_{(p17\ 77–85)}$	A2	0.15	--------- (20/20)
		$RLRPGGKKK_{(p17\ 20–28)}$	A3, A30, B42, Bw62	0.31	--------- (6/20)
					--------Q (11/20)
					--------R (3/20)
		$TAFTIPSI_{(pol\ 295–302)}$	B51	1.59	--------T (24/24)

[a] Initial response determined at week 14 of therapy.
[b] Mismatch with patient's HLA type.
[c] Dashes indicate identity with the optimized epitope sequence.

tained 500,000 cells in 1 ml of RPMI 1640 containing 10 μg of β_2-microglobulin/ml and between 0 and 100 μg of peptide/ml. After 9 h of incubation at 37°C in 5% CO_2, the cells were washed and spun down at 300 × *g* for 8 min in cold FACS buffer. The supernatant was removed, and the pellet was resuspended in a minimal volume and then stained for 30 min on ice with phycoerythrin-conjugated anti-HLA-A,B,C (clone W6/32) obtained from Serotec (Raleigh, N.C.). The cells were once again washed with FACS buffer and then were fixed with 1% paraformaldehyde and stored at 4°C until analysis. Analyses were performed with a FACSCalibur flow cytometer (BDIS).

RESULTS

Screening and initial sequencing. Responses to 1 to 3 of the 95 screening epitopes were found for each of the five subjects studied (Table 2), as previously reported (9). Sequencing of plasma viruses showed that there was a predominance of a sequence that did not match that of the screening peptide in three of the five subjects. In subjects B and F, a predominant sequence of YPLTFGWC***F*** was found rather than the sequence $YPLTFGWCY_{(nef\ 135-143)}$ used for screening (the variant residue is indicated in bold italics). In subject F, the viral sequence IRLRPGG***R***K was found at the HLA-B27 epitope, for which the peptide $IRLRPGGKK_{(p17\ 19-27)}$ was used for screening, and two viral sequences, ***D***IYKRWII and E***V***YKRWII, were found at the HLA-B8 epitope, for which the peptide $EIYKRWII_{(p24\ 128-135)}$ was used for screening. In subject R, two viral sequences, RLRPGGKK***Q*** and RLRPGG KK***R***, were found at the A3 epitope, for which the peptide $RLRPGGKKK_{(p17\ 20-28)}$ was used for screening, and a single viral sequence, TAFTIPS***T***, was found at the HLA-B51 epitope, for which the peptide $TAFTIPSI_{(pol\ 295-302)}$ was used for screening.

In patient F, the response to the peptide IRLRPGGK $K_{(p17\ 19-27)}$, a B27 optimized epitope, was discordant with the patient's HLA type. This patient did not have a detectable response to the overlapping HLA-A3-restricted optimized epitopes $RLRPGGKKK_{(p17\ 20-28)}$, $KIRLRPGGK_{(p17\ 18-26)}$, and $RLRPGGKKKY_{(p17\ 20-29)}$. Patient R (HLA type A31) responded to the HLA-A3-restricted epitope RLRPG $GKKK_{(p17\ 20-28)}$ but had no detectable response to the other overlapping optimized A3 epitopes in this region, i.e., KIRL $RPGGK_{(p17\ 18-26)}$ and $RLRPGGKKKY_{(p17\ 20-29)}$.

Responses to autologous virus sequences. To determine if autologous virus sequences could be recognized by $CD8^+$ T cells from each subject, we ran parallel incubations containing either a peptide representing the subject virus's autologous sequences or a peptide representing the optimized consensus epitope. These assays were performed with frozen PBMC from the closest date possible to the initiation of therapy. The frequency of $CD8^+$ T cells producing IFN-γ in response to the screening epitope and the autologous sequence were then compared.

For subject B, the $CD8^+$ T-cell response to the screening epitope $YPLTFGWCY_{(nef\ 135-143)}$ was 0.58% and the response to the autologous viral sequence, YPLTFGWC***F***, was 0.95%. For subject F, the frequency of response to YPLTFGWCY, 1.84%, was not significantly different from the 1.94% response observed for the autologous virus sequence YPLTFGWC***F***. For both subjects, only the YPLTFGWC***F*** epitope sequence was found in peripheral blood. For both subjects, assays of the responses to YPLTFGWCY and YPLTFGWC***F*** were done with cells taken from the patients immediately prior to the initiation of therapy. These results argue against viral escape at these epitopes.

We next examined cognate $CD8^+$ T-cell epitopes at which multiple autologous viral sequences were found. In all cases, the least frequent viral sequence at the time of initiation of treatment stimulated the largest $CD8^+$ T-cell population. Conversely, the most frequent viral epitope was the most poorly recognized in terms of the total number of responding $CD8^+$ T cells. Specifically, in subject F, the dominant viral sequence at epitope $IRLRPGGKK_{(p17\ 19-27)}$ was IRLRPGG***R***K, which was found in 12 of 15 clones. The sequence coding for IRLRPGGKK was found in only 3 of 15 clones. The $CD8^+$ T-cell response (0.75%) to the less frequent clone, IRLRPGGKK, was more than twice the response to the more frequent sequence, IRLRPGG***R***K (0.33%), when measured 12 weeks after the initiation of therapy. Both of the sequences found at the

HLA-B8 epitope EIYKRWII$_{(p24\ 128\text{-}135)}$, ***D***IYKRWII and E***V***YKRWII, were different from the peptide used to screen for a response to this epitope. Of the autologous viral sequences found at this epitope, E***V***YKRWII was found in 5 of 17 clones and ***D***IYKRWII was found in 12 of 17 clones. The frequency of IFN-γ production by $CD8^+$ T cells incubated with the less frequent sequence, E***V***YKRWII, was 3.69%, which was twice that of $CD8^+$ T cells incubated with the more frequent viral sequence, ***D***IYKRWII (1.86%), when measured 16 weeks after the initiation of therapy.

Sequencing of the epitope RLRPGGKKK$_{(p17\ 20\text{-}28)}$ in plasma viruses from subject R identified sequences corresponding to the screening peptide and two other sequences, one in which the anchor position lysine was replaced with an arginine (which has the same charge) (RLRPGGKK***R***) and another in which the anchor position was replaced by an amide-containing amino acid glutamine (RLRPGGKK***Q***). Similar to the results for patient F, the production of IFN-γ in response to the most frequently found variant, RLRPGGKK***Q***, was less than that observed for the less common variant, RL RPGGKK***R***, and the A3 optimized epitope RLRPGGKKK when measured 14 weeks after the initiation of therapy. Of the 20 autologous viral sequences from subject R, 30% contained the sequence RLRPGGKKK, 15% contained the sequence RLRPGGKK***R***, and 55% contained the sequence RLRPG GKK***Q***. The responses to RLRPGGKKK, RLRPGGKK***R***, and RLRPGGKK***Q*** were 0.09, 0.11, and 0.02%, respectively. For subject R, all 24 clones sequenced for the HLA-B51 epitope TAFTIPSI$_{(Pol\ 295\text{-}302)}$ showed the previously described HLA-B51 escape variant TAFTIPS***T*** (49). Again, the frequency of response to the less frequent viral sequence, TAFTIPSI (0.45%), was higher than that to the more frequent viral sequence, TAFTIPS***T*** (0.31%), when measured 14 weeks after the initiation of therapy.

Longitudinal virus sequencing at sites of $CD8^+$ T-cell pressure. We sequenced plasma viruses from three patients (subjects B, F, and R) at six targeted epitopes multiple times after the initiation of therapy. At four of these epitopes, there was evidence of viral evolution, as shown by multiple viral sequences within the targeted epitopes, a larger response to the least common sequence, and a lesser response to the most frequently observed sequence. There was no evidence of viral evolution at the other two epitopes. It is known that antiretroviral therapy causes dramatic changes in both the viral load and HIV-specific T cells (2, 9, 34–36). Due to the very low plasma virus loads after the initiation of therapy, not all regions of the plasma virus were successfully amplified and sequenced at each time point. Viral sequences were monitored for 12 to 44 weeks after the initiation of therapy (Tables 3, 4, and 5).

For subjects B and F, there was no evidence of immune pressure on the Nef$_{135\text{-}143}$ epitope before the initiation of therapy and no changes in the autologous plasma virus sequence within this epitope during the first 28 and 44 weeks of therapy (Table 3). Similarly, for epitopes p17$_{19\text{-}27}$ and p24$_{259\text{-}267}$ in subject F, no substantial changes in the viral quasispecies within these targeted epitopes were found during the first 20 weeks of treatment (Table 4). For patient F, it was possible to evaluate the response to variant peptide epitopes in $CD8^+$ T cells that were present either concurrently or within 1 month of the viral quasispecies sequencing (Fig. 1). Once again, the most frequently seen viral sequences at the optimized epitopes IRL RPGGKK$_{(p17\ 19\text{-}27)}$ and EIYKRWII$_{(p24\ 128\text{-}135)}$ were those which stimulated the smallest specific $CD8^+$-T-cell response. This was not unexpected since no dramatic changes in sequence frequency were seen after the initiation of therapy.

TABLE 3. Frequencies of optimized epitope YPLTFGWCY$_{(nef\ 135\text{–}143)}$ and of variant epitope before and after the initiation of therapy in patients B and F based on plasma viral RNA sequencing

Patient and time (wks) of therapy	Viral load[a]	No. of clones encoding YPLTFGWCY/***F***/ no. of clones	No. of clones encoding YPLTFGWC/***F***/ no. of clones
B			
0	245,000	0/10	10/10
8	ND	0/14	14/14
20	563	0/18	18/18
28	< 400	0/17	17/17
36	< 400	0/19	19/19
44	< 400	0/14	14/14
F			
0	23,620	0/15	15/15
12	ND	0/16	16/16
16	<400		
20	ND	0/16	16/16
24	<400		
28	ND	0/15	15/15
32	<400		

[a] ND, not determined.

A different pattern was observed for subject R. The majority of the autologous plasma virus sequences found for epitope p17$_{20\text{-}28}$ prior to the initiation of therapy encoded the peptide RLRPGGKK***Q***, which was different from the screening peptide RLRPGGKKK (Table 5). At week 14 of antiretroviral therapy, there was a major change in the viral quasispecies such that the plasma virus sequences present were either RL RPGGKKK (14 of 16 clones) or RLRPGGKK***R*** (2 of 16 clones), both of which were epitopes that were well recognized by HIV-specific $CD8^+$ T cells. By week 19, the viral quasispecies had reverted back to the sequence RLRPGGKK***Q***, which was not well recognized.

Similarly, prior to the initiation of therapy, only the sequence TAFTIPS***T*** was seen at the Pol$_{295\text{-}302}$ epitope (Table 5). After 14 weeks of treatment, this viral sequence was completely replaced within the plasma virus compartment by viruses that contained the sequence TAFTIPSI at this site. This sequence was not seen in this subject prior to antiretroviral therapy. By week 19 of therapy, the quasispecies was again replaced, this time by viruses that all had the sequence TAF TIPS***M*** at the Pol$_{295\text{-}302}$ epitope. The nucleotide sequences responsible for the changes in amino acid sequences from TAFTIPS***T*** to TAFTIPSI to TAFTIPS***M*** suggest a stepwise progression. Prior to HAART, the original codon for the C-terminal threonine in this epitope was ACA (24 of 24 clones). At week 14, a single C-to-T change in this codon (ACA to ATA) was responsible for a change to isoleucine at the C terminus of the epitope (14 of 14 clones). At week 19, a further single nucleotide change in this codon (ATA to ATG) was responsible for a change to methionine at the C terminus (18

TABLE 4. Frequencies of optimized epitope and variant epitope sequences in subject F before and after initiation of therapy for epitopes IRLRPGGKK$_{(p17\ 19–27)}$ and EIYKRWII$_{(p24\ 128–135)}$ based on viral RNA sequencing

Time (wks) in therapy	Viral load[a]	Sequence (no. of clones/total)	
		IRLRPGGKK	EIYKRWII
0	19,627	IRLRPGGKK (3/15), -------R- (12/15)	EIYKRWII (0/17), D------- (12/17), -V------ (5/17)
4	ND	IRLRPGGKK (6/19), -------R- (13/19)	EIYKRWII (0/22), D------- (22/22), -V------ (0/22)
12	<400	IRLRPGGKK (6/14), -------R- (8/14)	EIYKRWII (0/14), D------- (14/14), -V------ (0/14)
16	ND	IRLRPGGKK (8/17), -------R- (9/17)	
20	ND	IRLRPGGKK (0/17), -------R- (17/17)	
24	<400		

[a] ND, not determined.

of 18 clones). In PBMC collected 19 weeks after the initiation of therapy, when TAFTIPS***M*** was the only viral sequence found, the frequency of a response to TAFTIPS***M***, 0.63%, was only slightly less than that to TAFTIPSI (0.73%). The response to TAFTIPS***T*** was 0.55%. No evidence of a significant change in the relative response to TAFTIPSI versus that to TAFTIPS***T*** at weeks 12 and 19 or a change in the relative response to TAFTIPSI versus that to TAFTIPS***M*** at weeks 19 and 25 could be seen (Fig. 2).

Evidence for CD8$^+$ T-cell escape at Pol$_{295-302}$. In a population-based study, Moore et al. (33) reported that substitution of the consensus sequence isoleucine at position 135 of the B51-restricted epitope TAFTIPSI occurred in 39 of 40 HLA B51 individuals compared to 127 of 431 non-HLA-B51 individuals. This HLA-dependent substitution of isoleucine in this epitope suggests that substitution of a dissimilar amino acid at the B51 anchor point may affect the ability of variant peptides to bind to HLA-B51. Although fewer CD8$^+$ T cells produced IFN-γ when stimulated with TAFTIPS***T*** or TAFTIPS***M*** than when stimulated with TAFTIPSI, the differences observed were small. Despite the modest differences in IFN-γ production, CD8$^+$ T cells showed marked differences in the downregulation of CD3 when incubated with TAFTIPS***T*** or TAF TIPS***M*** compared to that observed with TAFTIPSI (Fig. 3a). The mean geometric CD3 fluorescence intensity in the nonstimulated CD8$^+$-T-cell population was 160 to 165 in these assays. In comparison, the mean CD3 fluorescence intensities were 24.8 in CD8$^+$ T cells responding to TAFTIPSI, 57.9 in CD8$^+$ T cells responding to TAFTIPS***M***, and 87.8 in CD8$^+$ T cells responding to TAFTIPS***T***. A similar, although less marked pattern, was seen with CD8 staining (Fig. 3b). These data suggest that fewer T-cell receptors are triggered by the formation of the TCR/pMHCI complex when TAFTIPS***M*** and TAFTIPS***T*** are incubated with PBMC than when TAFTIPSI is used at a concentration of 2 μg/ml (53).

To define differential recognition in more detail, we measured ex vivo IFN-γ production in response to both TAFTIPSI and TAFTIPS***T*** over a range of peptide concentrations. As shown in Fig. 4a, a 50% maximal response to TAFTIPS***T*** required a sixfold higher concentration of peptide (90 nM) than that required for TAFTIPSI (15 nM), clearly demonstrating the fact that TAFTIPS***T*** was less well recognized by the HIV-specific CD8$^+$ T cells of subject R. A similar titration curve showed decreased responses to TAFTIPS***M*** at 23 nM, 230 nM, and 2.3 μM compared to the responses to TAFTIPSI (Fig. 4b). The limited number of cells did not allow an estimation of a half-saturation point for TAFTIPS***M***.

HLA class I stabilization studies were performed with 174 × CEM.T2 (T2) cells to investigate the binding of TAFTIPSI, TAFTIPS***T***, and TAFTIPS***M*** to HLA-B51 (Fig. 5). The cytomegalovirus pp65 HLA-A2 immunodominant peptide NLVP MVATV (acting as a positive control), the HLA-B51 epitope TAFTIPSI, the variants TAFTIPS***T*** and TAFTIPS***M***, and the HLA-B27 epitope IRLRPGGKK (acting as a negative control) were tested for the ability to stabilize HLA-A2 and -B51 molecules. Incubations with TAFTIPSI and NLVPMVATV showed the most HLA class I stabilization, as measured by the surface staining of HLA with an anti-HLA-A,B,C monoclonal antibody (W6/32). The HLA stabilization level with TAFT IPS***T*** and TAFTIPS***M*** was lower than that observed with TAF

TABLE 5. Frequencies of optimized epitope and variant epitope sequences in subject R before and after initiation of therapy for epitopes RLRPGGKKK$_{(p17\ 20–28)}$ and TAFTIPSI$_{(pol\ 295–302)}$ based on viral RNA sequencing

Time (wks) in therapy	Viral load	Sequence (no. of clones/total)	
		RLRPGGKKK	TAFTIPSI
0	98,597	RLRPGGKKK (6/20)	TAFTIPSI (0/24)
		--------Q (11/20)	-------T (24/24)
		--------R (3/20)	-------M (0/24)
9.5	478		
14	ND[a]	RLRPGGKKK (14/16)	TAFTIPSI (14/14)
		--------Q (0/16)	-------T (0/14)
		--------R (2/16)	-------M (0/14)
19	<400	RLRPGGKKK (0/15)	TAFTIPSI (0/18)
		--------Q (15/15)	-------T (0/18)
		--------R (0/15)	-------M (18/18)

[a] ND, not determined.

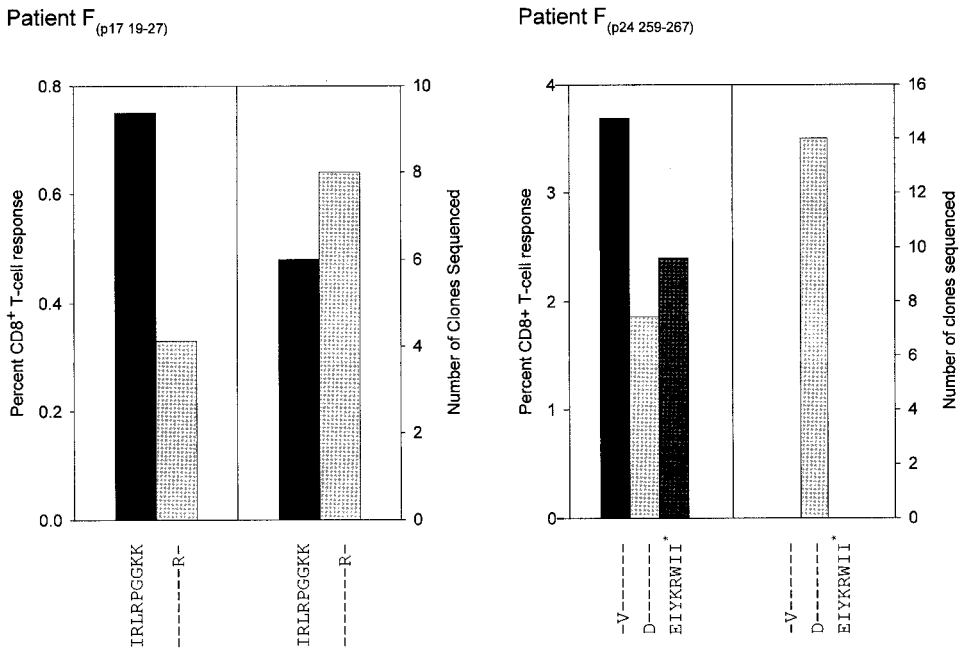

FIG. 1. Epitope-specific $CD8^+$ T-cell responses and autologous virus sequences from subject F. $CD8^+$ T-cell responses to screening peptides and peptide variants are shown to the left in each graph. Epitope frequencies, determined by nucleic acid sequencing of viral clones, are shown to the right in each graph. Screening peptides and variant amino acid sequences are shown on the abscissa. None of the sequenced clones contained the screening peptide EIYKRWII. Sequencing data and response data for the epitope IRLRPGGKK and its variant are from week 12 of therapy. Sequencing data are from 12 weeks and response data are from week 16 of therapy for EIYKRWII and its variants. An asterisk indicates that no nucleotide sequence corresponding to the screening epitope EIYKRWII was found in any viral sequence determined either prior to or after the initiation of therapy.

TIPSI and NLVPMVATV but higher than that observed with the HLA-B27 epitope IRLRPGGKK.

These data explain the similarities in the production of IFN-γ in response to stimulation by 2-μg/ml TAFTIPSI, TAF TIPS***T***, and TAFTIPS***M*** despite the substitution of a polar $-CHOHCH_3$ group in the case of TAFTIPS***T*** and a $-C_2H_4SCH_3$ group in the case of TAFTIPS***M*** for the $-CH(CH_3)C_2H_5$ group of TAFTIPSI. At saturating concentrations of peptide, the effects of the differences in binding avidity for TAFTIPSI, TAFTIPS***T***, and TAFTIPS***M*** were minimized. This masked the effects of substitutions of threonine and methionine for isoleucine at the carboxy-terminal anchor residue.

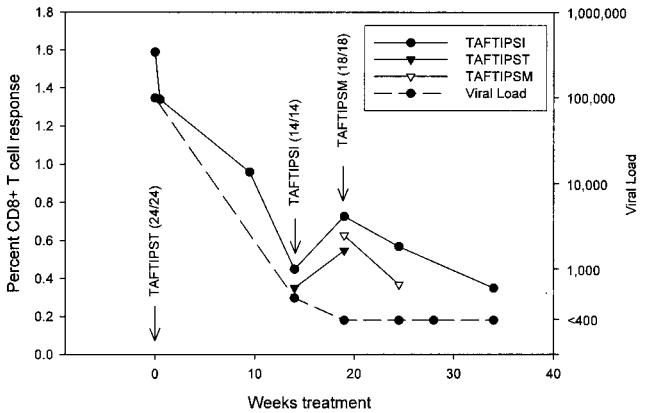

FIG. 2. Plot of viral load and $CD8^+$ IFN-γ production in response to TAFTIPSI, TAFTIPST, and TAFTIPSM in patient R. $CD8^+$-T-cell responses to individual peptides and viral loads are plotted against the time after the initiation of treatment. Responses to either TAFTIPSI, TAFTIPST, or TAFTIPSM were measured at the same time. The arrows and text indicate the numbers of viral clones sequenced and the sequence found at Pol 295–302 at the indicated times. The peptide concentration in all assays was 2 μg/ml.

Lack of nucleoside reverse transcriptase resistance mutations. The primers used to sequence the *pol* gene allowed the sequencing of amino acids 241 to 347 (HXB2 sequence), which correspond to amino acids 86 to 191 of reverse transcriptase (Fig. 6). In this 105-amino-acid sequence, the only point where the sequence changed over the 19 weeks of therapy in subject R was at the terminal anchor position for the epitope TAFT IPSI. Subject R's treatment regimen included combivir (zidovudine and lamivudine) and nevirapine. No evidence of drug resistance mutations for either lamivudine or nevirapine was found during this period. While not all potential sites corresponding to zidovudine resistance were sequenced, there was no evidence of a resistance mutation to zidovudine found at the V118 position. In addition, prior to the initiation of therapy, eight plasma virus clones were sequenced, and there was no evidence of zidovudine resistance mutations (M41L, E44D, D67N, or K70R) or multinucleoside reverse transcriptase complex mutations (A62V or the 69 insertion complex) (data not shown).

Lack of response to autologous peptides at other HLA matched epitopes. To assess whether screening with peptide epitopes that differed from the autologous virus sequences led to a failure to detect specific responses, we sequenced plasma

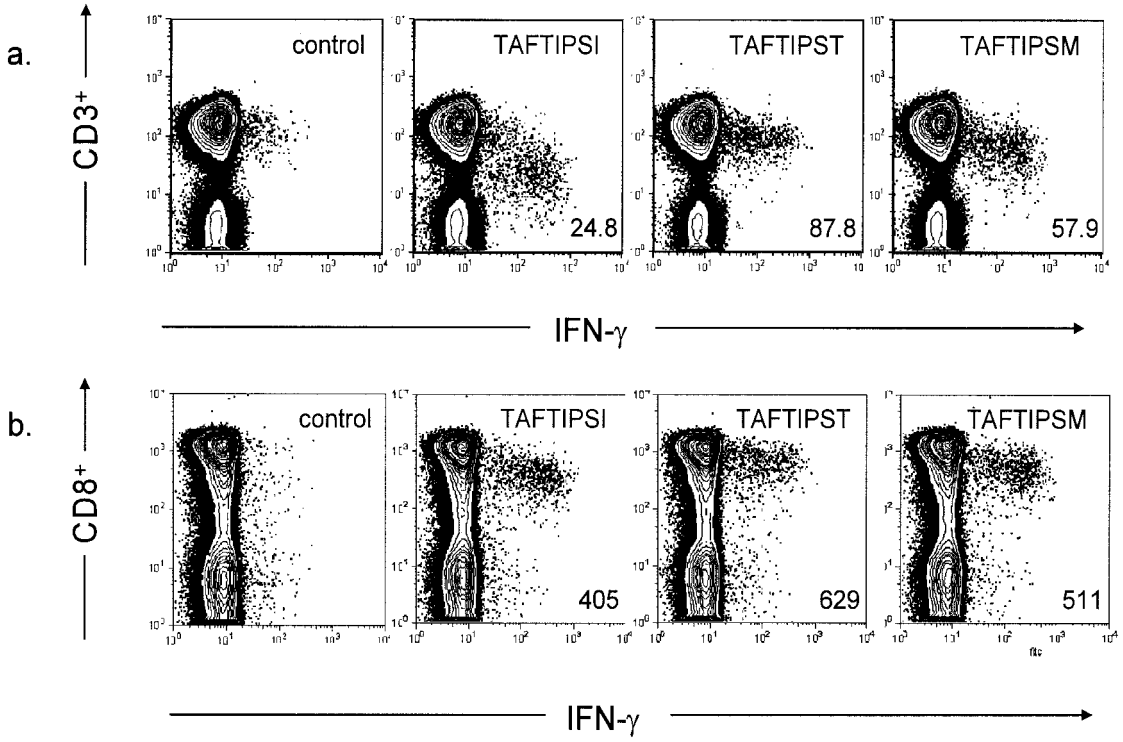

FIG. 3. Contour plots showing peptide-specific CD3 and CD8 down-regulation in $CD8^+$ T cells from patient R in response to TAFTIPSI and other autologous viral epitopes 19 weeks after the initiation of HAART. PBMC were gated sequentially on small lymphocytes (a) and on small lymphocytes and CD3 expression (b). Plots show CD3 or CD8 surface staining versus intracellular IFN-γ staining. Cells were incubated in the absence or presence of added peptide, as indicated. The peptide concentration was 2 μg/ml throughout. The geometric mean fluorescence for CD3 or CD8 expression in responding $CD8^+$ T cells is given in the lower right corner of each contour plot.

viral RNAs from subject B that had been collected prior to the initiation of therapy at the five regions for which we had PCR primers (Table 1). These regions corresponded to amino acids in p17 (4-149), p24 (232-348), Pol (240-345), Env (773-824), and Nef (1-161), or approximately 10% of the total HIV genome. Based on the HLA type of subject B, eight potential epitopes were identified for this subject. Seven variant sequences were found at those sites (Table 6). Each of these peptides was synthesized and used to assay $CD8^+$ T-cell responses. As with the peptides used for the initial screen, no response was found to any of the variant peptides for this subject (data not shown). Therefore, it is unlikely that the use of nonautologous virus sequences in this subject affected our ability to detect epitope-specific $CD8^+$ T cells.

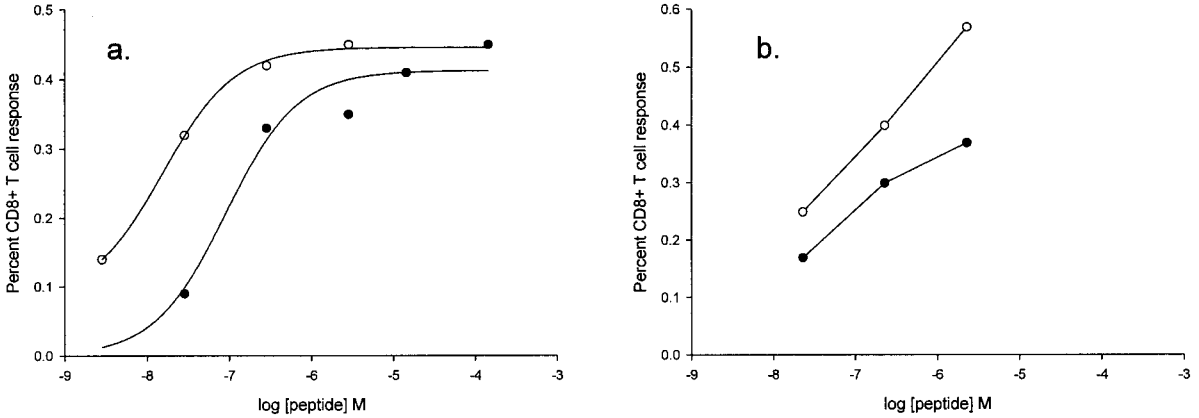

FIG. 4. Plot of data showing frequency of $CD8^+$-T-cell IFN-γ production in response to various concentrations of TAFTIPSI, TAFTIPST, and TAFTIPSM. Plot a shows a sigmoidal fit of $CD8^+$ IFN-γ production in response to various concentrations of TAFTIPSI (○) and TAFTIPST (●). The half-saturation point for TAFTIPSI was 15 nM and that for TAFTIPST was 90 nM. Responses shown are from PBMC prepared from blood drawn during week 14 of treatment. Plot b shows $CD8^+$ IFN-γ production in response to various concentrations of TAFTIPSI (○) and TAFTIPSM (●). Responses shown are from blood drawn during week 25 of treatment.

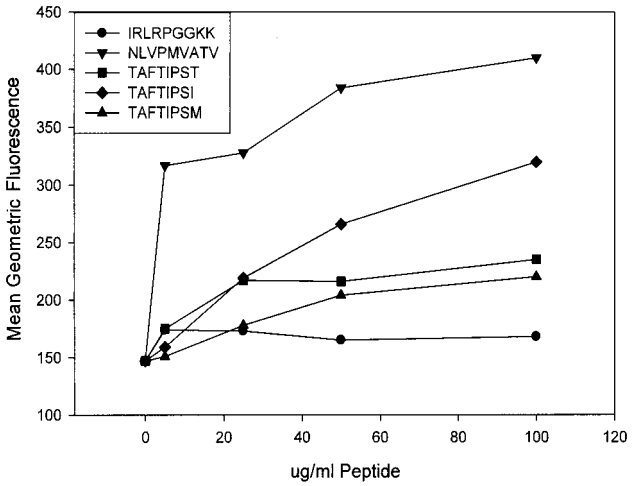

FIG. 5. Peptide-induced surface expression of HLA-A2 and -B51 in T2 cells. T2 cells were incubated for 9 h in RPMI supplemented with 100 U of penicillin/ml, 100 μg of streptomycin sulfate/ml, 1.7 mM sodium glutamate, and 10 μg of β2-microglobulin/ml, exposed to NLVPVATV, TAFTIPSI, TAFTIPST, TAFTIPSM, and IRLRPG GKK at the indicated concentrations, and then stained with anti-HLA-A,B,C (W6/32). Results are shown as mean geometric fluorescence intensities versus peptide concentrations.

DISCUSSION

The initiation of HAART dramatically reduces the antigenic load and results in a rapid decrease in HIV-specific $CD8^+$ T cells (2, 9, 34–36). During effective therapy, the viral load is reduced to such a low level that the progression of drug resistance appears to almost stop, even though low-level viral replication continues. For this setting, it was not known if immunologic pressure continues to drive viral evolution. In this study, we showed evidence of continued evolution in a recently infected individual during the first 19 weeks of therapy, even when the viral load was <400 copies/ml.

In our cohort of five patients, we found evidence of $CD8^+$-T-cell-dependent antiviral pressure in two HIV-infected subjects. For patient F, evidence of CTL-driven viral evolution was found at the HLA-B27 optimized epitope IRLRPGGK $K_{(P17\ 19\text{-}27)}$ and the HLA-B8 epitope EIYKRWII$_{(P24\ 259\text{-}267)}$. For patient R, evidence of CTL-driven viral evolution was found at the HLA-B51 epitope TAFTIPSI$_{(Pol\ 295\text{-}302)}$ and the HLA-A3 epitope RLRPGGKKK$_{(P17\ 20\text{-}28)}$. The appearance of a new variant at the targeted epitope TAFTIPSI$_{(Pol\ 295\text{-}302)}$ at week 19 in patient R represents evidence of CTL-driven evolution during antiretroviral therapy. Unlike the data for patient R for the targeted epitope RLRPGGKKK$_{(p17\ 20\text{-}28)}$, at which the recurrence of RLRPGGKK***Q*** in all 15 clones sequenced at week 19 of treatment may represent either continued evolution during therapy or amplification of a single viral clone due to sampling variability at low levels of starting template, TAFT IPS***M*** was not found before therapy was initiated and therefore represents further viral evolution at this epitope.

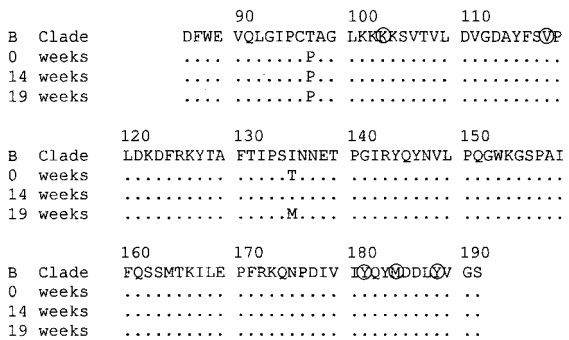

FIG. 6. Clade B consensus sequence and consensus sequences for plasma viruses from weeks 0, 14, and 19 of HAART for subject R. Twenty-four, 16, and 18 plasmids containing amplified viral cDNAs were sequenced, respectively. Dots indicate identity with the clade B consensus sequence. The locations of common drug resistance mutations for zidovudine (V118I), lamivudine (M184V), and nevirapine (K103N, Y181C/I, and Y188C/L/H) are circled in the consensus B clade sequence.

TABLE 6. Presence of variant sequences at HLA matched optimized epitopes in HIV isolated from blood of patient B prior to initiation of therapy

Screening peptide	Autologous sequence[a]
KIRLRPGGK(p17 18–26)	***R***IRLRPGGK
KIRLRPGGKK(p17 18–27)	***R***IRLRPGGKK
RLRPGGKKK(p17 20–28)	RLRPGGKK***T***
RLRPGGKKKY(p17 20–29)	RLRPGGKK***T***Y
AIFQSSMTK(pol 325–333)	AIFQ***C***SMTK
QVPLRPMTYK(nef 73–82)	QVPLRPMT***HS***
VPLRPMTY(nef 74–81)	VPLRPMT***H***

[a] Variant residues are indicated in bold italics.

Subject R was a recent seroconverter when HAART was initiated. Subject F had been infected for at least a year and a half, and patient B had been infected for at least 10 months prior to the initiation of therapy. Since viral sequences were monitored longitudinally for only three patients, the fact that evidence of viral evolution was observed only in the recent seroconverter might not be significant; however, viral evolution does occur rapidly soon after infection (6, 41). Viral replication rates are highest before CTL responses develop, and viruses exposed to the immune system immediately after infection represent a "clean slate" against which the developing responses can drive viral evolution. This, coupled with a CTL response focused on a smaller number of epitopes in acute HIV (54), may explain the relatively rapid changes in viral sequence at Pol 295-302 in patient R compared to those in patients B and F, who were chronically infected at the time of initiation of therapy (31).

These data do not explain the reason for the appearance of the optimized epitope TAFTIPSI 14 weeks after the initiation of therapy. Rapid shifts in different viral quasispecies due to preferential suppression of the most replication-capable (CTL escape) variant, the emergence of a previously suppressed quasispecies due to the decrease in HIV-specific $CD8^+$ T cells, or the emergence of different quasispecies from different reservoirs during the initiation of therapy are all possible explanations (39, 40). The emergence of TAFTIPS***M*** at week 19 occurred under somewhat different circumstances. Decreases in viral load do not occur as rapidly during the second phase of viral decay during HAART (39). As a result, dramatic shifts in viral populations are less likely to result in the appearance of

minor quasispecies. It is also unlikely that the presence of TAFTIPS***M***, a rarer sequence than TAFTIPS***T***, would be due to a preexisting sequence in a recently infected individual, especially as this sequence was not detected prior to the initiation of HAART. This, coupled with the stepwise change in nucleotide sequence observed in the transition from TAFT IPS***T*** to TAFTIPSI to TAFTIPS***M***, suggests that the emergence of TAFTIPS***M*** represents a new variant.

Any mutation in the reverse transcriptase gene carries the possibility of effecting antiretroviral drug resistance. No evidence of a clinically significant drug resistance mutation was seen in patient R either by the criteria of the Drug Resistance Mutations Group of the International AIDS Society—USA (22) or from clinical data. Patient R has maintained an undetectable viral load for >4 years on the same treatment regimen. Nonetheless, Brown et al. (7) have suggested that both TAFTIPST and TAFTIPSM may decrease the sensitivity to nonnucleoside reverse transcriptase inhibitors (NNRTIs). These authors screened 110 patients who had moderate reductions in sensitivity to NNRTIs. Twenty-seven of these individuals had an I135T mutation. Only one patient had an I135M mutation. In this patient, the I135M mutation occurred in combination with an E138A mutation, a site where mutations are known to affect the sensitivity to NNRTIs (47). Resistance test vectors using the consensus reverse transcriptase sequence with either an I-to-T or I-to-M mutation at amino acid 135 of the reverse transcriptase gave a 2- or 2.6-fold change in resistance, respectively. A review of the Stanford HIV Drug Database (43) showed 10 patients with an I135M mutation and no major nevirapine resistance mutations based on sequencing results. Resistance test vectors for these 10 patients showed a median change in sensitivity of 1.3. These data suggest that an I135M mutation has a minimal or undetectable effect on drug sensitivity in patients with nonconsensus RT sequences.

Two recent papers have assessed the HIV-specific $CD8^+$ T-cell response based on autologous viral sequences rather than consensus sequences. Lee et al. (28) suggested that the response to autologous sequences was lower than that observed with sequences closely related to the consensus B clade sequence. Altfeld et al. (3) suggested the opposite, i.e., that responses to autologous sequences occurred with a higher frequency than those to consensus sequences. With individual epitopes, we found both situations. However, our data also demonstrated the difficulty with using saturating peptide concentrations in ex vivo assays to determine the physiologic significance of a change in sequence at any epitope. Despite the relatively small difference in the responses to TAFTIPSI, TAF TIPS***M***, and TAFTIPS***T*** at 2 μg of peptide/ml, three lines of evidence suggest that TAFTIPS***T*** and TAFTIPS***M*** are escape variants. First, the down-regulation of CD3 expression was larger in response to the optimized epitope TAFTIPSI than it was for either of the variant peptides (TAFTIPS***M*** and TAF TIPS***T***). Second, titration experiments showed decreased responses to TAFTIPS***T*** and TAFTIPS***M*** compared to those to TAFTIPSI. Third, HLA stabilization experiments showed a weaker binding of TAFTIPS***T*** and TAFTIPS***M*** to HLA-B51 than that observed with the optimized epitope TAFTIPSI. These data, in combination with the viral sequencing data presented, indicate that TAFTIPS***T*** and TAFTIPS***M*** represent escape variants in subject R. Such a conclusion would be hard to make based on an isolated test. Our data show that using peptides at saturating concentrations increases the ability of assays to identify responses to variants that differ from the consensus sequence. Therefore, the best use of screening assays with overlapping peptides based on consensus sequences may be to identify epitopes to which $CD8^+$ T cells are directed in vivo rather than to quantify these responses accurately. Our failure to find any additional responses when eight peptides using autologous sequences were substituted for consensus optimized epitope sequences suggests that the consensus-based approach does identify most epitopes to which HIV-specific $CD8^+$ T cells are directed (Table 6).

A recent paper by Allen et al. (1) may explain the absence of a response to the frequently recognized A3 epitopes KIRLR $PGGKK_{(p17\ 19\text{-}27)}$ and $RLRPGGKKK_{(p17\ 20\text{-}28)}$ in patient F. Allen et al. (1) showed that a K28Q mutation results in an eightfold increase in the 50% inhibitory concentration for binding of this variant to the HLA-A3 molecule and also interferes with proteasome processing of the overlapping A3 epitope $KIRLRPGGKK_{(p17\ 19\text{-}27)}$. At each of the five time points observed and in 82 viral sequences, a K28Q mutation was found in the viruses sequenced from patient F (data not shown), suggesting that the K28Q mutation may be responsible for the absence of these common A3 responses.

Evidence of viral evolution at targeted CTL epitopes during effective antiretroviral treatment suggests that therapeutic vaccination of recently infected individuals may be more successful than structured treatment interruptions. The development of escape variants occurs rapidly in the first months after infection (6, 41). It is therefore important to limit viral evolution as quickly as possible in those who were recently infected. Structured treatment interruption does appear to enhance the immunologic control of viral replication under some circumstances (44), but as shown here, conditions exist long after the initiation of treatment that allow the outgrowth of quasispecies containing escape variants. Therapeutic vaccination might limit this possibility by decreasing the opportunity for further viral evolution, although even with prolonged HAART, this may not be absolute (16, 20, 55).

ACKNOWLEDGMENTS

This research was supported in part by NIH grant ROI-AI47603. D.A.P. is a Medical Research Council (UK) Clinician Scientist.

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