Determinants of HIV-1 Mutational Escape From Cytotoxic T Lymphocytes

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Abstract

$CD8^+$ class I–restricted cytotoxic T lymphocytes (CTLs) usually incompletely suppress HIV-1 in vivo, and while analogous partial suppression induces antiretroviral drug-resistance mutations, epitope escape mutations are inconsistently observed. However, escape mutation depends on the net balance of selective pressure and mutational fitness costs, which are poorly understood and difficult to study in vivo. Here we used a controlled in vitro system to evaluate the ability of HIV-1 to escape from CTL clones, finding that virus replicating under selective pressure rapidly can develop phenotypic resistance associated with genotypic changes. Escape varied between clones recognizing the same Gag epitope or different Gag and RT epitopes, indicating the influence of the T cell receptor on pressure and fitness costs. Gag and RT escape mutations were monoclonal intra-epitope substitutions, indicating limitation by fitness constraints in structural proteins. In contrast, escape from Nef-specific CTL was more rapid and consistent, marked by a polyclonal mixture of epitope point mutations and upstream frameshifts. We conclude that incomplete viral suppression by CTL can result in rapid emergence of immune escape, but the likelihood is strongly determined by factors influencing the fitness costs of the particular epitope targeted and the ability of responding CTL to recognize specific epitope variants.

Key words: cellular immunity • T cell receptor • antigenic variation • T cell receptor specificity

Introduction

Cellular immunity is believed to have an important role in the pathogenesis of HIV-1 infection. Several clinical correlations strongly suggest that $CD8^+$, MHC class I–restricted CTLs specific for HIV-1 contribute to the control of disease: temporal correlation of CTL induction to the drop in peak viremia in acute infection (1, 2), inverse correlations of CTL activity with clinical progression (3, 4) and viral load (5) in chronic infection, and the finding of antiviral CTL activity in some exposed yet uninfected individuals (6–8). Data from in vitro studies further indicate that CTL are capable of exerting potent antiviral effects (9–11).

Despite often vigorous CTL responses against HIV-1, however, most infected persons fail to contain and clear the infection. Many mechanisms for CTL failure have been proposed (for reviews, see references 12–17), and a leading hypothesis is escape through epitope mutation. In evaluations of viral sequences in infected persons, evolving epitope mutations have clearly suggested escape in a few longitudinal studies (18–23). Data in acute SIV (24, 25) and HIV-1 (1, 22) infection have more clearly demonstrated the emergence of viral CTL escape mutations, suggesting that CTL of specificities in early versus late infection (26) may differ in their propensity for escape mutations. Nevertheless, the issue of whether CTL exert immune pressure leading to viral escape has remained controversial, with other studies failing to find any evidence for epitope escape mutation in HIV-1 (27–32). Based on the assumption that immune pressure and escape mutation are directly correlated, several of these studies have concluded that CTL do not exert antiviral pressure in vivo.

The emergence of HIV-1 escape mutation depends upon the net balance between multiple factors that are poorly understood in vivo. Selective pressure exerted by the CTL may be influenced by numerous determinants, including CTL frequency, epitope specificity, T cell receptor

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properties, and functional state of the CTL. In turn, viral constraints on the generation of epitope mutations include the viral fitness cost of the mutations, through either direct loss of replicative capacity or interference with functions important for viral persistence. Indirect factors such as access of CTL to infected cells, the level of viral replication and diversity, the types of cells infected, the interplay between CTL of multiple specificities, and viral immune-modulatory strategies (such as MHC down-regulation) likely play a role as well. For an epitope mutation to predominate in vivo the sum of these factors must favor overall fitness of the viral mutant.

It is unclear to what extent the lack of the escape observed for some CTL responses in vivo reflects lack of CTL pressure or the influence of other factors. Absence of escape mutations could reflect the selective persistence of CTL that recognize highly constrained epitopes where change is not tolerated, inadequate CTL function and immune pressure, or a combination of these and/or other factors. Therefore, we have evaluated the ability of CTL to induce escape mutations in HIV-1 in vitro where conditions can be readily standardized. We used a panel of HIV-1–specific CTL clones characterized for epitope specificity and HLA restriction, cocultured with acutely infected HIV-1–permissive T cell lines, to evaluate the evolution of escape mutations while controlling for factors that cannot be manipulated feasibly in vivo.

Materials and Methods

HIV-1–permissive Cell Lines. T1 (33), T2 (34), and H9 (35) cells were maintained as described previously (10). T1 cells expressed HLA A2 and B60 as determined by serological typing and were therefore HLA matched at the restricting class I allele with all the CTL clones in this study. T2 cells (class I antigen transport-deficient) or H9 cells (not expressing HLA A2 or B60), being unable to present antigen to the CTL studied, served as negative control cells for all the selection experiments described below.

HIV-1 Molecular Clone Point Mutants. NL4–3 (36) Gag p17 point mutations were produced by PCR-based point mutagenesis (37) of the p83–2 plasmid (38) with sequence confirmation. These mutants are indicated in Table I.

Virus Stocks. HIV-1 IIIB was produced by serial passaging in H9 cells. NL4–3 and NL4–3 Gag mutant virions were produced by coelectroporation of H9 cells with p83–2 derivatives and p83–10 plasmid DNA linearized with EcoR1 (38). NL4–3.1 differed from wild-type NL4–3 by point mutations in p17 Gag, altering the NL4–3 sequence from SLYNTIAVL to the consensus SLYNTVATL sequence (Table I). Low passage virus stocks were frozen in aliquots at −80°C until use. Viral titer ($TCID_{50}$/ml) was determined by endpoint dilution with C8166 indicator cells as described previously (39).

CTL Clones. HIV-1–specific CTL clones were obtained from the blood of infected individuals by cloning of PBMC at limiting dilution, and characterized for specificity and class I restriction as described previously (40). Three clones (161JxA14, 18030D23, 115DEC4) recognized the HLA A2–restricted epitope SLYNTVATL (SL9) in Gag (p17 amino acids [a.a.]* 77–85, SL9). One (68A62) recognized the A2-restricted epitope ILKEPVHGV (IV9) in reverse transcriptase (RT a.a. 309–317). One (161JD27) recognized the B60-restricted epitope IEIKDTKEAL (IL10) in Gag (p17 a.a. 92–101). One (161Jx12) recognized the B60-restricted epitope SEGATPQDL in Gag (p24 a.a. 44–52). Two (STD11, KM3) recognized the B60-restricted epitope KEKGGLEGL (KL9) in Nef (Nef a.a. 92–100; all positions are numbered in relation to HXB2 sequences). Of note, clones 161JxA14, 161JD27, 161Jx12, and STD11 were derived from a single infected long-term nonprogressing individual, subject 161J. All CTL were maintained as described previously (10).

Passaging of HIV-1 Under Selection by CTL Clones. 5×10^6 T1 and control H9 or T2 cells were initially infected with HIV-1 at a multiplicity of 10^{-1} $TCID_{50}$/cell for 4 h, followed by two washes and resuspension in RPMI supplemented with 10% fetal calf serum and 50 U/ml IL-2 (NIH AIDS Reference and Reagent Repository). 5×10^5 CTL were then added in a total volume of 10 ml R10–50, followed by culture in a T25 flask in a humidified incubator with 5% CO_2. The cultures were then fed twice over the next week, followed by harvesting of the cell pellet and supernatant virus, which were both saved at −80°C. The process was then repeated in a second round of passage using the supernatant virus, at an initial input of 500 pg p24 antigen (quantitated by commercial p24 ELISA kit; Dupont) per 10^6 target cells. The second round was otherwise performed in identical fashion to the first, with cell pellets and supernatant virus again saved for subsequent analyses.

Analysis of HIV-1 for Susceptibility to Inhibition by CTL. HIV-1 was tested for susceptibility to inhibition using a coculture system we developed previously (10). T1 cells (HLA matched at A2 or B60 with all the clones used in this study) were infected with the passaged supernatant virus at 500 pg p24/10^6 cells, washed twice, and cocultured with CTL at a ratio of 5×10^5 T1 cells with 1.25×10^5 CTL. At two to four day intervals, 1 ml of supernatant was removed for quantitative p24 ELISA (Dupont) and replaced with fresh medium.

Chromium Release Assays. CTL were assessed for specific lysis of peptide-loaded target cells as described previously (41). Briefly, target cells were labeled with $Na_2{}^{51}CrO_4$ (New England Nuclear) for 1 h with or without synthetic peptide at 100 μg/ml unless otherwise indicated. Cytolytic activity against these target cells was then determined by adding effector CTL at a ratio of 5:1 (with appropriate controls for spontaneous and maximal lysis) in a 96-well U-bottom microtiter plate and measuring ^{51}Cr release in duplicate after 4 h. For determination of the 50% sensitizing dose of peptides (SD_{50}), the target cells were preincubated with serial 10-fold concentrations of the peptide, and the approximate concentration resulting in 50% of maximal killing by the CTL was extrapolated.

Analysis of Passaged Virus for Epitope Mutations. DNA was isolated from the cell pellets after each round of passaging (Puregene DNA isolation kit; Gentra Systems). Proviral sequences were amplified using nested PCR (25 cycles each for two amplifications), under limiting dilution conditions predetermined by dilution series to yield approximately one amplification per five reactions. Positive reactions were then PCR-sequenced using the same primer set as the secondary amplification.

Sequences Around the SLYNTVATL (SL9) and IEIKDTKEAL (IL10) Epitopes. A sequence spanning potions of Gag p17 and p24 was amplified using outer primers GCGGAGGCTAGAAGGAGAGAG (5LTR768) and TGCTGTCATCATTTCTTCTAGTGT (3gag1047), and inner primers ATGGGTGCGAGAGCGTCAGTAT (5gag1) and TCTATCCCATTCTGCAGC-

*Abbreviation used in this paper: a.a., amino acid(s).

Table I. *NL4-3–based SL9 Variant Viruses*

DNA sequence	A.A. sequence	
TCA TTA TAT AAT ACA GTA GCA ACC CTC	SLYNTVATL	(Consensus SL9 sequence, NL4-3.1)
TCA TTA TAT AAT ACA ATA GCA GTC CTC	-----I-V-	(Wild-type NL4-3)
TCA TTA TTT AAT ACA GTA GCA ACC CTC	--F------	
TCA TTA TTT AAT ACA ATA GCA ACC CTC	--F--I---	
TCA TTA TAT AAT CTA GTA GCA GTC CTC	----L--V-	

The panel of SL9 mutant viruses used in Fig. 1 is indicated. Point mutagenesis was performed on NL4-3 to alter the epitope; changed nucleotides are underlined. Amino acid sequences of the epitope relative to the consensus SL9 sequence are also indicated.

TTC (3gag642). This amplified HIV-1 nucleotides 812–1410 (HXB2 numbering), containing the coding sequences for SL9 (1018–1044) and IL10 (1063–1092).

Sequences Around the ILKEPVHGV (IV9) Epitope. A sequence spanning a portion of reverse transcriptase was amplified using outer primers AGAACCTCCATTCCTTTGGA (RT3218F) and TGGGCCTTATCTATTCCATCTAAAAATAGT (4255R), and inner primers TACAGCCTATAGTGCTGCCAG (RT3271F) and GTGCTGGTACCCATGCCAGATAGAC (4164R). This amplified HIV-1 nucleotides 3292–4139, containing the coding sequences for IV9 (3474–3500).

Sequences Around the KEKGGLEGL (KL9) Epitope. A sequence spanning almost all of Nef was amplified using outer primers AGAGCTATTCGCCACATACC (NEF8736) and TAGTTAGCCAGAGAGCTCCCA (NEF9589R), and inner primers CTATAAGATGGGTGGCAAGTG (NEF8780F) and TTATATGCAGCATCTGAGGGC (NEF9495R). This amplified HIV-1 nucleotides 8810–9498, containing the coding sequences for KL9 (9069–9095).

Results

CTL Clones Recognizing the Same Epitope Exhibit Differential Tolerance for Epitope Mutation. HIV-1–specific CTL clones vary in their ability to recognize epitope mutants as determined by synthetic peptide titrations, as has been shown for a comprehensive study (31) of CTL recognizing a common HLA A2–restricted epitope in Gag p17 a.a. 77–85, SLYNTVATL (SL9). To evaluate whether this poor recognition of exogenously added peptide variants affects the antiviral function of CTL, two SL9-specific clones from different persons were tested for their ability to suppress a panel of HIV-1 mutants differing in SL9 sequence (Fig. 1). Clones 161JxA14 and 18030D23 demonstrated clear differences in their ability to act upon HIV-1 with variation in SL9. Whereas 161JxA14 still suppressed virus with the --F------ variant of SL9, 18030D23 did not. Conversely, the --F--I--- and -----I-V- variants were suppressed by 18030D23 but not by 161JxA14. Previously published (31) 50% sensitizing dose peptide concentrations (SD_{50}) for recognition of exogenously added peptide variants by 18030D23 indicated that SL9 and variants --F--I--- and -----I-V- were relatively well-recognized ($SD_{50} < 50$ ng/ml), whereas the other poorly inhibited variants were inefficiently recognized ($SD_{50} > 500$ ng/ml). Although 161JxA14 was not evaluated in that study, two other SL9-specific clones from the same subject exhibited good recognition of SL9 and the --F------ variant ($SD_{50} < 10$ ng/ml) and poor recognition of the other variants ($SD_{50} > 400$ ng/ml). Our findings therefore indicated that CTL clones targeting the same epitope can vary significantly in their antiviral activity against HIV-1 containing epitope variants, providing functional confirmation for prior findings using exogenously added synthetic peptide.

HIV-1 Can Rapidly Escape from CTL Pressure through Epitope Mutation. To examine whether HIV-1 could respond to selective pressure against CTL under controlled conditions, a molecular clone virus was passaged in the presence of the SL9-specific CTL clone 161JxA14 under conditions favoring vigorous viral replication (Fig. 2). The

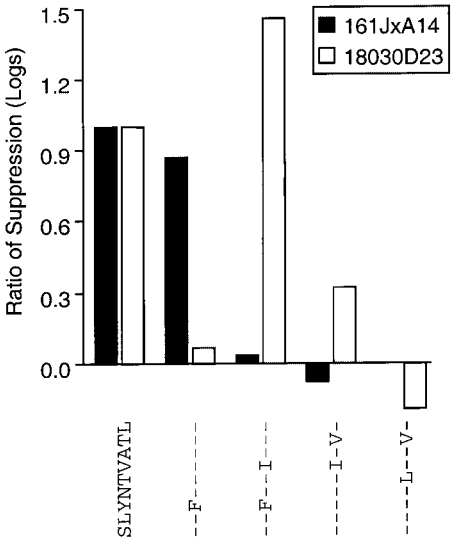

Figure 1. HIV-1–specific CTL clones differentially inhibit replication of HIV-1 point mutants. A panel of HIV-1 NL4–3–based viruses containing point mutations in the SL9 epitope was screened for inhibition of viral replication by the SL9-specific CTL clones 161JxA14 and 18030D23. Viral replication as assessed by quantitative p24 ELISA was measured 7 d after addition of CTL to acutely infected T1 cells. Inhibition of each virus by each clone (in $\log_{10}$ units) was determined by comparing p24 production in the presence and absence of CTL. Plotted in this figure are the ratios of suppression of HIV-1 with each SL9 mutation relative to virus with the index SL9 sequence SLYNTVATL.

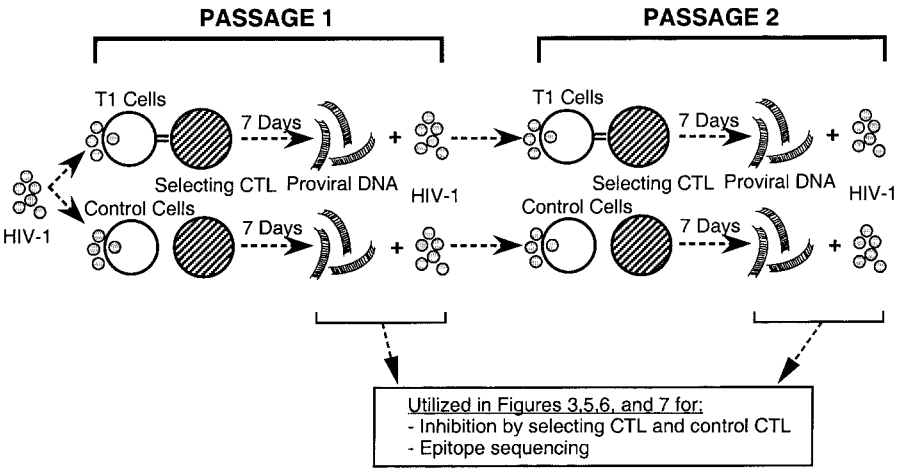

Figure 2. Scheme for passaging HIV-1 under selective pressure by CTL. Defined starting virus was grown in T1 cells (able to present antigen to the selecting CTL clone) or a control cell line (unable to present antigen to the CTL due to HLA mismatching or antigen transport defect) in the presence of a selecting CTL clone for 7 d. After this first passage, proviral DNA and supernatant HIV-1 were harvested. These were then used for the analyses of sensitivity to CTL and epitope sequence (Figs. 3, 5, 6, and 7). The virus was also further passaged under the same conditions for a second passage, after which proviral DNA and supernatant HIV-1 were again harvested for analysis.

viruses resulting from this passaging were then screened for development of resistance to that clone and sequenced for epitope changes (Fig. 3). Selected virus and negative control virus (passaged in parallel in cells unable to present antigen to the clone) were tested for phenotypic sensitivity to that Gag-specific clone or a control HLA A2–restricted RT-specific clone (68A62). Virus that had previously undergone one week of selective passaging remained sensitive to the selecting CTL (Fig. 3, bottom left), but virus that had undergone two weeks of selective passaging developed resistance (Fig. 3 bottom right, see also Fig. 6), as indicated by inhibitory activity relative to the control RT-specific CTL clone. Negative control virus from both passages remained sensitive (Fig. 3 top row). Limiting dilution sequencing revealed that the control virus remained unaltered in the Gag epitope, while CTL-selected virus was also unaltered after 7 d of culture (Fig. 3 bottom left), but contained a predominance of a mutation in the epitope to -----I--- after the second round of passage (Fig. 3, bottom right; see also Fig. 6). Mutation was confined to the SL9 epitope and not observed in flanking sequences (Table II, and unpublished data). This epitope variant was poorly recognized by 161JxA14 in a peptide titration chromium release assay, with an SD_{50} of >100 μg/ml (Fig. 4). These data indicated that a specific monoclonal genetic escape mutation arose within two weeks of selective pressure by the Gag-specific CTL clone, conferring phenotypic resistance.

Different CTL Clones Recognizing the Same Epitope Vary in Their Susceptibility to HIV-1 Escape. Given these results, two other CTL clones recognizing the same Gag SL9 epitope (18030D23 and 115DEC4) derived from two additional HIV-1–infected persons were also tested in the same manner (Fig. 5 and Table II). Resistance was not observed for either Gag-specific clone after two passages (18030D23 Fig. 5, 115DEC4 unpublished data). Epitope sequencing revealed a minority epitope mutation only in virus that had previously undergone two passages under selection by 18030D23 (Fig. 5 and Table II) and none for 115DEC4 (Table II). The -----I--- escape mutation observed for 161JxA14 above therefore did not arise for either of these other two clones recognizing the same epitope. Consistent with the absence of this mutation after

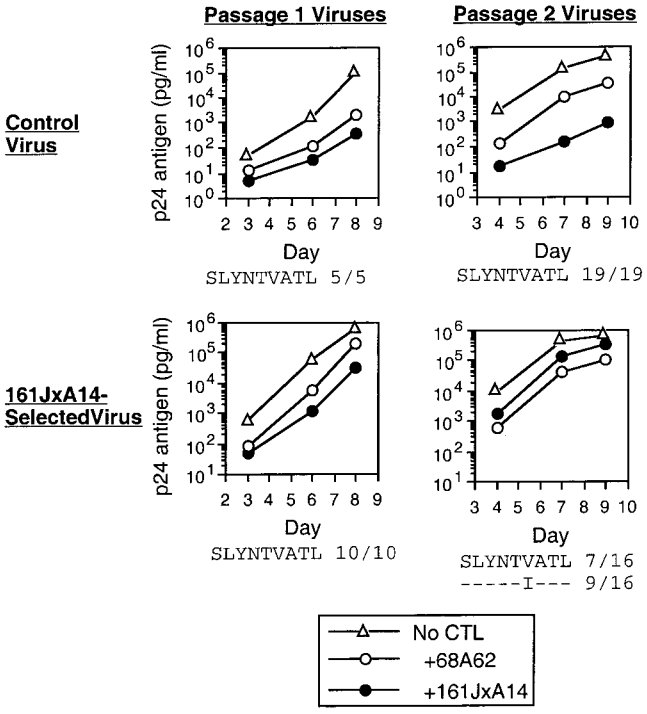

Figure 3. The SL9-specific CTL clone 161JxA14 is rapidly escaped by HIV-1. HIV-1 IIIB previously passaged (Fig. 2) under selective pressure in HLA A2-matched T1 cells (bottom row) or no selective pressure in HLA mismatched H9 cells (top row) by the CTL clone 161JxA14 (recognizing the Gag epitope SL9) for 1 (first column) or 2 (second column) wk of passage was tested for susceptibility to this clone or the control clone 68A62 (recognizing an RT epitope). Replication of the control and 161JxA14-selected viruses in T1 cells in the absence (open triangles) or presence (open and closed circles) of CTL is shown. These control and CTL-selected viruses were also sequenced for the SL9 epitope at limiting dilution, and the viral sequences are indicated below each graph. Similar results (decreased susceptibility to inhibition and -----I----- mutation observed after two weeks) were obtained in three experiments using HIV-1 IIIB and one using the molecular clone NL4–3.1 (Table II, Fig. 5, and unpublished data).

Table II. *Comparison of HIV-1 Escape from Multiple CTL Clones Recognizing Epitopes in Gag and RT*

CTL	Sequence	Frequency
161JxA14 (Gag p17, A2)	CRQILGQLQPSLKTGSEELR**SLYNTVATL**YCVHQRIDVKDTKEALDKIE	
	----------	1/10
	----------I----------	8/10
	----------I-V----------	1/10
115DEC4 (Gag p17, A2)	CRQILGQLQPSLQTGSEERR**SLYNTVATL**YCVHQRIEIKDTKEALDKIE	
	----------	16/16
18030D23 (Gag p17, A2)	CRQILGQLQPSLQTGSEELR**SLYNTVATL**YCVHQRIDVKDTKEALDKIE	
	----------	13/15
	----------A----------	2/15
68A62 (RT, A2)	LTEVVPLTEEAELELAENRE**ILKEPVHGV**YYDPSKDLIAEIQKQGQGQW	
	----------	6/12
	----------L----------	6/12
161JD27 (Gag p17, B60)	SEERRSLYNTVATLYCVHQR**IEIKDTKEAL**DKIEEEQNKSKKKAQQAAAD	
	----------	9/10
	----------K----------	1/10

HIV-1 passaged in the presence of CTL (protein specificity, HLA restriction) was sequenced for mutations in and around the recognized epitope. The index epitope and flanking sequences are given, and changes induced by passaging with the CTL are noted below. The frequency of the changes after the second passage (14 days) is given. The epitopes are in bold. Other flanking sequence mutations were not observed in regions sequenced (at least 150 nucleotides upstream and downstream of each epitope).

selection by 18030D23, this epitope variant was previously shown to be somewhat cross-recognized by this clone (with a peptide SD_{50} of ~100 ng/ml [31]), and the escape virus from 161JxA14 was also noted to be sensitive to inhibition by 18030D23 (Fig. 6). Of note, all three clones demonstrated different T cell receptor sequences (unpublished data). Together, these data indicated that different CTL recognizing the same epitope differ in susceptibility to viral escape mutation, depending on their individual ability to recognize specific epitope variants. Thus, properties specific to the T cell receptor of individual CTL play an important role in determining the repertoire of epitope mutations that can contribute to escape by differential pressure on the possible mutants.

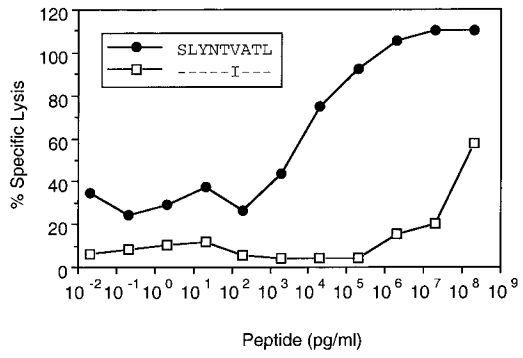

Figure 4. The SL9 variant -----I--- is poorly recognized by 161JxA14. Lysis of target cells labeled with serial dilutions of exogenously added SL9 or -----I--- variant peptides was assessed by standard chromium release assay.

HIV-1 Escape from Other Gag- and RT-specific CTL Clones Also Occurs Variably, and Tends to Be Monoclonal. Evolution of viral resistance against CTL recognizing other epitopes in Gag and RT also was evaluated (Table II). HIV-1 passaged under selective pressure by the HLA A2-restricted RT-specific clone (68A62) contained a single epitope point mutation, again only seen after the second week of passaging, leading to resistance after two weeks of selection, altering ILKEPVHGV to ----L---- (SD_{50} of 50 pg/ml versus >100 μg/ml, unpublished data). Two clones (161JD27 and 161Jx12) recognizing different HLA B60-restricted Gag epitopes failed to select for phenotypic resistance, or detected mutations (161JD27 in Table II, not done for 161Jx12) under these conditions. Thus, for the Gag- and RT-specific CTL tested in general, epitope escape mutation was observed after 2 wk of passaging if at all, and consisted of a single virus population differing by a single amino acid. Although multiple potential point mutations can ablate recognition for any given CTL clone (14, 31), only one or no escape mutations arose under pressure by the clones tested here, indicating that the combined fit-

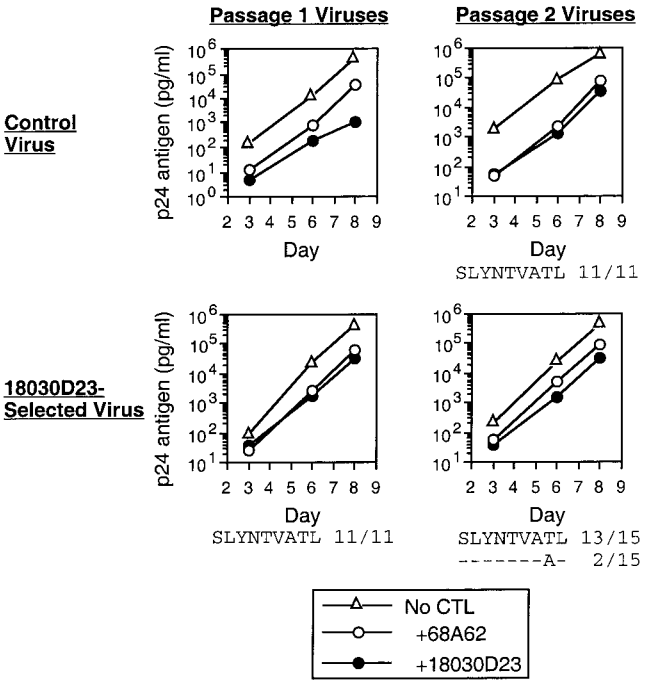

Figure 5. Another SL9-specific CTL clone 18030D23 does not rapidly select resistant virus. The experiment depicted in the Figure 3 was repeated with another clone 18030D23 (derived from another infected person, also recognizing the SL9 epitope) using HIV-1 NL4–3.1 (see also Table II). Again, control virus was passaged under no selective pressure (this time in TAP-deficient T2 cells) or with selective pressure (in HLA A2–matched T1 cells) by the selecting clone (18030D23). These viruses were tested for inhibition by 18030D23 or 68A62 as indicated, and SL9 sequences for these tested viruses are again shown. In a repeat experiment using strain IIIB, neither phenotypic resistance nor SL9 mutation was observed for 18030D23-selected virus (unpublished data).

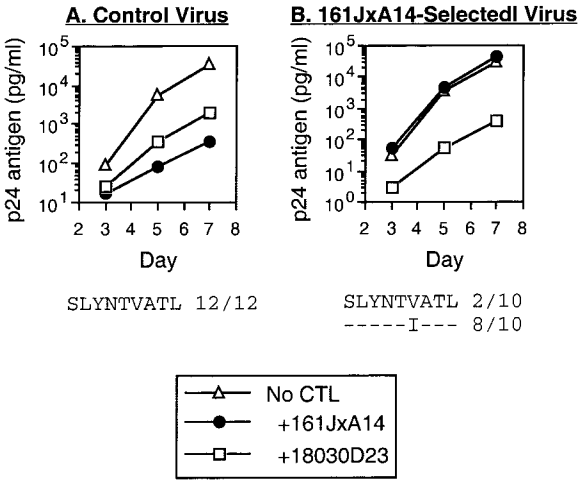

Figure 6. 18030D23 readily inhibits the escape virus for 161JxA14. HIV-1 NL4–3.1 viruses previously passaged (Fig. 2) for 2 wk under no selective pressure in TAP-deficient T2 cells (A) or selective pressure in HLA A2–matched T1 cells (B) by 161JxA14 were tested for susceptibility to viral inhibition by clones 161JxA14 and 18030D23. Below each graph are the SL9 sequences of the tested viruses.

ness costs and recognition of most potential escape variants outweighed the selective pressure exerted by the CTL on the original epitope. Furthermore, although epitope flanking mutation leading to altered epitope processing has been proposed as a mechanism of escape (42), such changes were not observed (Table II). In general, these findings suggested that escape is limited for CTL recognizing epitopes in structural proteins, due to the fitness constraints of particular epitope variants among potential escape mutations.

Nef-specific CTL Rapidly Select Polyclonal Mutations In Vitro. Previous observations that nonrecognized variants of the A2-restricted Gag epitope are uncommon in vivo (31) and that escape occurs readily against Tat- and Nef- but not Gag-specific CTL in acutely SIV-infected macaques (24, 25) prompted evaluation of other CTL clones specific for early expressed proteins. Analysis focused on Nef, a protein commonly targeted by CTL during acute HIV-1 infection (26). HIV-1 was passaged under selection by two CTL clones recognizing an epitope in Nef (STD11 and KM3), again with parallel negative control viruses. Passaging with these CTL produced a strikingly consistent pattern of rapid escape and polyclonal mutation compared with the RT-and Gag-specific CTL. Virus exposed to a single round of selective passage with the clones became highly resistant (Fig. 7, bottom left), compared with the negative control virus (Fig. 3, top left). Resistance was associated with a polyclonal mixture of substitution mutations in the epitope and upstream frameshift/stop mutations. Almost all of the frameshifts were due to single adenosine insertions in a stretch of adenosines at the beginning of the epitope (nt. positions -1 to $+3$ relative to the start of the epitope), or single thymidine deletions in a stretch of thymidines just before the epitope (nt. -6 to -2). Because the input virus was a molecular clone (NL4–3.1), these mutations arose de novo within the seven days of selection (three to seven replicative cycles, given an estimated HIV-1 generation time of 1 to 2 d [43, 44]). Downstream epitope flanking mutations were not observed, and multiple upstream mutations disrupted epitope expression. Comparison of the number of mutations induced by the Gag/RT-specific CTL clones compared with the Nef-specific CTL clones indicated a significant difference ($P < 0.0001$). Moreover, a Nef-specific CTL line recognizing a B*15-restricted epitope induced a similar pattern of polyclonal epitope mutations and upstream frameshifts (unpublished data). These data therefore indicated that the tested Nef-specific CTL more readily induce escape mutations, including reading frame disruptions, compared with RT- and Gag-specific CTL in vitro. This was further evidence of a key role for fitness constraints in determining escape, given the dispensability of Nef for viral growth in this culture system (45).

Discussion

In contrast to antiretroviral drugs, which readily induce escape mutations in their target proteins under conditions of persistent viremia, escape mutations against CTL responses have not been consistently observed in vivo. Lack

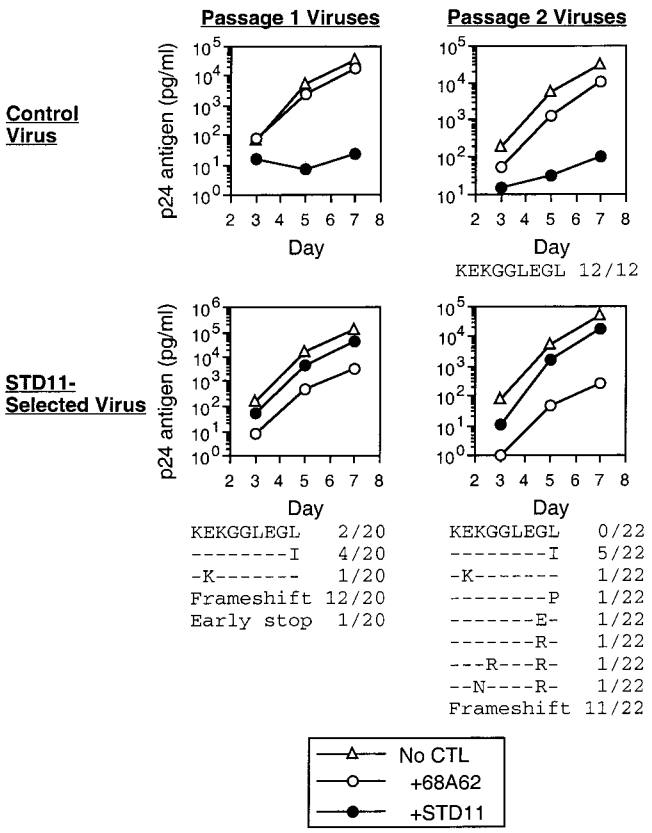

Figure 7. The Nef-specific CTL clone STD11 very rapidly selects resistant virus that contains multiple mutations. HIV-1 NL4–3.1 previously passaged (Fig. 2) under selection by STD11 (recognizing the Nef epitope KEKGGLEGL) was tested for phenotypic sensitivity to STD11 or 68A62, as well as genetic changes in the Nef epitope recognized by STD11. Frameshift = frameshift mutations upstream of the epitope. Early stop = substitution mutation upstream of the epitope leading to a stop codon. Similar results were obtained in another experiment using STD11 and HIV-1 IIIB (unpublished data) and two experiments using another clone KM3 of the same specificity (also with both NL4–3.1 and IIIB). Similar results (polyclonal epitope mutations and upstream frameshifts) were also obtained with Nef-specific CTL recognizing an HLA B15-restricted CTL clone and H9 target cells (unpublished data).

of observed escape often has been interpreted to reflect a lack of antiviral pressure by CTL, given the high mutation and replication rates of HIV-1 (46) that should ensure tremendous diversity in CTL epitopes (14). The degree of antiviral pressure by CTL is clearly an important consideration, but escape probably is determined by the relative balance of selective pressure and fitness costs of potential escape mutations. One study has demonstrated reversion of escape mutations after loss of CTL pressure (47), supporting this concept. The replicative capacity of any given virion is determined by its intrinsic fitness and the pressure applied by the immune system. Thus, for any given epitope mutation to gain predominance over the initial epitope within a viral population facing recognition by CTL, its net balance of recognition by CTL and replicative fitness must outweigh that of the wild-type. Lack of observed CTL epitope escape mutations in vivo therefore could be due to any combination of insufficient pressure on the wild type epitope, good pressure on variants, or high fitness costs to variants.

These interactions are difficult to evaluate in vivo, where it is impossible to isolate factors such as CTL specificity and function, and input viral sequence. To study HIV-1 escape from CTL in a controlled manner, we devised a strictly defined in vitro system. Previously, in vitro CTL escape mutations had been shown for murine lymphocytic choriomeningitis virus (48) and HIV-1 (11). Thus we systematically examined a panel of HIV-1–specific CTL clones for their ability to drive escape mutations in HIV-1 in vitro, under conditions that allowed vigorous viral replication in the face of weakly inhibitory concentrations of CTL. This system allowed us to evaluate defined factors that influence the interaction of CTL with HIV-1.

We first examined three CTL clones specific for the SL9 epitope in p17 Gag, a common and well-described epitope that appears to develop escape mutations infrequently in vivo (31). These clones exhibited consistently different susceptibility to escape mutation. One clone (161JxA14) rapidly selected phenotypically resistant virus containing a monoclonal mutation (-----I---), after 14 d of selection. The ability of this variant to arise and predominate rapidly suggested that this mutation did not carry a substantial fitness cost, at least in the IIIB and NL4–3 strains used here in vitro. Two other clones, however, did not induce the escape mutation selected by the first. One clone variably selected a different monoclonal mutation (-------A-), and the other induced no detectable mutations. We further confirmed that the -----I--- variant was well recognized by at least one of these two clones, indicating that it escapes 161JxA14 and not the other clone. The variability between these clones was consistent with the finding that their T cell receptors (TCRs) are distinct in terms of Vβ usage (unpublished data). These data indicate that selective pressure on various epitope mutants is clone-specific, and thus the selective pressure applied on any given potential escape mutant can markedly differ between clones.

Another interesting aspect of these data was the clonality of escape when it did occur. Further experiments with other Gag- and RT-specific CTL revealed the same pattern of monoclonal or no escape. Because CTL nonrecognition should be readily achievable through single point mutations abrogating TCR binding (as demonstrated for SL9-specific clones previously [31]), epitope processing, or MHC-I nonbinding, this narrowness of escape suggested that the replicative fitness constraints for potential escape mutations usually outweighed the replicative advantage gained by nonrecognition by the CTL. For example, MHC-I presentation of epitopes is highly dependent on individual anchor amino acids (49) and should be easily disrupted by any of dozens of single point mutations, yet no such mutations were seen after CTL selection here in vitro or in extensive studies of SL9 sequences in vivo (31, 50). These data suggest that fitness costs may play a substantial role in determining whether escape occurs in SL9 and other epitopes in structural proteins, and may partially ex-

plain why SL9 escape mutations are uncommon in vivo (31). A study of another Gag epitope has suggested more directly that fitness constraints are an important determinant of escape (47). In sum, these findings strongly suggest that fitness considerations play a central role in determining whether escape mutations arise within a CTL-pressured HIV-1 population, particularly in the setting of a polyclonal response to viral epitopes (51).

When Nef-specific CTL were evaluated, escape was highly polyclonal and arose much more rapidly and completely. Furthermore, the mutations occurred in multiple sites of the epitope (TCR binding and HLA-binding anchor residues) and were dominated by upstream frameshifts resulting in functional deletion of the epitope. At least two factors likely contributed to the higher degree of escape we observed with Nef-specific CTL clones. First, unlike Gag and RT, Nef is entirely dispensable for viral replication in our culture system (45) and thus changes in Nef (including functional deletion through frameshifts) probably occur with no fitness cost to HIV-1 in vitro. Second, Nef-specific CTL may exert more immune pressure than Gag- or RT-specific CTL. This is seen in the markedly more efficient suppression of viral replication by Nef-specific CTL (Fig. 7), further supported by our observation that Nef-specific CTL are reproducibly more inhibitory for HIV-1 replication than Gag- or RT-specific CTL in vitro (unpublished data), and recent data that the earlier epitope expression in Nef allows more efficient CTL clearance of infected cells (52). Intriguingly, autologous infusion of exogenously expanded Nef-specific CTL has been reported to result in epitope deletion in vivo (19), although generally the *nef* reading frame is remarkably conserved in vivo (53, 54). Because Nef-specific CTL are common, these findings underscore the functional importance of Nef for HIV-1 pathogenesis, as reflected by the apparently high fitness costs in vivo (but not in vitro) favoring its conservation.

Our data may be relevant to several further in vivo observations concerning CTL specificity. During the course of HIV-1 infection, specificity appears to evolve from targeting accessory proteins such as Nef initially to targeting structural proteins in chronic infection (26). Moreover, elegant studies in the SIV model have shown that such early CTL recognizing Tat and Nef induce escape mutations at far higher rates than those recognizing Gag (24, 25, 55). A hypothesis consistent with our data and these findings is that the most antiviral CTL arise early in infection, preferentially targeting early expressed proteins such as Tat and Nef and being of the highest avidity. However, these CTL are rapidly escaped by nonrecognized epitope mutations, leading to loss of antigen to drive persistence of these CTL. Other less efficient CTL recognizing structural proteins can then predominate, and those recognizing the most constrained epitopes continue to persist into chronic infection. This hypothesis would explain the observations of shifting CTL specificities and more consistent findings of escape mutations against early CTL versus late CTL, although further study will be required to support or disprove this scenario. Another aspect concerning the difference between early and late CTL is the suggestion that the high avidity of early CTL may be a determinant of immune pressure and escape (55). However, the Gag SL9-specific clones 161JxA14 and 18030D23 had SD_{50} measurements of 20 ng/ml versus 1 ng/ml, and the lower avidity clone 161JxA14 appeared to be more prone to escape. Although the Nef-specific clones were of high avidity ($SD_{50} < 100$ pg/ml) and induced more escape, it is difficult to isolate the effects of avidity and epitope mutation fitness costs, and further study will be required to delineate the potential role of avidity in escape.

Another topic of intense interest has been the issue of CTL function in vivo. The clones used for this study were highly selected as potent killers in cytolysis assays. Numerous investigators have suggested that CTL function is diminished in vivo, in reports of lacking CD4 T helper responses (56, 57), diminished CTL production of cytolytic enzymes (58), lack of effector phenotype (59), loss of important signaling molecules such as CD3ζ (60), and impaired homing to sites of viral replication (61). Our data hint that CTL pressure in vivo may be suboptimal. Although clone 161JxA14 rapidly and reproducibly selected the -----I--- escape mutation in vitro, this mutation appeared to be in the minority or lacking in the person (Subject 161J) from whom this clone was isolated (1/4 --F--I---, 3/4 --F------; reference 31), consistent with suboptimal selective pressure in vivo. Although suggestive, we cannot exclude that this is due to factors other than suboptimal pressure, such as other CTL exerting pressure on the -----I--- variant, or differing fitness costs between the virus in vivo and the IIIB/NL4–3.1 isolates in our study. Of note, the SL9-specific CTL response in 161J appears to be nearly monoclonal (unpublished observation). Clone STD11 recognizing the Nef epitope KEKGGLEGL also was isolated from 161J, and the predominant epitope sequence in vivo was -K------- (62), which was a minor sequence selected by this clone in vitro. Again, this difference could be due to inadequate selective pressure, differential pressure on the variants by other CTL, or different fitness costs. Since Nef is dispensible in vitro, the latter possibility could be particularly significant.

In conclusion, we find that HIV-1 can readily escape from some virus-specific CTL, indicating that CTL can exert selective pressure that exceeds viral fitness costs. Not all CTL clones are equally susceptible to escape mutations. The interplay of multiple factors affecting immune pressure and viral fitness determines the likelihood of viral escape. Both immune pressure and fitness costs may be influenced by the particular epitope targeted, the protein targeted, and the TCR structure of the selecting CTL clone. Study of escape mutations in vitro may permit a focused evaluation of important issues regarding the role of CTL specificity and function in the immune control of HIV-1. Such an approach may be useful in dissecting the determinants of CTL success and failure in immunopathogenesis and vaccine development, where it may be crucial to optimize the specificity of antiviral responses.

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