Cytotoxic T Lymphocyte Lysis Inhibited by Viable HIV Mutants

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Immune evasion by the human immunodeficiency virus (HIV) is unexplained but may involve the mutation of viral antigens. When cytotoxic T lymphocytes engaged CD4-positive cells that were acutely infected with HIV bearing natural variant epitopes in reverse transcriptase, substantial inhibition of specific antiviral lysis was observed. Mutant viruses capable of these transactive effects could facilitate the persistence of a broad range of HIV variants in the face of an active and specific immune response.

Cytotoxic T lymphocytes (CTLs) capable of recognizing HIV are detectable throughout the asymptomatic period of infection (*1*). However, the immune response fails to clear the virus in the vast majority of patients for reasons that are not fully understood (*2*). The destruction of virally infected cells by CTLs relies on the successful engagement of the T cell receptor (TCR) with a complex formed by viral peptide bound to a human leukocyte antigen (HLA) class I molecule on the infected cell surface (*3*). Recent findings indicate that such interactions do not necessarily activate a full response in the T cell. For $CD4^+$ T cells, small changes in the amino acid sequence of major histocompatibility complex (MHC) class II bound peptides altered activation signals transmitted by the TCR (*4*). MHC class II restricted T cells became anergic after exposure to antigen-presenting cells that bore a mixture of the wild-type peptide and a higher concentration of an altered peptide ligand (APL) or antagonist (*5*). Similarly, alterations in HLA class I–bound peptides influenced CTL activation when target cells were sequentially pulsed with peptides that differed by one or more amino acids at putative TCR contact points. Some peptides severely inhibited the lytic effect of CTLs in a clone-dependent fashion (*6*); these peptides were active at concentrations as low as 20 to 200 fM, which suggested that epitopes derived from mutant viruses could influence the CTL response to the wild-type virus. Natural variation in HIV *gag* (*7*) and in the core protein of hepatitis B virus (*8*) produced epitope mutants capable of potent inhibition of antiviral CTLs. In these experiments, epitope variants were initially represented by synthetic peptides pulsed onto Epstein-Barr virus–transformed B cell (BCL) targets that had previously been exposed to wild-type peptide. Variant antigen processed from recombinant vaccinia virus was also capable of this inhibitory or antagonist effect on T cell activation (*7*). Thus these experiments implied, but did not establish, that mutant viruses could subvert the immune response to the wild-type virus.

While tracking the CTL response of four HIV-1–seropositive individuals (*9*) to an HLA-B8–restricted epitope in reverse transcriptase (RT), we found amino acid variation in viral sequences encoding the highly conserved epitope GPKVKQWPL (Fig. 1) (*10, 11*). A CTL line from patient 84 failed to recognize four of these viral peptides—1S ($Gly^1 \rightarrow Ser^1$), 3R ($Lys^3 \rightarrow Arg^3$), 6R ($Gln^6 \rightarrow Arg^6$), and 6E ($Gln^6 \rightarrow Glu^6$)—in a standard chromium lysis assay (*12*). Two of these variants, 1S and 3R, also inhibited the lysis of target cells prepulsed with wild-type peptide (Fig. 2). The x-ray structure of the HLA-B8–Gag(25–32) complex (*13*) reveals that changes at position 1 of the bound peptide are likely to make direct contact with the TCR. Position 3 is an anchor residue, but a K3R mutation could have a secondary effect on the conformation of residues that come into contact with the TCR. Mutant RT containing these amino acid changes (1S and 3R) showed no impairment of in vitro enzyme function (Table 1). We now could construct mutant viruses that contained the variants 1S and 3R (chosen for their potency in peptide experiments) to test whether whole viable HIV could inhibit CTL recognition. Although the presence of the 1S and 3R sequences in the patients' proviruses had suggested that virions with these mutations would be viable, it was important to confirm

Fig. 1. Variation in an HLA-B8–restricted epitope of HIV-1 RT (*11*). DNA encoding the NH_2-terminal region of HIV-1 RT was amplified from peripheral blood mononuclear cells (PBMCs) of infected patients and cloned into M13 with the use of a nested PCR (*7, 24*). In patient 84, RNA was amplified by means of a modification of this method with complementary DNA preparation, as described (*25*). In each case, 20 to 35 M13 clones were sequenced; variations encoding amino acid changes are shown if they were detected in two separate PCR reactions. Patients 008 (HLA A1/2, B8), 065 (A1/2, B8), and 007 (control) (A1/32, B27/44) are hemophiliacs infected in 1983 or 1984; patients 84 (A1/2, B8/44) and 85 (A2/24, B8/35) have sexually acquired infection and seroconverted in 1989 and 1985, respectively. At the time of the first blood sample shown, CD4 counts were 530, 360, 350, 450, and 320 cells per microliter, respectively.

Epitope	^{18}G	P	K	V	K	Q	W	P	L^{26}	Frequency (%)	Date of sample
	^{517}GGC	CCA	AAA	GTT	AAA	CAA	TGG	CCA	TTG		
Patient											
008	G	P	K	V	K	Q	W	P	L	100	19 November 1991
	G	P	K	V	K	Q	W	P	L	100	7 April 1992
	G	P	K	V	K	Q	W	P	L	92	6 January 1993
	G	P	R (AGA)	V	K	Q	W	P	L	1	
	G	P	K	V	K	R (CGA)	W	P	L	1	
	G	P	K	V	K	E (GAA)	W	P	L	6	
065	G	P	K	V	K	Q	W	P	L	31	28 February 1993
	S (AGC)	P	K	V	K	Q	W	P	L	63	
	G	P	R (AGA)	V	K	Q	W	P	L	3	
	G	P	K	V	K	E (GAA)	W	P	L	3	
84	G	P	K	V	K	Q	W	P	L	96	1 February 1993
DNA	S (AGC)	P	K	V	K	Q	W	P	L	2	
	G	P	E (GAA)	V	K	Q	W	P	L	2	
RNA	G	P	K	V	K	Q	W	P	L	96	1 October 1993
	D (GGT)	P	K	V	K	Q	W	P	L	4	
85	G	P	K	V	K	Q	W	P	L	95	2 May 1994
	G	P	E (GAA)	V	K	Q	W	P	L	5	
Control	G	P	K	V	K	Q	W	P	L	100	

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this suggestion because defective proviruses are common (*14*). Using recombinant techniques (*15*), we introduced the amino acid changes that represented the 1S and 3R sequences by site-directed mutagenesis into an RT clone that was transfected into a T cell line (CEM) along with a proviral clone lacking much of RT. Sequencing confirmed that the viruses emerging from this culture bore the desired changes, and these viruses were used for subsequent CTL experiments.

To establish a cellular model of acute HIV variant infection, we inoculated a cell line (C8166) with either mutant or wild-type viruses. The growth of wild-type and mutant viruses in this system, as measured by p24 antigen production in the supernatant, was comparable in terms of growth kinetics and final titer reached (Fig. 3). Similar results were obtained by analysis of virus tissue culture infectivity and by a kinetic analysis of reverse transcription within cells by the polymerase chain reaction (PCR) for long terminal repeat DNA transcripts after infection (*16, 17*). C8166 cells have HLA-B8 on the cell surface, and a polyclonal CTL line from patient 84 that killed ^{51}Cr-labeled target cells infected with only wild-type virus showed minimal recognition of cells infected with variants 1S or 3R alone (Fig. 4); this observation confirmed that these APLs are weak or minimally active agonists when processed during intracellular viral replication.

Viruses with mutant epitopes would initially be expected to expand into separate cell populations (*18*). To simulate this phenomenon, we infected distinct C8166 cell cultures with pure preparations of a variant virus. After 1 or 2 days, a CTL line was incubated for 1 hour with unlabeled (cold) cells that either were uninfected or expressed the 1S or 3R variant. ^{51}Cr-labeled (hot) wild-type virus–infected or uninfected control targets were then added at a cold/hot ratio of 5:1 and specific lysis was measured after 4 hours. Exposure to variant virus–infected cells caused a substantial inhibition of lysis compared with that caused by incubation with uninfected cold targets alone (Fig. 5A). This effect was enhanced by increasing the cold target/hot target ratio (Fig. 5, B and C). Although superficially like a classic cold target inhibition experiment (*19*), these results are crucially different because the cold cells are not themselves lysed by the CTLs. Also, although these CTLs were polyclonal, their anti-HIV activity was inhibited by exposure to a cell population infected by a virus with a single amino acid change. This dominant or transactive mechanism could enhance the persistence of not only the mutant but also a wider set of wild-type viruses. It is not clear why the appearance of epitope variants does not always elicit a novel CTL response capable of controlling these potential escape sequences (*20*). Possibly the initially predominant wild-type viral clones could inhibit, by a reciprocal antagonist effect, any new CTL response to epitope mutants. Multiple-epitope mutants with inhibitory effects could further enhance wild-type virus persistence. Although a decline in CD4 cell function may deprive the CD8 precursor cell population of help for clonal expansion, there is evidence in vitro that APLs may drive proliferation of CTLs that retain

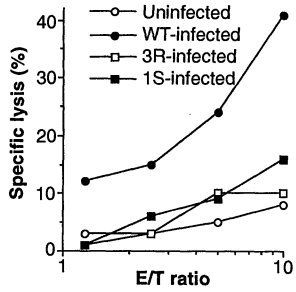

Fig. 4. CTL recognition of cells infected with mutant viruses. C8166 cells were infected with viruses bearing wild-type or mutant (3R or 1S) epitopes at a MOI of 0.02 and cultured for 3 days. Cells were ^{51}Cr-labeled, and lysis by CTLs from patient 84 that were specific for HLA-B8 complexed with RT was tested at the E/T ratios shown.

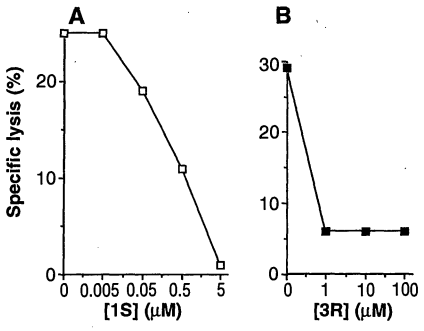

Fig. 2. Natural epitope variants in RT inhibit CTL lysis. Autologous ^{51}Cr-labeled BCLs were pulsed with 100 nM wild-type peptide (GPKVKQWPL), washed, and plated in 96-well plates (*6–8*). Variant peptide was then added to the wells at the final concentrations shown, followed by a CTL line specific for the wild-type RT epitope (derived from HIV-1–infected patient 84). Specific lysis was determined at 4 hours. (**A**) Peptide 1S; specific lysis without peptide, 1%; effector/target (E/T) ratio, 10:1. (**B**) Peptide 3R; specific lysis without peptide, 5%; E/T ratio, 20:1.

Table 1. Effect of epitope mutations on RT function. Activities are expressed as percentage of wild-type activity ± SD. RT-WT, RT-3R, and RT-1S contain the HXB2 1.7-kb RT gene bearing wild-type, 3R, and 1S mutant epitope sequences, respectively (*11*). Pol-WT, Pol-1S, and Pol-3R constructs bear the same mutations within the entire 2.55-kb *pol* gene. Pol-AZT-DDI and Pol-AZT-DDI-NV bear drug resistance mutations; Pol-AZT-DDI is resistant to AZT and ddI, and Pol-AZT-DDI-NV is resistant to AZT, ddI, and Nevirapine. Pol-AZT-DDI-1S bears the two drug resistance mutations and epitope mutation 1S. The differences between the original RT mpHXB2 and Pol mpRTH1 construct backgrounds are Leu^{13} → Ala^{13}, Val^{21} → Ile^{21}, Thr^{58} → Ser^{58}, and Val^{436} → Leu^{436} (*26*).

Construct	Genotype						Activity (%)
	18	20	41	74	106	215	
Wild type	G	K	M	L	V	T	
RT-WT	–	–	–	–	–	–	100
RT-1S	S	–	–	–	–	–	96 ± 14
RT-3R	–	R	–	–	–	–	206 ± 34
Pol-WT	–	–	–	–	–	–	100
Pol-1S	S	–	–	–	–	–	85 ± 18
Pol-3R	–	R	–	–	–	–	226 ± 84
Pol-AZT-DDI	–	–	L	V	–	Y	106 ± 7
Pol-AZT-DDI-NV	–	–	L	V	A	Y	126 ± 15
Pol-AZT-DDI-1S	S	–	L	V	–	Y	96 ± 9

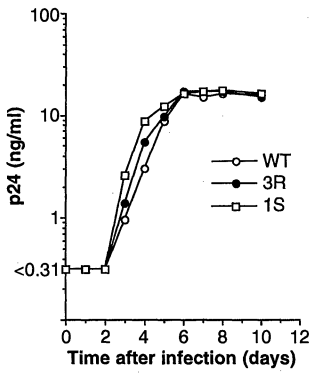

Fig. 3. Growth of recombinant HIV-1. Viruses bearing mutations in the HLA-B8 RT epitope were prepared as described (*15*): pHIVDRTBstEII (a plasmid vector bearing HIV-1 with a deletion in RT) was digested with Bst EII and Bcl I; mpHXB2 (M13 bearing HIV-1 HXB2 *pol*) was subjected to site-directed mutagenesis (*26*) to create G18S or K20R mutations. Digested pHIVDRTBstEII (10 μg) or Hind III– and Eco RI–digested mpRTH1 (10 μg)—bearing wild-type (WT) or mutant epitopes, respectively—were transfected into 2 × 10^6 CEM cells, and cultures were maintained by addition of further CEM cells. After 14 days, supernatant was used to infect further fresh cells; virus from these cultures was sequenced, and viral stocks were prepared and frozen at −80°C before use. Titration of stock viruses was performed as described (*16*). Infections of subsequent cultures were performed at a multiplicity of infection (MOI) of 0.01 to 0.02, as indicated. Production of p24 in the supernatant was measured by specific ELISA; 0.31 ng/ml is the lower limit of detection of this assay (*27*).

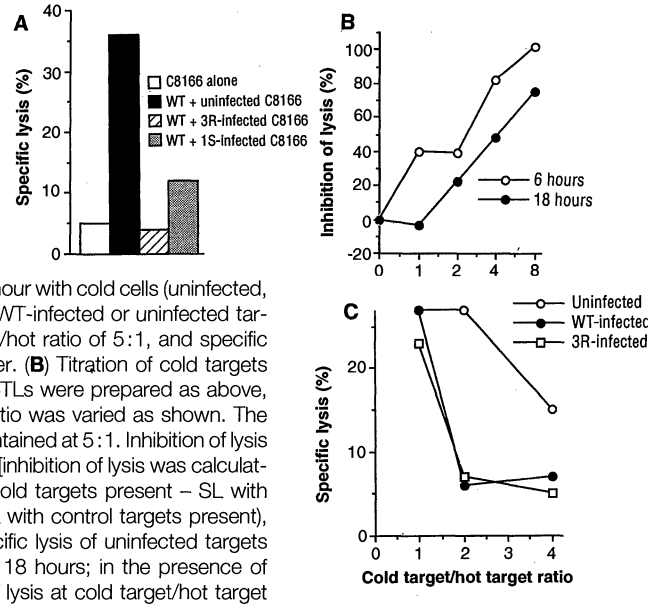

Fig. 5. (**A**) Inhibition of lysis by virally infected cells. C8166 cells were infected and cultured as in Fig. 4. Wild-type virus (WT)–infected and uninfected control target cells were ^{51}Cr-labeled (hot cells); cells infected with 3R and 1S viruses or uninfected were left unlabeled (cold cells). CTLs from patient 84 were incubated for 1 hour with cold cells (uninfected, 3R, or 1S) at E/T 1:1. Labeled WT-infected or uninfected targets were then added at a cold/hot ratio of 5:1, and specific lysis was measured 4 hours later. (**B**) Titration of cold targets bearing 3R virus. Targets and CTLs were prepared as above, but the cold target/hot target ratio was varied as shown. The effector/hot target ratio was maintained at 5:1. Inhibition of lysis of WT-infected targets is shown [inhibition of lysis was calculated as 100 × (SL with control cold targets present – SL with 3R-infected targets present)/(SL with control targets present), where SL is specific lysis]. Specific lysis of uninfected targets was 3% at 6 hours and 6% at 18 hours; in the presence of uninfected cold targets, specific lysis at cold target/hot target ratios of 1, 2, 4, and 8 was 26, 22, 22, and 18%, respectively, at 6 hours and 45, 50, 40, and 32%, respectively, at 18 hours. (**C**) Inhibition of lysis of WT-infected cells by targets infected with wild-type or 3R virus. A BCL from patient 90 bearing HLA-B8 (*7*) was infected with wild-type and 3R viruses at a MOI of 0.1, which resulted in sustained chronic virus production. WT-infected targets were labeled as above, and lysis by CTLs from patient 84 that were specific for HLA-B8 complexed with Pol (E/T 5:1) was measured at 6 hours in the presence of uninfected, WT-infected, or 3R-infected cold targets at the cold target/hot target ratios shown. Lysis of uninfected cells was 7%.

specificity for the wild-type epitope and do not lyse cells pulsed with the APLs (*21*). This effect, if active in vivo, could distort the CTL repertoire and overwhelm a minor response to the new variant.

The potent ability of naturally occurring viable HIV to inhibit CTL activity by causing an unproductive but specific engagement with the TCR constitutes a protective feature of these mutant viruses that might exceed the advantage conferred by simple epitope escape. Under these circumstances, mutant viruses with weak agonist T cell epitopes of the type shown here may aid the survival of a wide range of viral variants. This mechanism of immune evasion would not be evoked by mutations of anchor positions in peptides that prevent HLA class I binding altogether (*22*), by flanking changes that block processing (*23*), or by epitope variants that have no agonist properties at all. Viruses that mutate and effectively lose epitopes altogether might contribute less to HIV persistence than has previously been proposed (*1*). Although the emergence of inhibitory viral epitopes late in the clinical progression might be favored because CD4$^+$ T lymphocyte function is by then weak or defunct, we have detected these epitope mutations throughout the course of infection (*7*, *24*). Their fluctuation may reflect complex viral dynamics (*9*), but the transactive qualities of partial agonist sequences such as 1S and 3R may give such viruses enhanced survival in the hostile environment that prevails in the immunocompetent host.

Viral persistence in the face of active and potentially effective immune responses demands explanation. Our study describes a pervasive mechanism of immune escape by a mutable pathogen. In HIV infection, genetic variation within CTL epitopes arises, which causes both radical and subtle changes in the configuration of the HLA class I peptide complex (*7*, *13*, *20*, *24*). Some of these APLs retain the ability to interact with TCRs specific for the wild-type sequence, but the engagement fails to trigger CTL lysis. CTLs that encounter antigenic decoys of this sort are distracted and perhaps are actively inhibited from killing wild-type virus–infected cells. Cell populations protected by this CTL inhibition could harbor and permit the propagation of an array of viruses that would normally be eliminated. The simplicity and potency of this dominant form of immune escape suggests that it may be used by other pathogens capable of mutating T cell epitopes, irrespective of the class of the presenting molecule.

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11. Abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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26. All constructs used were M13-based vectors that express RT under a *tac* promoter [H. A. De Boer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 21 (1983)]. The epitope changes were introduced by oligonucleotide-directed mutagenesis (T7 gene mutagenesis kit, U.S. Biochemical; U-DNA mutagenesis kit, Boehringer). The two mutant epitope coding sequences each differed from the wild-type sequence by one amino acid (Gly^{18} → Ser^{18} and Lys^{20} → Arg^{20}, respectively). Mutagenesis was performed with oligonucleotides 5′-GGAATGGATAGCCCAAAAGTTA-3′ (1S) and 5′-GGATGGCCCAAAAGTTAAACAA-3′ (3R). The region of the RT genome that contained the mutated epitope in a Bsr GI–Bsr GI restriction fragment (nucleotides 1021 to 6120) was subcloned into a construct with an azidothymidine (AZT) and 2′,3′-dideoxyinosine (ddI) RT genotype [B. A. Larder, P. Kellam, S. D. Kemp, *Nature* **365**, 451 (1993)] to generate a dual CTL- and drug-resistant construct (Pol-AZT-DDI-1S). The constructs were expressed by infecting *Escherichia coli* TG1 at high multiplicity with bacteriophage clones. Bacterial cultures (2 ml) were grown to an optical density of 0.6 at 600 nm. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were harvested after 15 hours of growth at 300°C. The cells were lysed by lysozyme treatment (5 mg/ml) and low-salt buffer [25 mM tris-HCl (pH 8.0), 5 mM mercaptoethanol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride] followed by sonication. The supernatant contained the active enzyme and was used in RT assays. The RT activity was assayed with the use of poly(A)·oligo(dT) as primer templates and two assay systems [J. Eberle and R. Seibl, *J. Virol. Meth.* **40**, 347 (1992); N. Bosworth and P. Towers, *Nature* **341**, 167 (1989)]. The enzyme-linked immunosorbent assay (ELISA, Boehringer) was done in triplicate, the tritiated thymidine–based assay (Amersham) in duplicate. The mutants were assayed on four separate occasions from bacterial cultures grown independently. The extracts were analyzed by protein immunoblotting with the monoclonal antibody ADP3019 (MRC AIDS Directed Reagent Programme), which reacts with p66 and p51.
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