Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus

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The HIV-1-specific cytotoxic T lymphocyte (CTL) response is temporally associated with the decline in viremia during primary HIV-1 infection, but definitive evidence that it is of importance in virus containment has been lacking. Here we show that in a patient whose early CTL response was focused on a highly immunodominant epitope in gp160, there was rapid elimination of the transmitted virus strain and selection for a virus population bearing amino acid changes at a single residue within this epitope, which conferred escape from recognition by epitope-specific CTL. The magnitude (>100-fold), kinetics (30–72 days from onset of symptoms) and genetic pathways of virus escape from CTL pressure were comparable to virus escape from antiretroviral therapy, indicating the biological significance of the CTL response *in vivo*. One aim of HIV-1 vaccines should thus be to elicit strong CTL responses against multiple codominant viral epitopes.

The development of effective vaccines and immune-based therapeutic strategies to combat human immunodeficiency virus type 1 (HIV-1) infection has been hampered by the lack of clear understanding of the contribution that humoral and cell-mediated immune responses make to virus containment, and also of why the immune response fails to control virus replication more completely in the early stages of the infection[1,2]. In many other virus infections, the virus-specific CD8$^+$ CTL response plays a key role in viral clearance. Likewise, recent studies in animal models suggest that vaccine-induced protection against simian and feline immunodeficiency viruses is correlated with the induction of strong antigen-specific CTL responses[3,4]. HIV-1-specific CTL activity has also been demonstrated in exposed seronegative individuals[5,6], although the relative contributions made by the cell-mediated immune response and genetically conferred resistance[7] to the lack of infection in these individuals remain unclear. Further suggestive evidence that virus-specific CD8$^+$ CTLs make an important contribution to containment of virus replication during infection with HIV-1 comes from the observations that HIV-1-infected long-term nonprogressors have high levels of HIV-1-specific CTL precursor (CTLp) cells[8,9], whereas HIV-1-specific CTL precursors are lost during progression to AIDS (ref. 8–11). However, the immunosuppressive nature of HIV-1-associated disease makes it difficult to assign cause and effect when a loss of immune function is observed in association with increasing viral titers in the end stages of the infection.

Study of the events taking place early after infection with HIV-1 has shown that virus-specific CD8$^+$ CTL responses are induced before seroconversion and are temporally associated with the fall in viremia during acute infection[1,2]. Although these observations are consistent with the antiviral CTL response playing a role in containment of virus replication early after infection, it has been suggested that the decline in primary viremia may simply be a result of population dynamics, with virus replication decreasing because of a diminishing pool of activated lymphocytes[12]. To provide definitive evidence that the early CTL response does have an impact on virus replication *in vivo*, we performed an in-depth analysis of CTL–virus interactions at early times post infection in a patient (WEAU; subject no. 1 in Clark *et al.*[13]) who presented with acute symptomatic HIV-1 infection. This particular patient was chosen for study because preliminary analysis[2,14] suggested that his early CTL response may have been of limited epitope specificity. In other virus infections, antiviral CTLs reach very high frequencies at early times post infection[15]. We reasoned that if the antiviral CTL response is indeed an important controlling force restricting HIV-1 replication during the acute phase of the infection, then in a patient where the early HIV-1-specific CTL response was directed against a small number of viral epitopes, selection for HIV-1 variants bearing mutations conferring escape from CTL recognition might be observed[16], analogous to viral mutations that lead to escape from antiretroviral drugs.

Table 1 Clinical course of patient WEAU

		HIV-1 antibody[a]		Plasma viremia[b]		
DFOSx[c]	CD4	ELISA	WB	Infectivity	RNA	p24 Ag
15	358	–	–	1000	216,400	80
16		–	–	1000		103
20		+	–	1	355,200	299
23		+	+	0	355,400	258
27	748	+	+	0	146,800	32
34		+	+	0	100,900	11
44	972	+	+	0	34,700	0
72		+	+	0	11,400	0
136		+	+	0	17,322	0
212	197	+	+	0	90,109	0
391	89[d]	+	+	5	55,268	0

[a]Presence (+) or absence (–) of HIV-1-specific antibody as determined by enzyme-linked immunosorbent assay (ELISA) or western blot (WB)[13].
[b]Plasma viremia as determined by infectivity titers (tissue culture infectious doses per milliliter of plasma[13]); plasma viral RNA (molecules per milliliter[31]); and HIV-1 p24 core antigen after antigen–antibody dissociation (picograms per milliliter[31]).
[c]Days following onset of symptoms (DFOSx) of the acute retroviral syndrome.
[d]CD4 counts declined to 30/mm³ 772 DFOSx and to 5/mm³ 1099 DFOSx. The patient received antiretroviral therapy beginning 540 DFOSx but died 1601 DFOSx.

The results presented in this paper show that CTLs directed against a highly immunodominant epitope in the viral glycoprotein were present at extremely high frequency in patient WEAU 16 days after the onset of the acute retroviral syndrome, and that this CTL response was associated with rapid and complete replacement of the plasma virus population with mutant viruses bearing amino acid changes at a single residue in gp160, which conferred escape from recognition by primary CTLs. These findings demonstrate that the early HIV-1-specific CTL response exerts a strong selective pressure on virus replication *in vivo* and illustrate a mechanism that may contribute to the inability of the antiviral CTL response to achieve more complete control of virus replication during acute and chronic infection.

Characterization of the very early CTL response in patient WEAU

Patient WEAU was a homosexual male who presented with symptomatic primary HIV-1 infection 20 days after a single sexual encounter with a patient with AIDS, shown by viral DNA sequence analysis to represent the source of infection[13]. Infectious virus was present in the plasma of WEAU at the earliest time point tested [15 days following the onset of symptoms (DFOSx)], but the titer declined rapidly, reaching undetectable levels by 23 DFOSx when seroconversion occurred (Table 1). Plasma virion-associated RNA and viral p24 antigen each peaked between 20 and 23 DFOSx. Plasma viral RNA reached its nadir at day 72 and thereafter increased coincident with a decline in $CD4^+$ lymphocyte count. Initial analysis of the CTL response mounted by this patient revealed that HIV-1-specific, major histocompatibility complex (MHC)-restricted $CD8^+$ CTL activity could be detected at the earliest time point available for study, 16 DFOSx, and that this early CTL response was directed predominantly against the viral envelope glycoprotein, gp160 (ref. 2).

To allow a more detailed characterization of the specificity of the primary CTL response, we constructed recombinant vaccinia viruses that expressed full-length gp160 derived from the autologous HIV-1 strain in patient WEAU 15 DFOSx, as well as serial sections of this protein truncated at the amino and carboxy termini. As shown in Fig. 1, polyclonal CTLs cryopreserved from patient WEAU 20 DFOSx mediated MHC-restricted lysis of target cells expressing the full-length autologous virus gp160, but not other HIV-1 proteins (Gag, Pol, Tat or Nef). Within the autologous gp160 protein, all the epitope(s) recognized were located within the N-terminal 110 amino acids (Fig. 1). When a panel of ten gp160-specific MHC-restricted CTL lines/clones derived from patient WEAU 20 DFOSx were screened, they were also all found to recognize only epitope(s) within amino acids 1–110 (not shown). To determine the location of these epitope(s), we used overlapping synthetic peptides corresponding to the N-terminal 110 amino acids of the day 15 autologous virus gp160 sequence from patient WEAU. As representative results in Fig. 2*a* illustrate, all clones tested recognized a single peptide corresponding to gp160 amino acids 25–41. By using a panel of target cells known to have one or more human leukocyte antigen (HLA) A or B alleles in common with patient WEAU (whose HLA type is A1, A29; B8, B44), the restricting HLA molecule for these clones was shown to be HLA-B44 (Fig. 2*b*). A peptide binding motif has been defined for HLA-B44: the anchor residues are Glu (E) at position 2 and Tyr (Y) or Phe (F) at position 9, with a second Y or F residue at position 10 forming an auxiliary anchor[17]. A sequence corresponding to this motif was contained in gp160 amino acids 25–41, at position 30–39. Peptides of differing lengths based on the gp160 amino acid 29–39 sequence of patient WEAU's day 15 autologous virus were tested for their relative ability to sensitize autologous target cells for lysis by day 20 WEAU CTLs. Both polyclonal CTLs and CTL clones were able to recognize a peptide corresponding to gp160 amino acids 29–39 (Fig. 2*c* and data not shown), confirming that, as predicted, the epitope was contained in this sequence. Different CTL clones were found to prefer either amino acids 30–38 or amino acids 30–39 as the optimal length of epitope (examples are shown in Fig. 2*c*). Polyclonal CTLs from patient WEAU 23 DFOSx recognized both peptides efficiently, giving 50% maximal lysis of target cells presenting each peptide at 5×10^{-6} to 10^{-7}M (not shown).

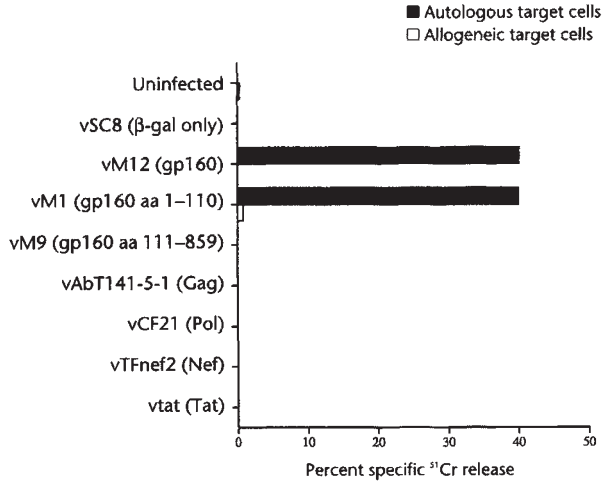

Fig. 1 All detectable HIV-1-specific CTL activity mediated by PBMCs cryopreserved from patient WEAU 20 DFOSx is directed against epitope(s) in the first 110 amino acids (aa) of gp160. The results shown are the percent specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTLs at an effector:target cell (E:T) ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding β-galactosidase (β-gal) only (vSC8); full-length gp160 (vM12) or sections thereof (vM1 and vM9) derived from the autologous virus in this patient 15 DFOSx; or other HIV-1 proteins (vAbT 141-5-1, vCF21, vTFnef2 and vtat) as indicated.

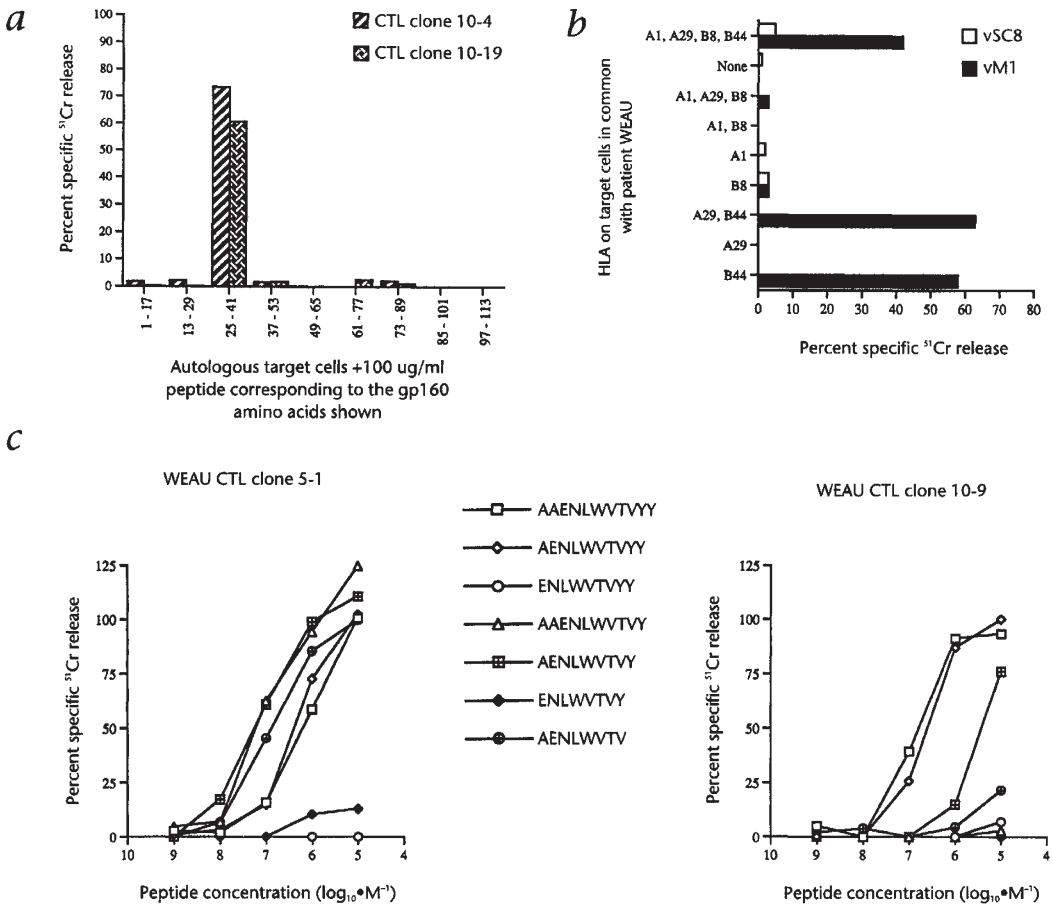

Fig. 2 Epitope mapping studies showing that CTL clones derived from patient WEAU 20 DFOSx recognize gp160 amino acids 30–38(9) in association with HLA-B44. *a*, Percent specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of 100 µg/ml of a series of synthetic peptides (each 17 amino acids long and overlapping by 5 amino acids) corresponding to the gp160 sequence of the autologous virus of patient WEAU 15 DFOSx. The amino acid 25–41 peptide recognized by both clones (and others tested) has the sequence MICSAAENLWVTVYYGV. *b*, Lysis mediated by CTL clone 10-4 at an E:T ratio of 10:1 of autologous EBV-B-LCL target cells (HLA-A1, A29; B8, B44) and EBV-B-LCL sharing between zero and three HLA-A or HLA-B molecules with patient WEAU as indicated, following infection with recombinant vaccinia viruses vM1 (expresses autologous gp160 amino acids 1–110) or vSC8 (expresses β-gal only). Target cells sharing HLA-B44 with patient WEAU were recognized by this and other day 20 WEAU CTL clones (not shown) after infection with vM1. *c*, Percent specific ^{51}Cr release mediated by two CTL clones at an E:T ratio of 10:1 from autologous target cells in the presence of different concentrations of the synthetic peptides shown, which represent the gp160 amino acid 29–39 sequence of the autologous virus of patient WEAU 15 DFOSx (AAENLWVTVYY), and N- or C-terminal truncated versions of this peptide. The optimal epitope length for recognition by the two clones differs, clone 5-1 preferring (A)AENLWVTVY, whereas clone 10-9 prefers (A)AENLWVTVYY.

Immunodominance of the gp160 epitope 16 DFOSx

Limiting dilution analysis indicated that the frequency of CTL precursors recognizing the gp160 amino acids 30–38 epitope in recombinant vaccinia virus vM1 was approximately 1 per 17 peripheral blood mononuclear cells (PBMCs) in a sample taken 16 DFOSx (not shown). By contrast, the frequency of CTLp recognizing epitopes elsewhere in gp160 (recombinant vaccinia virus vM9) was below the limit of detection in this assay (<1 per 2000 PBMCs), as was the frequency of CTL precursors directed against vSC8, the control vaccinia virus. All detectable HIV-1-specific CTL activity mediated by PBMCs cryopreserved from patient WEAU 16 DFOSx was thus directed against a strongly immunodominant viral epitope, gp160 amino acids 30–38(9). From the very high frequency 16 DFOSx, the frequency of CTL precursors directed against this epitope subsequently declined to 1 per 49 PBMCs 23 DFOSx, and 1 per 7400 PBMCs by 136 DFOSx.

Analysis of sequence changes in gp160 in early infection

To determine whether the early CTL response mounted by patient WEAU exerted a biologically significant selective pressure on the virus population, we performed a quantitative analysis of sequence changes occurring throughout gp160 over time. Because circulating plasma virus most accurately represents the actively replicating virus population *in vivo*[18,19], we sequenced and compared serial specimens of *uncultured* plasma virus RNA (cDNA). At the initial time point (16 DFOSx), plasma virus was essentially homogeneous as assessed by direct population sequencing of virion RNA (cDNA) (example shown in Fig. 3*a*) and by sequence analysis of individually cloned envelope genes (Fig. 3*b*). In other studies (not shown), *gag*, *vpu*, *env* and *nef* genes were amplified by polymerase chain reaction (PCR) from uncultured PBMC DNA of this patient (10–20 sequences per gene) and found to be similarly homogeneous (<0.5% variation) at this time point. Analysis of the *env* gene sequences from plasma virus of patient WEAU between 16 and 136 DFOSx revealed progressive replacement of the initial virus population by one differing primarily at amino acid 31 (Fig. 3). This turnover in the plasma virus population was rapid and complete by day 136 (Fig. 3*a*). Further evidence for the strong selective forces acting on this CTL epitope (amino acids 30–38) is depicted in Fig. 3*b*, which shows that changes within the epitope were numerous and accumulative, whereas changes outside it were less frequent and sporadic. For example, within the CTL epitope of the 48 *env* clones shown in Fig. 3*b* (boxed area), there were a total of 432 (9 amino acids × 48 clones) potential sites for amino acid substitution: 37 (8.6%) were changed. In the immediately adjacent sequences shown, there were 2688 (56 amino acids × 48 clones) potential sites for amino acid substitution but only 11 (0.4%) were

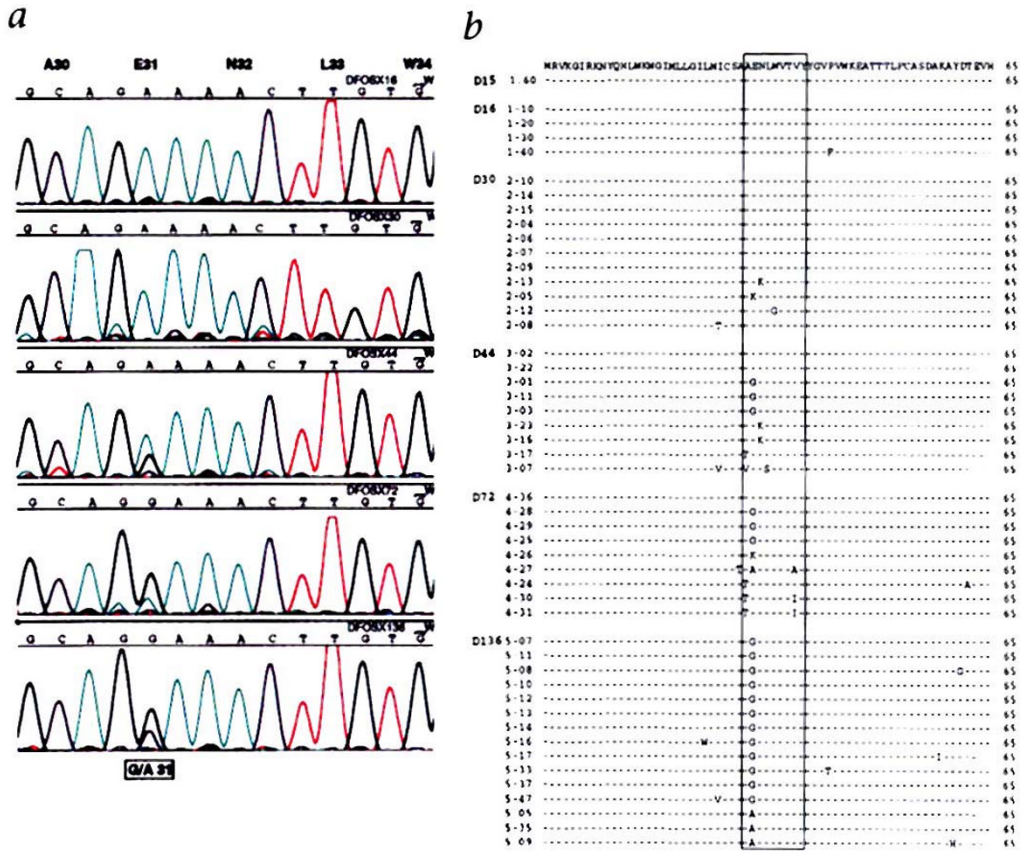

Fig. 3 Quantitative detection of HIV-1 CTL escape mutations by automated sequence analysis of plasma viral RNA (cDNA). *a*, Population sequences of codons 30–34 of the gp160 gene are shown, demonstrating the emergence of mutant virus [GAA(E) to GAA/GGA (E/G) at codon 31] on day 44 and with complete replacement of wild-type virus (AENLW) by mutant virus (AGNLW/AANLW) by day 136. *b*, Deduced amino acid sequences of individual plasma viral RNA (cDNA) clones corresponding to the N-terminal 65 amino acids of gp160. The CTL epitope at position 30–38 is boxed.

changed. Thus, changes within the CTL epitope exceeded by 21-fold those in surrounding areas of *env*. Moreover, the proportion of nonsynonymous nucleotide changes within the CTL epitope (37/38; 97.4%) exceeded that in the surrounding *env* regions (11/16; 68.8%) by 1.4-fold. To further evaluate whether the frequency and rapidity of sequence changes observed at positions 30–38 were unique, direct viral RNA (cDNA) population sequencing of the entire N-terminal half of gp160 (430 amino acids) was performed on plasma specimens corresponding to days 16, 30, 44 and 72 DFOSx; the *only* changes observed by population sequencing were those at position 31 [E to Gly (G) or Ala (A)]. To complete the analysis of HIV-1 quasispecies evolution in this patient, population sequencing of the full-length gp160 (860 amino acids) from plasma viral RNA (cDNA) from day 136 DFOSx, together with sequence analysis of two full-length gp160 molecular clones derived from the same viral RNA (cDNA), were compared with the viral sequences from day 16. Here, for the first time, changes in addition to those occurring at codon 31 were identified. These included substitutions at amino acids 356 [Lys (K) to E], 400 [His (H) to Y], 402 [Asn (N) to Asp (D)], 407 (N to D), 815 [Val (V) to Ile (I)], 819 (N to D) and 857 [A to Thr (T)]. (Sequences submitted to GenBank, accession nos. GSDB:S: 1166004–1166056 and U21135).

Virus escape from CTL recognition

To determine whether the observed changes in the gp160 amino acid 30–38 epitopic sequence affected CTL recognition of this epitope, synthetic peptides corresponding to amino acids 30–38 of the predominant mutant virus gp160 sequences were tested for their ability to sensitize autologous target cells for lysis by polyclonal CTLs and CTL clones derived from patient WEAU 16–20 DFOSx. Unlike the wild-type peptide, peptides corresponding to the amino acid 31 mutant virus sequences, which first emerged 30–72 DFOSx and became predominant by 136 DFOSx, were not recognized by polyclonal CTLs cryopreserved from patient WEAU 16 DFOSx (not shown), or by CTL clones derived from the patient 20 DFOSx (Fig. 4). This could either be because of failure of these mutant peptides to bind to HLA-B44 or lack of recognition of the peptides by CTLs following MHC binding: the former seems more likely given that the gp160 amino acid 31 E residue forms one of the dominant MHC anchor residues in the HLA-B44 peptide binding motif[17]. It is interesting that virus variants with an amino acid substitution from A to T at position 30 were transiently observed at 44 and 72 DFOSx but were not selected for by 136 DFOSx, and that the corresponding peptide was efficiently recognized by epitope-specific CTLs (Fig. 4). Thus, unlike the early (day 16) or transient (A30T; day 44) viral population, mutants at position 31 (E31G; E31A) were able to escape recognition by the primary CTL response mounted by this patient and accumulated progressively through day 136.

Subsequent evolution of the CTL response in patient WEAU

The emergence of a mutant virus population able to escape recognition by the primary HIV-1-specific CTL response in patient WEAU was not accompanied by a dramatic increase in the plasma viral load in this patient (Table 1). Analysis of the CTL activity mediated by PBMCs cryopreserved from patient WEAU 30 DFOSx (Fig. 5) revealed that, even by this relatively early time, responses were detectable to several new viral epitopes, including at least one in gp160 amino acids 111–860, plus epitopes in Gag, Pol and Nef. CTLs of these novel specificities would be capable of recognizing their antigens associated with the gp160 amino acid 31 mutant virus population and thus could account for its continued containment.

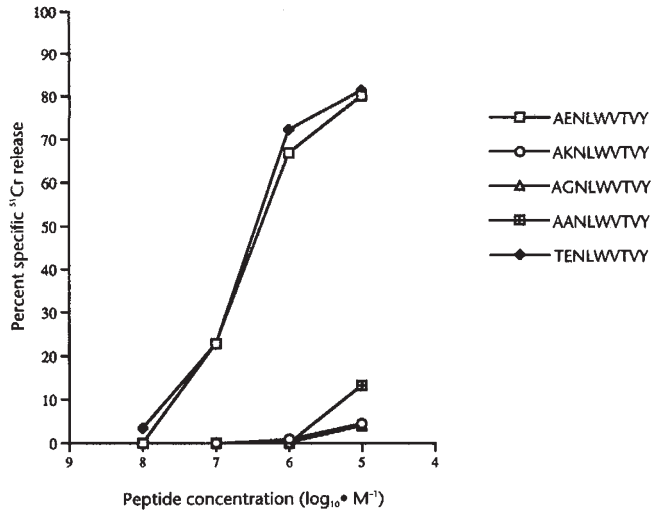

Fig. 4 Relative abilities of synthetic peptides corresponding to gp160 amino acids 30–38 of the virus population in patient WEAU 16 DFOSx, as well as the predominant mutant populations that emerged later, to sensitize autologous target cells for lysis by early WEAU CTLs. The results shown are the percent specific ^{51}Cr release mediated by day 20 WEAU CTL clone no. 5-1 at an E:T ratio of 10:1 from autologous EBV-B-LCL target cells in the presence of different concentrations of synthetic peptides, as indicated.

Discussion

Here we provide evidence that the early HIV-1-specific CTL response exerts a substantial controlling pressure on virus replication *in vivo*. The present study is unique in that it represents a detailed molecular analysis of CTL–virus interactions very early after HIV-1 infection, before seroconversion and even before viral RNA had achieved peak titers. The first time point analyzed, 15 DFOSx, was exactly 35 days after the patient was infected by HIV-1 (ref. 13). The kinetics of decline in plasma viremia coincided temporally with the appearance, first, of a strong CTL response focused on a highly immunodominant epitope at gp160 amino acids 30–38 and, shortly thereafter, of broader CTL reactivity. Evidence for a strong and biologically important selective pressure exerted by CTLs was provided by the observation that by 30–44 DFOSx, CTL escape mutants were detectable. By 136 DFOSx, there had been complete replacement of the transmitted virus strain (which had initially replicated to high titers) by a mutant population that differed primarily at amino acid 31. The cellular compartments harboring replicating virus early in infection were thus largely eliminated and replaced by cells infected with CTL escape variants by this time point. The magnitude of wild-type virus decline, the kinetics of mutant virus appearance, and the genetic pathways by which virus escaped CTL recognition bear certain similarities to viral dynamics in the setting of antiretroviral drug therapy. For example, between 16 and 72 DFOSx, wild-type virus in the plasma (defined by amino acids 30–38 sequence AENLWVTVY) declined from 200,000–300,000 RNA molecules per milliliter to approximately 1,000 (10% of 11,400). Mutant virus first appeared at 30 DFOSx and evolved in a complex pattern until a best-fit population not recognized by HLA B44-restricted CTLs came to predominate at day 136. Viral evolution to the amino acids 30–38 escape variant would likely have occurred even more rapidly, and plasma virus titers reached higher levels, had not CTL responses directed against other viral gene products developed.

HIV-1 variants that are not recognized by autologous CTLs have previously been observed in longitudinal studies of HIV-seropositive patients[21–23], but it has generally been difficult to demonstrate that CTL escape mutant viruses have a clear selective advantage *in vivo*. One factor that may have facilitated the demonstration of CTL escape variants in this study may be our approach for identifying changes in the viral quasispecies by analyzing plasma viral RNA (cDNA). Plasma virus exhibits a circulating half-life of approximately 6 hours[20] and the cells producing most of this virus a half-life of approximately 2 days[18,19]. However, these virus-producing cells are underrepresented in the blood where latently infected cells and defectively infected cells (with half-lives as long as 80 days) predominate (G.M.S. *et al.* unpublished data). Analysis of plasma viral RNA gives a dynamic assessment of the most active viral compartments.

From an immunological perspective, the fact that the earliest CTL response in patient WEAU was predominantly focused on a highly immunodominant viral epitope and that the CTLp frequency was so high early after infection also may explain why CTL-mediated selection of escape-conferring mutations was more evident in this patient than in previous studies[21–25]. If CTL pressure is simultaneously directed against several codominant epitopes, the outgrowth of virus variants with escape-conferring mutations in only one of these epitopes may be controlled by CTLs directed against the other epitopes[26,27] (unless the variant epitopes have strongly antagonistic properties)[28]. At 16 DFOSx, limiting dilution assays showed the frequency of epitope-specific CTL precursors to be approximately 1 per 17 PBMCs, and even this value is likely to be an underestimate because the PBMCs had been cryopreserved before testing. This frequency is similar to the CTLp frequencies measured at the peak of the acute response to lymphocytic choriomeningitis virus (LCMV) infection in mice[15,29]. The primary CTL response in patient WEAU may thus have been near its peak around 16 DFOSx. Whether HIV-1-specific CTL precursors generally reach such

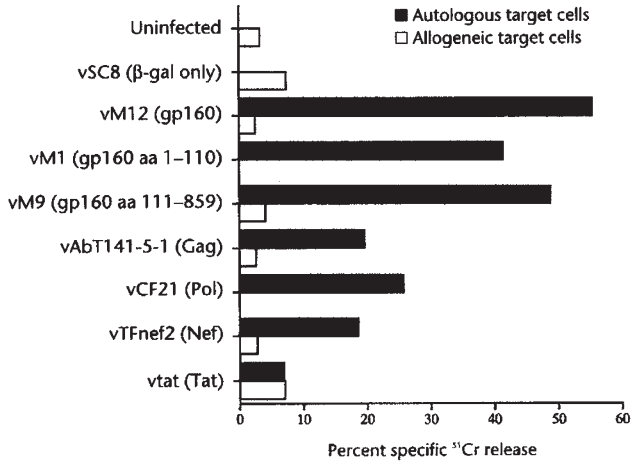

Fig. 5 Peripheral blood mononuclear cells cryopreserved from patient WEAU 30 DFOSx mediate CTL activity directed against multiple HIV-1 epitopes. The results shown are the percent specific ^{51}Cr release mediated by *in vitro* restimulated polyclonal CTLs at an E:T ratio of 40:1 from autologous and allogeneic EBV-B-LCL target cells infected with recombinant vaccinia viruses encoding β-galactosidase only (vSC8); gp160 derived from the plasma virus in patient WEAU 15 DFOSx and sections thereof (vM12, vM1 and vM9); or other HIV-1 proteins (vAbT 141-5-1, vCF21, vTFnef2 and vtat) as indicated.

high frequencies at the peak of the early immune response is currently unclear.

As patient WEAU obviously had the capacity to mount CTL responses to multiple HIV-1 epitopes, it remains unclear why the earliest CTL response in this patient was so predominantly focused against the gp160 amino acids 30–38(9) epitope. One possibility is that prior unrelated infections in this patient left him with a population of memory T cells that cross-reacted on this epitope and that these cells, being present at higher frequency and more readily activated than naive T cells directed against other HIV-1 epitopes, dominated the initial HIV-1-specific immune response. That the CTL response mounted to a virus infection may be modified by the host's prior immune experience with unrelated pathogens has been demonstrated in murine virus infections[30]. Alternatively (or in addition), the gp160 amino acids 30–38(9) epitope may have been particularly immunogenic. In this context, it is of interest that during the natural processing of gp160, signal peptide cleavage occurs between amino acids 29 and 30, thus generating the same N terminus as the amino acid 30–38 CTL epitope. This may have favored production of the amino acid 30–38 peptide at higher levels than other epitopic peptides and may have resulted in preferential presentation of this epitope especially at very early times after infection when levels of viral antigens were limiting. The immunogenicity of a peptide is also influenced by its affinity of binding to MHC and the affinity of peptide–MHC complexes for the T-cell receptor. The gp160 amino acid 30–38(9) epitope may have been "strong" in these respects, and the epitopes recognized later may have been "weaker." The shift in the response to weaker epitopes may have reduced the long-term efficiency of containment of virus replication[23] and may have been one of the factors that contributed to the subsequent rapid rate of disease progression in patient WEAU[31]. Recent studies[32] suggest that the viral load established early in HIV infection is a predictor of the subsequent clinical course, with higher viral loads after seroconversion predicting shorter survival. The shift in CTL specificity, which occurred around the time of seroconversion in patient WEAU, may also have influenced the viral "setpoint" established in this patient, again contributing to his rapid rate of disease progression.

Future in-depth analysis of virus–CTL interactions in larger numbers of patients is needed to reveal how commonly CTL escape virus variants are selected for during acute HIV-1 infection and whether their selection is correlated with the establishment of a high viral setpoint and a rapid rate of subsequent disease progression. Preliminary results we have obtained in a second patient show that here, too, rapid selection occurred within the plasma virus population for mutants bearing amino acid changes in a gp160 epitope recognized by the early CTL response. It is noteworthy that this patient was also a rapid disease progressor. The events we report here in patient WEAU are thus not unique and may in fact prove to represent a mechanism that HIV-1 commonly uses to evade control by the early antiviral immune response.

In summary, the results presented here provide the first direct demonstration of the substantial and biologically relevant pressure exerted by the early CD8$^+$ CTL response on HIV-1 replication *in vivo*. Together with the large body of suggestive evidence already in the literature that virus-specific CD8$^+$ CTLs make an important contribution to containment of virus replication during infection with HIV-1 (ref. 5, 6, 8–11), these findings suggest that stimulation of this arm of the antiviral immune response should be an important goal of future prophylactic and therapeutic strategies to combat HIV-1 infection.

Methods

Patient samples. The clinical profile of patient WEAU has been reported[13]. After obtaining informed consent, blood specimens were collected in acid-citrate-dextrose. PBMCs were isolated and cryopreserved as previously described[2]; plasma was also cryopreserved at each time point.

Recombinant vaccinia viruses. Recombinant vaccinia viruses vSC8, which expresses only *Escherichia coli* β-galactosidase; vPE16, which expresses gp160 from HIV-1 IIIB; vCF21, which expresses Pol from HIV-1 HxB2; vTFnef2, which expresses Nef from HIV-1 NL43 and vtat, which expresses Tat from HIV-1 IIIB were obtained from Bernard Moss [National Institutes of Health (NIH), Bethesda, MD]; and recombinant vaccinia virus vAbT 141-5-1, which expresses the full-length p55 Gag protein from HIV-1 IIIB (ref. 33) was obtained from Dennis Panicali and Gail Mazzara (Therion Biologics Corp., Cambridge, MA).

Recombinant vaccinia viruses expressing full-length gp160 (vM12) or sections thereof (vM1 and vM9) derived from the autologous HIV-1 in patient WEAU 15 DFOSx were produced by homologous recombination into the thymidine kinase gene of vaccinia virus by using the vaccinia transfer plasmid pNVV3, a modified version of pSC11, as described[34]. The clone expressing full-length gp160 used for the construction of the recombinant vaccinia viruses was obtained from a full-length replication-competent proviral clone (1.60) derived by lambda phage cloning of an isolate obtained 15 DFOSx (ref. 35). The envelope gene was subcloned into plasmid pCR II (Invitrogen Corp., San Diego, CA), from which it was subsequently excised and ligated into the *Sma*I and *Not*I sites of pNVV3 to yield pM12. Sections corresponding to nucleotides (nt) 1–330 and 334–2580 of the *env* gene were amplified from the full-length clone by PCR using oligonucleotide primers that incorporated the necessary start and stop codons as well as a *Sma*I site at the 5' end and a *Not*I site at the 3' end. The PCR products were then ligated into pNVV3 to yield pM1 (*env* nt 1–330) and pM9 (*env* nt 334–2580). pM12, pM1 and pM9 were used to produce recombinant vaccinia viruses vM12, vM1 and vM9, respectively. Protein expression was confirmed by western blotting (not shown).

CTL assays. In some assays the effector cells were polyclonal patient CTLs. Here, cryopreserved PBMCs were expanded *in vitro* by bulk culture for 10 days as previously described[2]. For use in other experiments, CTL lines/clones were established from PBMCs cryopreserved from patient WEAU 20 DFOSx by culture at limiting dilution as described[36]; these are operationally referred to as clones. CTL activity was assayed in a conventional 5-h ^{51}Cr release assay as described[2]. Target cells were autologous (HLA A1, A29; B8, B44) and allogeneic EBV-B-LCL, either uninfected or infected at a multiplicity of infection of 10 plaque-forming units (PFU) per cell 16 h previously with different recombinant vaccinia viruses. In some assays, synthetic peptides were added to the assay medium at different concentrations. Peptides were synthesized by the solid-phase method on an automated peptide synthesizer with fluorenyl methoxycarbonyl (FMOC) chemistry and purified, and their identity was confirmed as described[37]. CTL assay results are expressed as the percent specific ^{51}Cr release, calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Limiting dilution analysis of specific CTLp frequency. Precursor frequencies of specific CTLs were estimated by limiting dilution analysis as described[33]. Cryopreserved patient PBMCs were plated at a range of dilutions and were restimulated by *in vitro* culture in interleukin-2-containing medium with an anti-CD3 antibody and irradiated allogeneic PBMCs from a normal donor in order to allow expansion of CTLs. Wells were then split and assayed for cytotoxicity on ^{51}Cr-labeled autologous target cells infected with different recombinant vaccinia viruses or coated with synthetic peptide AENLWVTVY. The fraction of nonresponding wells was calculated by determining the number of wells in which ^{51}Cr release did not exceed 10% specific lysis. Precursor frequencies were then estimated by single hit model Poisson distribution analysis[38].

Gp160 sequence analysis. The gp160 sequence of the autologous virus from patient WEAU 15 DFOSx was determined by automated DNA sequencing of the WEAU 1.60 provirus and is reported as the HIV-1 reference sequence in the 1995 HIV Molecular Immunology Database[35]. Quantitative detection of gp160 mutations occurring in the uncultured plasma virus population over time was performed as described[18]. HIV-1 RNA was isolated from virions pelleted from plasma specimens, and cDNA prepared by using antisense oligonucleotide primers corresponding to either 5'–TTGCTACTTGTGATTGCTCCATGT–3' (nt 8920–8943), 5'–TCTTATGAGTGTGGTGACATTGAAAGA–3' (nt 6706–6732), or 5'–CAGAGTGGGGTTAATTTTACACATGG–3' (nt 6571–6596) [numbering according to HIV-1 proviral clone WEAU 1.60 (ref. 39)]. Full-length and partial gp160 gene sequences were amplified by nested PCR as described[18] by using the primers listed above along with primers at positions 5851–5875, 5956–5981, 6204–6226, 6436–6455, 6571–6596 and 8889–8911. Primers incorporated the universal M13 sequence for subsequent dye-primer sequence analysis of the gp160 amino acid 30–39 region. For sequence analysis of the complete gp160 gene, dye-labeled dideoxynucleotide terminators were used. A total of four to six separate PCR amplifications of plasma viral RNA/cDNA (750 molecules/reaction) was done for each time point. Double-strand sequence analysis was performed by using an automated ABI 373A Sequenator and Dye Primer/Dye Terminator Cycle sequencing kits (Applied Biosystems Inc., Foster City, CA). Sequences were analyzed by using Sequencher (Gene Codes Corp., Ann Arbor, MI) and Microgenie (Beckman Instruments, Fullerton, CA) software packages, and base-pair mixtures were quantified by measuring relative peak-on-peak heights[18]. Relative proportions of wild-type and mutant sequences were determined independently by subcloning the amplified gp160 envelope products (above) into pCR3 (Invitrogen) and subjecting the individual clones to double-stranded automated DNA sequencing.

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