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Overlapping Epitopes in Human Immunodeficiency Virus Type 1 gp120 Presented by HLA A, B, and C Molecules: Effects of Viral Variation on Cytotoxic T-Lymphocyte Recognition

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Human immunodeficiency virus (HIV)-specific cytotoxic T lymphocytes (CTL) are thought to exert immunologic selection pressure in infected persons, yet few data regarding the effects of this constraint on viral sequence variation in vivo, particularly in the highly variable Env protein, are available. In this study, $CD8^+$ HIV type 1 (HIV-1) envelope-specific CTL clones specific for gp120 were isolated from peripheral blood mononuclear cells of four HIV-infected individuals, all of which recognized the same 25-amino-acid (aa) peptide (aa 371 to 395), which is partially contained in the CD4-binding domain of HIV-1 gp120. Fine mapping studies revealed that two of the clones optimally recognized the 9-aa sequence 375 to 383 (SFNCGGEFF), while the two other clones optimally recognized the epitope contained in the overlapping 9-aa sequence 376 to 384 (FNCGGEFFY). Lysis of target cells by the two clones recognizing aa 375 to 383 was restricted by HLA B15 and Cw4, respectively, whereas both clones recognizing aa 376 to 384 were restricted by HLA A29. Sequence variation, relative to the IIIB strain sequence used to identify CTL clones, was observed in autologous viruses in the epitope-containing region in all four subjects. However, poorly recognized autologous sequence variants were predominantly seen for the A29-restricted clones, whereas the clones specific for SFNCGGEFF continued to recognize the predominant autologous sequences. These results suggest that the HLA profile of an individual may not only be important in determining the specificity of CTL recognition but may also affect the ability to recognize virus variants and suppress escape from CTL recognition. These results also identify overlapping viral CTL epitopes which can be presented by HLA A, B, and C molecules.

Cytotoxic T lymphocytes (CTL) are thought to play an important role in immune control of acute and chronic viral infections (reviewed in reference 21). Increasing evidence suggests that CTL specific for human immunodeficiency virus type 1 (HIV-1) in humans, and simian immunodeficiency virus-specific CTL in rhesus macaques, are critical in controlling the initial viremia following acute infection with these pathogenic retroviruses and in suppressing replication to varying degrees during the asymptomatic phase of infection (1, 19, 26, 35). Evidence of a strong and broadly directed HIV-specific CTL response in HIV-infected persons who fail to progress to AIDS despite many years of infection further underscores the potential importance of the cellular immune response in controlling infection (8, 10, 17, 29). However, despite the presence of HIV-specific CTL, in most individuals virus continues to replicate during the asymptomatic phase, eventually leading to an increase in viral load, profound $CD4^+$ lymphocyte depletion, and the development of AIDS (reviewed in reference 11). The mechanisms which allow HIV-1 to persist remain poorly understood.

$CD8^+$ CTL recognize target cells through the binding of their T-cell receptor (TCR) to 8- to 11-amino-acid (aa) antigenic peptides complexed with major histocompatibility complex (MHC) class I molecules. These MHC-peptide complexes arise from the processing of endogenous viral antigens, and their composition is determined by both protein processing and MHC-binding constraints. Elution of naturally processed peptides as well as epitope mapping of antigenic peptides recognized by CTL has defined MHC allele-specific binding motifs with fixed anchor residues for a number of MHC class I molecules (reviewed in reference 28). The diversity of CTL responses and their ability to control viral replication during HIV infection may, in part, be determined by the range of viral peptides presented by the MHC molecules expressed in a given individual. It is well established that single-amino-acid substitutions within a class I-restricted epitope can abrogate CTL recognition, either by decreasing binding affinity of peptide for the MHC molecule or by altering the peptide-MHC complex so that it is no longer recognized by the TCR (4, 27). The in vivo immunologic impact of viral sequence variation through an epitope would thus be expected to vary depending on the available TCR repertoire and the restricting MHC class I an-

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tigen. Population studies of HIV-infected individuals suggest a correlation between certain HLA haplotypes and slow or rapid disease progression, supporting the hypothesis that the array of MHC class I molecules available to present epitopic peptides may play a role in determining disease outcome for each infected individual (16).

In this study, we have evaluated four CTL clones that were found to recognize distinct overlapping epitopes within a 10-aa sequence in a relatively conserved region of HIV-1 gp120 (23). The CTL epitopes contained within this region could be presented in the context of three different HLA class I molecules. Sequence evaluation within this region was conducted to determine the effects of immune selective pressure mediated through these three class I molecules and to compare the in vivo virus variants in persons with immune responses targeting the same region but restricted by different class I molecules.

MATERIALS AND METHODS

Subjects. Four HIV-1-infected subjects who demonstrated clonal CTL responses to a 25-aa peptide in the HIV-1 envelope protein (aa 371 to 395) were studied. Subjects 08109 and 19142 are HIV-1-infected women with CD4 counts of 320 and 645, respectively. Subjects 010-035i (referred to hereafter as 035i) and 18026 are HIV-1-infected men with CD4 counts of 145 and 300, respectively, at the time of study. Plasma HIV-1 RNA levels were determined for each subject at the time of T-cell cloning by the AMPLICOR HIV-1 Monitor Test (Roche Diagnostic Systems, Inc., Branchburg, N.J.) per the manufacturer's instructions, with the following results: subject 08109, 510 RNA copies/ml; subject 19142, 7,741 copies/ml; subject 035i, 94,800 copies/ml; and subject 18026, 10,300 copies/ml.

Cell lines. Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were established from the peripheral blood mononuclear cells (PBMC) of each subject and maintained as previously described (31) in RPMI 1640 medium (Sigma, St. Louis, Mo.) containing 20% heat-inactivated fetal calf serum (Sigma). RPMI 1640 medium used for all cell lines was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and HEPES (10 mM). Additional allogeneic B-LCL used in HLA restriction experiments were established and maintained in a similar fashion.

HLA typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory using a standard lymphocytotoxicity assay.

Recombinant vaccinia viruses. Recombinant vaccinia viruses expressing the control *lacZ* gene (vsc8) and the full-length HIV-1 gp160 gene (VPE16) as well as serial truncations of the HIV-1 envelope gene (VPE17-22) were provided by P. Earl and B. Moss (5). Stocks of recombinant vaccinia viruses were adjusted to approximately 10^9 PFU/ml, stored in aliquots at −80°C, and thawed immediately prior to use.

Synthetic HIV-1 peptides. Synthetic peptides corresponding to the HIV-1 envelope PV22 sequence were synthesized and purified by Multiple Peptide Systems (San Diego, Calif.) as described previously (13) and consisted of a series of peptides 25 aa in length, overlapping by 8 aa. Peptides were synthesized as COOH-terminal amides unless otherwise noted. Smaller peptides of 8 to 15 aa used for fine mapping were synthesized as free acids on an automated peptide sequencer (Applied Biosystems Model 420A). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethyl sulfoxide (Sigma) and 1 mM dithiothreitol (Sigma).

Isolation of HIV-1 envelope-specific CTL clones and lines. CTL clones were isolated and maintained as previously described (14, 32). Briefly, PBMC were obtained by separation of whole blood on a Ficoll-sodium diatrozoate gradient (Sigma) and were plated at concentrations ranging from 10 to 100 cells per well of a 96-well plate with 200 μl of feeder solution containing 10^6 irradiated allogeneic PBMC per ml from HIV-1-seronegative subjects in RPMI 1640 with 10% heat-inactivated fetal calf serum (R10) supplemented with 100 U of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, N.J.) per ml. The CD3-specific monoclonal antibody (MAb) 12F6 (34) was added at 0.1 μg/ml to stimulate T-cell proliferation. Cells from wells demonstrating growth were further restimulated as described previously (13) and were tested for cytolytic activity against autologous target cells infected with recombinant vaccinia viruses expressing either the control *lacZ* or the HIV-1 envelope gene approximately 4 to 6 weeks following the initial cloning. Clones exhibiting envelope-specific CTL activity were restimulated every 14 to 21 days with anti-CD3 MAb and irradiated allogeneic PBMC. CTL lines were established from active wells in a limiting dilution assay from subject 19142 in which anti-CD3 MAb was used as a stimulator for T-cell proliferation (15).

Flow cytometric analysis. Cells were incubated with fluorescent probe-conjugated MAbs, singly or in combination as follows: anti-CD3–anti-CD4, anti-CD3–anti-CD8, and anti-mouse immunoglobulin G2b-immunoglobulin G1 as controls (Coulter Electronics, Hialeah, Fla.). Samples were analyzed with a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, Calif.) as previously described (14).

Cytotoxicity assay. Target cells consisted of B-LCL infected with recombinant vaccinia viruses or preincubated with synthetic HIV-1 peptides. Target B-LCL were infected with recombinant vaccinia virus as previously described (12), labelled with 65 to 100 μCi of $Na_2(^{51}CrO_4)$ (New England Nuclear, North Billerica, Mass.) overnight, and then washed three times with RPMI 1640 medium. Peptide-sensitized target cells were obtained by incubating 2×10^6 to 3×10^6 B-LCL cells with peptide for 60 min during ^{51}Cr labelling. Cytolytic activity was determined in a standard 4-h ^{51}Cr-release assay (31) using U-bottomed microtiter plates containing 5×10^3 targets per well. Assays were performed in duplicate. Supernatant fluids were harvested onto 96-well plates containing solid scintillate, allowed to dry overnight, and counted in a TopCount Microplate Scintillation Counter (Packard Instrument Co., Meriden, Conn.). Maximum release was determined by lysis of targets in detergent (1% Triton X-100; Sigma). Percent lysis was determined by the formula: % lysis = 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release values were less than 30% of maximal release for all reported assays.

PCR amplification and sequence analysis. PBMC were lysed in 1 ml of lysing buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.5% sodium dodecyl sulfate) containing proteinase K (100 μg/ml) and incubated at 37°C overnight. High-molecular-weight DNA was extracted twice with phenol-chloroform (1:1) and once with chloroform, precipitated with 2 volumes of absolute ethanol, and dissolved in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). About 0.2 to 1.0 μg of DNA was used for each PCR. An envelope fragment containing the epitope coding region was amplified with first-round primers (env C, 5′-TGTCAGCACAGTACAATGTACACATGGAAT-3′, and env K, 5′-AGTAGTGGTGCAGATGGTTTTCCAGAGC-3′) and second-round primers (env D, 5′-CAACTGCTGTTAAATGGCAGTCTAGCAGAA-3′, and env J, 5′-CAGCCAGGACTCTTGCCTGGAGCTG-3′). PCR amplification was carried out in a total volume of 100 μl, containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM deoxynucleoside triphosphates, 20 pmol of each primer, and 2.5 U of *Taq* polymerase. Samples were subjected to 30 cycles of denaturing (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 2 min), with a 10-min additional extension at 72°C after all cycles. PCR products were visualized by agarose gel electrophoresis, purified from agarose gels with the QIAEX Gel Extraction Kit (Qiagen Inc., Chatsworth, Calif.), and subcloned into pCRII by T/A overhang (Invitrogen, San Diego, Calif.). Sequence analysis was performed by cycle sequencing and dye terminator methods on an automated DNA sequenator (model 373A; Applied Biosystems, Inc.) according to the manufacturer's recommendations. Final sequences were analyzed and aligned with Eugene (Baylor College of Medicine, Houston, Tex.) and MASE (7).

RESULTS

Isolation of HIV-1 envelope-specific CTL clones. PBMC from HIV-1-infected subjects 19142, 08109, and 18026 were cloned at limiting dilution and screened for the ability to lyse autologous B-LCL infected with recombinant vaccinia virus expressing the entire HIV-1 envelope gene. Clones 19142-5, 08109-37, and 18026-12 were all found to vigorously lyse VPE16-infected B-LCL expressing the HIV-1 gp160 Env protein. These clones were phenotypically $CD3^+$ $CD8^+$ $CD4^-$ as determined by flow cytometric analysis (data not shown). Clones were further tested for the ability to lyse autologous B-LCL expressing serial truncations of the HIV-1 envelope glycoprotein. All clones lysed target cells expressing the NH_2-terminal 393 aa of the envelope glycoprotein but not truncations expressing fewer than the first 287 aa, localizing the epitope to the region spanning aa 287 to 393 of HIV-1 gp120 (data not shown). In a previous study, clone 035i-J27 from a fourth subject had been shown to recognize this same region of HIV-1 Env (13).

Identification of optimal epitopes recognized by Env-specific CTL clones. Further fine mapping of the epitope specificity of these envelope-specific clones was performed with B-LCL incubated with a series of overlapping peptides of 25 aa in length and overlapping by 8 aa which spanned aa 287 to 393 of HIV-1 gp120. Surprisingly, all CTL clones recognized target cells incubated with peptide env/116 (aa 371 to 395, sequence IVTHSFNCGGEFFYCNSTQLFNSTW) as depicted in Fig. 1 and as reported by Johnson et al. for clone 035i-J27 (13).

Epitope specificity was then defined for each clone by eval-

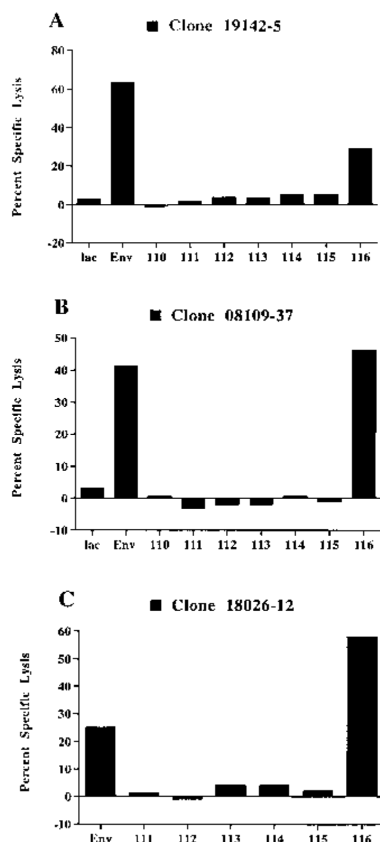

FIG. 1. Epitope mapping of the specificity of HIV-1 envelope-specific clones 19142-5 (A), 08109-37 (B), and 18026-12 (C) using synthetic overlapping peptides. Target cells were prepared by incubating autologous B-LCL with 50 μg of the indicated peptide (env/110 to env/116) per ml or infecting B-LCL with recombinant vaccinia viruses expressing the control *lacZ* (lac) or HIV-1 envelope (Env). Clones were incubated with autologous target B-LCL at effector/target ratios of 5:1 to 10:1. The indicated peptides are 25 aa in length, overlap by 8 aa, and span aa 269 (EVVIR. . ., env/110) through aa 395 (. . .FNSTW, env/116) of the HXB2R envelope. The full sequence of peptide env/116 is IVTHSFNCGG EFFYCNSTQLFNSTW (aa 371 to 395).

uating recognition of shorter peptides spanning this region of gp120. All clones recognized targets labelled with the 11-aa peptide, SFNCGGEFFYC (116B), with high levels of specific lysis (Table 1) (13). However, each clone showed a slightly different pattern of recognition when serial truncations of single amino acids were made at either end of this 11-aa peptide, suggesting that the epitopes, although overlapping, required different N- and C-terminal amino acids.

This differential recognition was further confirmed in experiments using limiting peptide concentrations to define optimal epitopes. Figure 2 shows the concentration of individual peptides which were able to sensitize autologous target cells for half-maximal lysis by the representative clones 19142-5 and 08109-37. Peptide 116L (SFNCGGEFF) was able to sensitize target cells for killing by clone 19142-5 (Fig. 2A) at lower concentrations than all other peptides tested. Half-maximal lysis of target cells (15) labelled with peptide 116L occurred at 0.0001 μg/ml, whereas peptide 116K (FNCGGEFFY) was unable at any concentration to sensitize targets for lysis at values approaching half of the maximal value obtained against peptide 116L. This indicates that the sequence SFNCGGEFF is the optimal epitope for clone 19142-5. In contrast, peptide 116K (FNCGGEFFY) was able to sensitize targets for killing

TABLE 1. Fine mapping of the epitope specificity of the envelope-specific CTL clones 19142-5, 08109-37, and 18026-12

Env peptide	Sequence	% Specific lysis[a]		
		19142-5	08109-37	18026-12
116A (aa 374–385)	HSFNCGGEFFYC	64	81	NT[b]
116B (aa 375–385)	SFNCGGEFFYC	72	83	97
116C (aa 376–385)	FNCGGEFFYC	43	89	54
116D (aa 377–385)	NCGGEFFYC	1	32	NT
116E (aa 378–385)	CGGEFFYC	5	16	NT
116F (aa 374–384)	HSFNCGGEFFY	69	72	58
116G (aa 374–383)	HSFNCGGEFF	84	12	56
116H (aa 374–382)	HSFNCGGEF	3	7	56
116I (aa 374–381)	HSFNCGGE	31	13	67
116J (aa 376–383)	FNCGGEFF	46	70	NT

[a] Targets were ^{51}Cr-labelled autologous B-LCL incubated with the indicated peptide (env/116 A to J) at a concentration of 50 μg/ml for 1 h. Clones were incubated with target cells at an effector/target ratio of 10:1.

[b] NT, not tested.

by clone 08109-37 at much lower concentrations than the other peptides tested (Fig. 2B), with half-maximal lysis occurring at 0.0001 μg of peptide per ml, indicating that the 9-aa sequence FNCGGEFFY is the optimal epitope recognized by this clone.

Comparison of all four clones for recognition of these two 9-aa peptides with limiting peptide concentrations is shown in Fig. 3. Clones 19142-5 and 035i-J27 recognized peptide 116L (SFNCGGEFF) but not peptide 116K (FNCGGEFFY), and the reverse was found for clones 08109-37 and 18026-12. These results also indicate that clones 19142-5 and 035i-J27 share the optimal epitope SFNCGGEFF and clones 18026-12 and 08109-37 share the optimal epitope FNCGGEFFY. Although some variation was noted from assay to assay in the absolute peptide concentration at which half-maximal lysis occurred, peptide 116K was generally able to sensitize target cells for

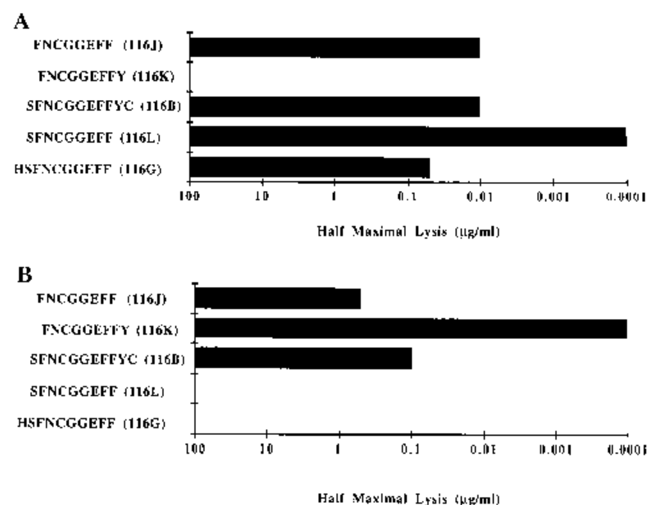

FIG. 2. Effects of varying peptide concentration on CTL recognition of truncated envelope peptides. Autologous B-LCL were sensitized with varying concentrations of synthetic peptides and incubated with CTL clone 19142-5 (A) or 08109-37 (B) at effector/target ratios of 5:1. Amino acid sequences for each peptide are as shown. Half-maximal lysis values were calculated as those peptide concentrations at which half-maximal target cell lysis (based on the highest percent lysis in a given assay) occurred. In panel A, half-maximal lysis values were not achieved by clone 19142-5 against peptide 116K even at peptide concentrations of 10 to 100 μg/ml. In panel B, half-maximal lysis values were not achieved by clone 08109-37 against peptides 116G and 116L at the highest peptide concentrations tested.

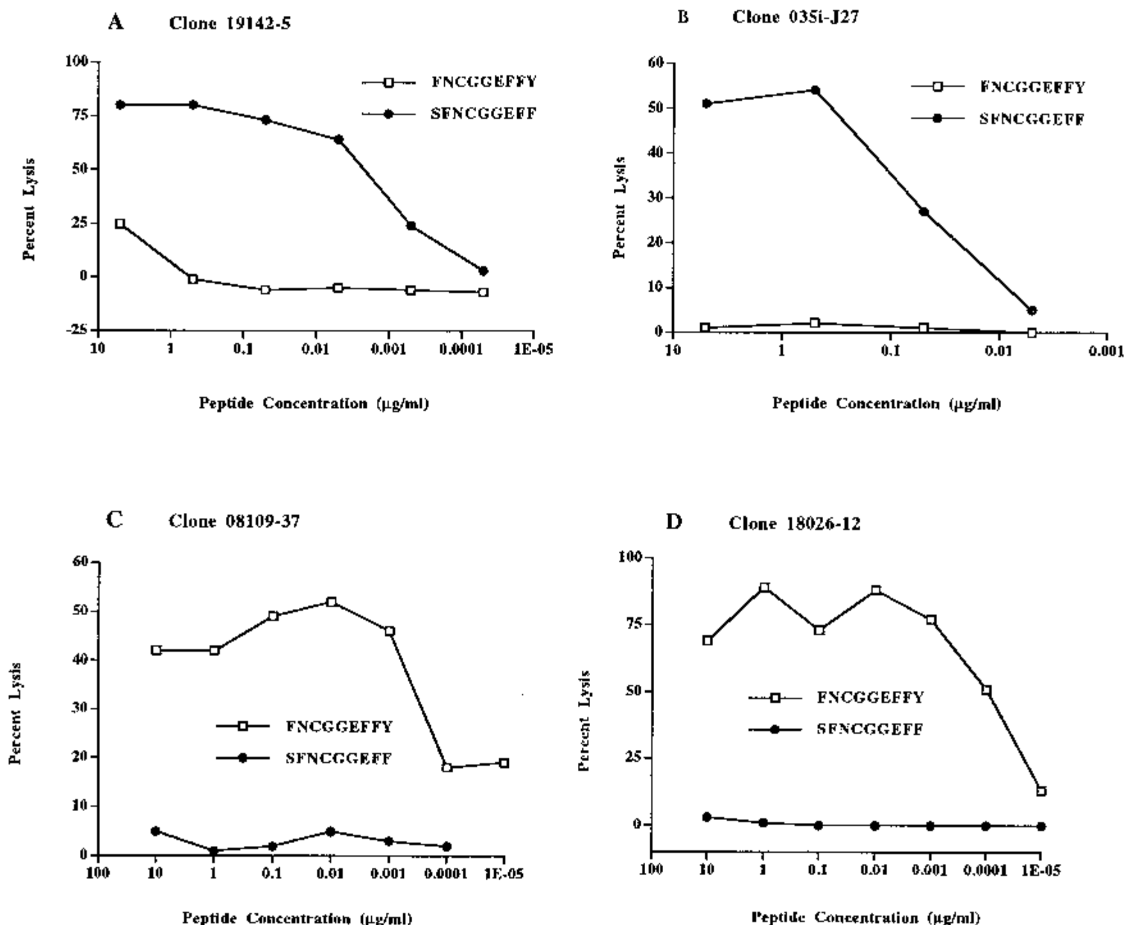

FIG. 3. Recognition of overlapping minimal epitopes by gp120-specific CTL clones. Clones 19142-5 (A), 035i-J27 (B), 08109-37 (C), and 18026-12 (D) were tested for the ability to lyse autologous B-LCL pulsed with varying concentrations of two peptides, SFNCGGEFF (116L, aa 375 to 383) and FNCGGEFFY (116K, aa 376 to 384). Clones were incubated with targets at effector/target ratios of 5:1.

lysis by the cognate CTL clones at lower peptide concentrations than peptide 116L.

HLA restriction of the envelope-specific CTL clones. To determine the HLA class I molecules responsible for presenting the recognized viral epitopes to each CTL clone, we evaluated the lysis of a panel of allogeneic B-LCL matched at one or more HLA class I alleles and expressing viral antigen. Target cells included those which had been infected with vaccinia viruses expressing HIV-1 envelope as well as those incubated with previously recognized envelope peptides. Figure 4 shows lysis by the three envelope-specific clones of autologous and allogeneic envelope-expressing target cells which share one or more HLA alleles.

For clone 19142-5, the restricting antigen could be identified as the B15 molecule (Fig. 4A), whereas for clones from subjects 08109 (Fig. 4B) and 18026 (Fig. 4C), HLA A29 was shown to restrict lysis. Clone 035i-J27 recognized envelope-expressing targets as well as target cells labelled with the SFNCGGEFF peptide in a HLA Cw4-restricted fashion as previously described (13). The epitope sequence is consistent with the peptide ligand HLA-Cw*0401 motif as described by Falk et al. (6). Peptide motifs for A29 and B15 are yet to be defined, but the data presented here indicate that tyrosine and phenylalanine, respectively, can serve as C-terminal anchor residues.

The manner in which the HLA class I restriction patterns relate to specificity of peptide recognition is further delineated in Table 2, in which peptide-pulsed B-LCL from the four subjects were used as targets for recognition by CTL clones obtained from three of the four subjects. Clone 19142-5 recognized only targets pulsed with 116L (SFNCGGEFF), the optimal epitope for this clone, and only in a B15-restricted manner. Despite the fact that Cw4-expressing target cells present this identical peptide, no cross-recognition was observed when clone 19142-5 was tested against the Cw4-expressing target cells from subject 035i when sensitized with peptide 116L. Clones 08109-37 and 18026-12 lysed only in an A29-restricted fashion. $A29^+$ B-LCL from subject 18026 were able to present both peptides 116K and 116L for killing whereas $A29^+$ 08109 B-LCL could present only the optimal epitope 116K, suggesting that there may be subtypes of the serologically defined HLA A29 molecule. Interestingly, whereas B15-restricted killing of target cells expressing envelope was noted in the case of 19142-5 above, no B15-restricted lysis was noted by the CTL clone from subject 08109, although the B15 allele is common to both. In addition, no envelope-specific clones with B15 restriction were isolated from subject 08109 by standard limiting dilution cloning techniques (data not shown). These data underscore the complexity and specificity of the peptide-class I MHC-TCR interaction in lysis by CTL.

Recognition of naturally occurring sequence variants by the envelope-specific clones. Clone 19142-5 was tested for the ability to recognize six major published variant sequences of the

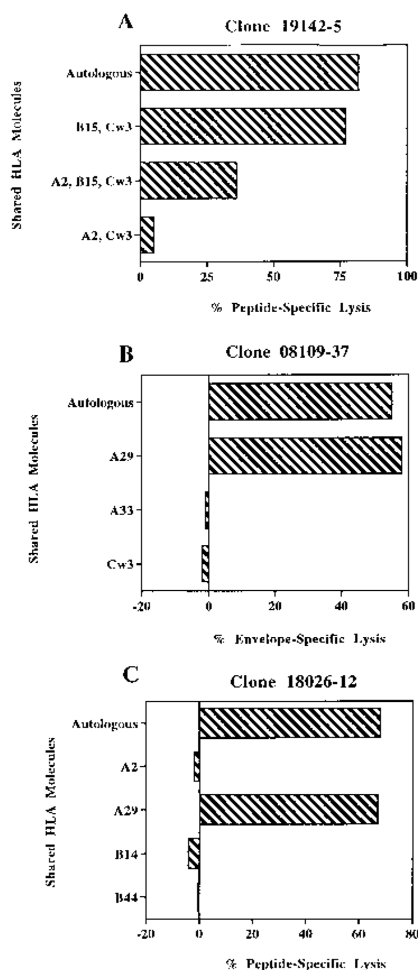

FIG. 4. HLA restriction analysis of gp120-specific CTL clones. (A) Lysis of peptide-sensitized B-LCL in a B15-restricted fashion by clone 19142-5 (A2, 26 B15, 40 Bw4, 6 Cw3). Target cells were pulsed with relevant (Env/116, aa 371 to 395) or irrelevant (Env/115, aa 354 to 378) peptides at a concentration of 50 μg/ml for 1 h prior to incubation with effector cells at an effector-to-target ratio of 5:1. Peptide-specific lysis was determined by subtracting lysis of targets sensitized with irrelevant peptide from lysis of those sensitized with the relevant envelope peptide. (B) Lysis of envelope-expressing B-LCL by clone 08109-37 (A29, 33 B15, Bw4, 6 Cw3) is A29 restricted. Effectors were incubated with autologous or allogeneic B-LCL infected with envelope or *lacZ*-expressing recombinant vaccinia virus. Allogeneic envelope-expressing B-LCL having the B15 allele in common with this clone were not specifically lysed in other assays (data not shown). (C) Clone 18026-12 (A2, 29, B14, 44) recognizes peptide-pulsed allogeneic target cells in an A29-restricted fashion. Allogeneic target cells were pulsed with env/116 as described above prior to incubation with effectors (effector-to-target ratio, 5:1).

TABLE 3. Natural strain variation in HIV-1 envelope affects recognition by CTL clones[a]

Strain	Sequence	Half-maximal lysis (μg/ml)		
		Clone 19142-5	Clone 08109-37	Clone 18026-12
IIIB (Index), Cons[b] A, B, D	SFNCGGEFF	0.0005		
SF2; Cons C, F	SFNC**R**GEFF	0.1		
HIVUGO6(A)	SFNC**V**GEFF	0.01		
HIVJFL(B)	**T**FNCGGEFF	1		
HIVSBC(B)	**N**FNCGGEFF	10		
Consensus E	**H**FNC**R**GEFF	>100[c]		
Consensus O	**H**FNC**H**GEFF	>100		
IIIB (Index), Cons A, B, D	FNCGGEFFY		0.00001	0.0001
SF2; Cons C, E, F	FNC**R**GEFFY		10	0.5
HIVUG06(A)	FNC**V**GEFFY		1	0.5
Consensus O	FNC**H**GEFFY		10	0.5
HIVBRVA(B)	FNCGGEFF**F**		0.0005	0.001
HIVNDK(D)	**L**NCGG**D**FFY		10	>100

[a] Envelope peptides corresponding to aa 375 to 383 and aa 376 to 384 of the indicated HIV-1 strain were incubated at varying concentrations to sensitize autologous B-LCL for lysis by CTL clones. Amino acids which differ from the index sequence of the HIV-1 IIIB strain are in boldface and underlined. Half-maximal lysis indicates the peptide concentration at which half-maximal lysis (based on maximal lysis of index peptide) of each peptide-labelled target cell occurred.

[b] Cons, consensus sequence according to the Los Alamos Database.

[c] >100, absence of specific lysis of target cells pulsed with peptide at the highest concentration tested (100 μg/ml).

epitope SFNCGGEFF (23) (Table 3). In this case, CTL recognition was maintained against target cells pulsed with the two variant peptides (SF2 and UG06 strains) containing substitutions at the fifth position (G-R, G-V), whereas lysis of targets bearing strain JFL and SBC variant peptides with single-amino-acid substitutions at position 1 (S-T, S-N) was greatly reduced. As reported previously by Johnson et al. (13), clone 035i-J27 was unable to recognize the epitopic peptide corresponding to the SF2 variant but could lyse targets incubated with the HIV JFL variant in a fashion similar to that observed with the index peptide. These different patterns of variant recognition may reflect different peptide binding constraints of the restricting HLA molecules, B15 and Cw4, or differences in TCR structure of the individual T-cell clones.

Half-maximal lysis values were determined for lysis of five strain variants of the A29-restricted epitope FNCGGEFFY by clones 08109-37 and 18026-12, again revealing marked differ-

TABLE 2. Restricted recognition of overlapping minimal epitopes to targets with shared HLA alleles[a]

Target cell	HLA type	% Specific lysis by CTL clone (restricting element):					
		19142-5 (B15)		08109-37 (A29)		18026-12 (A29)	
		116K	116L[b]	116K[b]	116L	116K[b]	116L
08109 B-LCL	**A29**, 33; **B15**; CW3	0	65	21	5	72	8
19142 B-LCL	A2, 26; **B15**, 40; CW3	2	79	4	3	0	3
18026 B-LCL	A2, **29**; B14, 44	0	12	21	16	75	32
035i B-LCL	A1, 11; B8; CW4	0	8	2	3	3	1

[a] B-LCL (from the same subjects from which envelope-specific clones were derived) were pulsed with peptides env/116K (FNCGGEFFY) or 116L (SFNCGGEFF) at a concentration of 50 mg/ml for 1 h during ^{51}Cr labelling prior to the addition of CTL clones. Clones 08109-37, 19142-5, and 18026-12 were tested for ability to lyse the peptide-pulsed autologous and allogeneic target cells, with the HLA allele determined to be the restricting agent noted in parentheses for each clone. Percent specific lysis was calculated by subtracting lysis of targets bearing an irrelevant peptide from lysis of targets bearing the relevant peptides, 116K and 116L. Clone 035i-J27 could not be tested due to poor growth.

[b] The peptide which corresponds to the optimal epitope for a given clone is indicated.

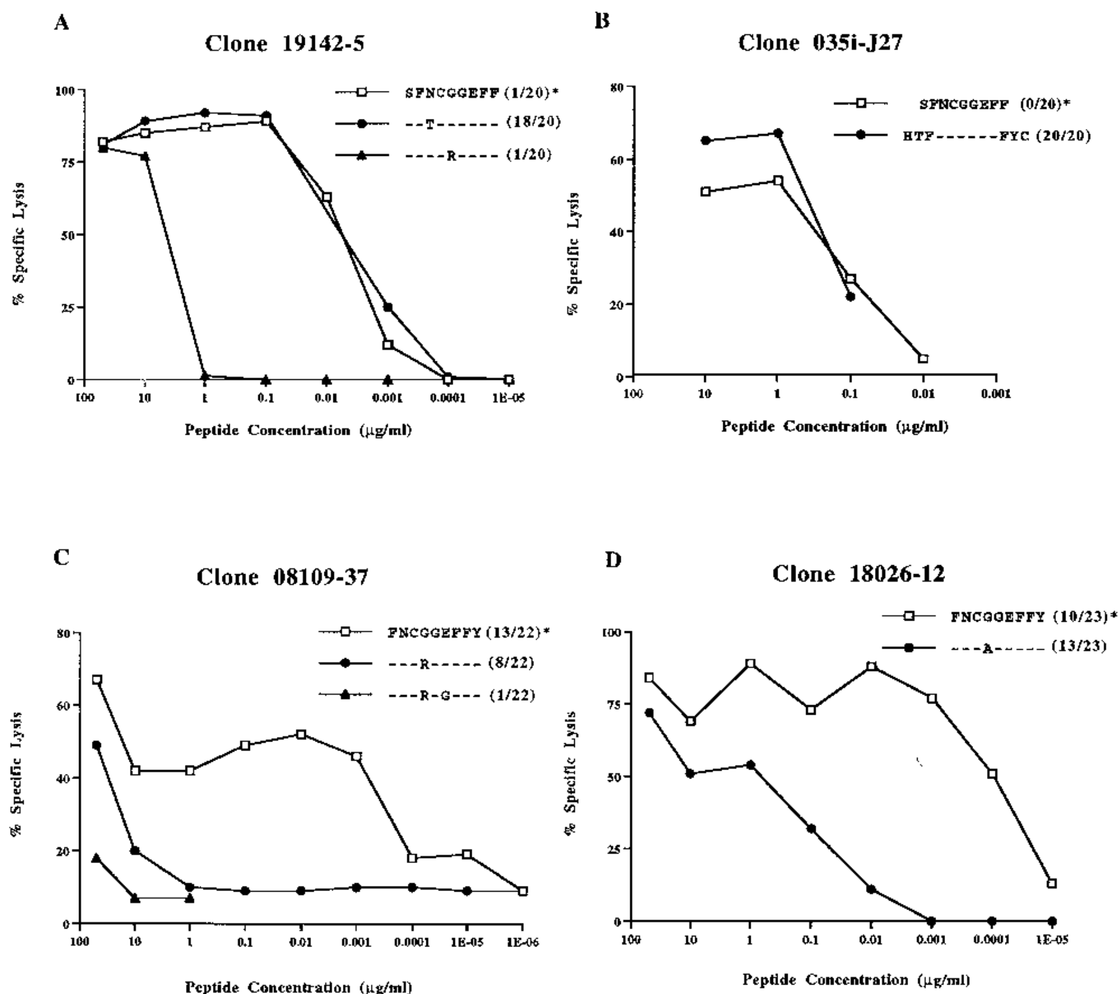

FIG. 5. Recognition of autologous HIV-1 envelope sequences by gp120-specific CTL clones. Peptides corresponding to autologous HIV-1 envelope DNA epitope sequences from PBMC of four infected subjects were pulsed on autologous B-LCL in varying concentrations. Recognition of the peptide-pulsed target cells by the following envelope-specific CTL clones was tested in a standard 4-h chromium-release assay: clone 19142-5 (A), 035i-J27 (B), 08109-37 (C), and 18026-12 (D). The relative frequency of each variant sequence in DNA from unstimulated PBMC is indicated in parentheses, and the HIV-1 IIIB strain index sequence is indicated by an asterisk. Recognition of a peptide corresponding to the autologous sequence for clone 10035i-J27 has previously been reported (13) and is presented here to facilitate comparison.

ences in recognition based on single-amino-acid substitutions (Table 3). The overall recognition patterns of these two independently derived A29-restricted CTL clones were quite similar, as has been seen in a similar analysis of B14-restricted clones (15).

Evaluation of autologous viral sequence variation in the Env epitopes and recognition by CTL clones. In order to determine whether immunologic pressure in the region of envelope was associated with sequence variation in autologous virus, PBMC from each of the subjects tested were obtained from the same time points at which the Env-specific clones were isolated and were used as a source for isolation and sequencing of HIV-1 DNA through the envelope region containing the epitopes. Figure 5 lists the amino acid sequences derived from nucleotide sequencing of the isolated DNA clones through the region of Env corresponding to aa 375 to 383 for subjects 19142 and 035i and aa 376 to 384 for subjects 08109 and 18026. Peptides corresponding to the autologous viral sequences were synthesized and used to label target cells for recognition by the envelope-specific CTL.

Sequence variation relative to the IIIB index sequence was observed for each subject, but the consequences of this variation with respect to CTL recognition differed. For the two subjects recognizing the epitope SFNCGGEFF, the dominant in vivo sequences were well recognized. The major autologous sequence detected (SFTCGGEFF, 18 of 20 cloned) and the IIIB sequence (SFNCGGEFF, 1 of 20) showed similar patterns of recognition when corresponding peptides were used to label targets for killing by the B15-restricted T-cell clone 19142-5 (Fig. 5A). The other minor variant seen in 1 of 20 clones was the sequence SFNCRGEFF, corresponding to the HIV-1 SF2 strain sequence as well as consensus sequences for clades C and F. This sequence was poorly recognized by 19142-5, probably due to the unconserved nature of the glycine-to-arginine substitution at position 5. All DNA clones sequenced (20 of 20) from subject 035i, who recognized SFNCGGEFF in the context of HLA Cw4, contained nucleotide sequences corresponding to the amino acid sequence TFNCGGEFF. This sequence was conserved in this subject over time, being the sole sequence isolated in 1991 and again in 1995 (data not shown). Clone 035i-J27 was previously tested for ability to lyse targets bearing the JFL variant in a slightly

longer form (aa 374 to 385, HTFNCGGEFFYC), and no abrogation of recognition was noted as a result of this conserved substitution (13) (Fig. 5B).

More sequence variation was noted through the epitope corresponding to Env aa 376 to 384 (index sequence FNCGG EFFY) in viral DNA obtained from the two HIV-infected subjects having A29-restricted CTL clones specific for this optimal epitope. A major variant (sequence FNCRGEFFY, 8 of 22) as well as a minor variant (sequence FNCRGGFFY, 1 of 22) was seen in addition to the index sequence in HIV DNA clones isolated from subject 08109. Comparison of lysis of peptide-coated target cells by the 08109-37 clone revealed that the minor variant was not recognized even at higher peptide concentrations (Fig. 5C). The major variant, the sequence of which corresponds to HIV-1 strain SF2 and consensus sequences for clades C, E, and F (Table 3), was recognized much less well than the index sequence, possibly due to the unconserved nature of the position 4 substitution (glycine to arginine).

The findings were different for the other A29-restricted clone. Over 50% (13 of 23) of the viral DNA clones obtained from subject 18026 contained the major variant sequence FNC AGEFFY, while the remainder consisted of IIIB index sequence (10 of 23). Despite the relatively conservative nature of the amino acid substitution at position 4, there was clearly a decrease in the ability of the A29-restricted clone 18026-12 to recognize targets pulsed with the variant peptide, especially at lower peptide concentrations (Fig. 5D). Overall, these results indicate that the quasispecies which arise in vivo can differ significantly, even in persons with CTL directed at the same epitope and which appear to be equally cross-reactive when tested against laboratory strains of virus.

When each of the A29-restricted clones was tested for ability to lyse autologous targets pulsed with viral variant peptides derived from the other A29$^+$ subject, similar recognition patterns emerged (data not shown). Both clones recognized the FNC**A**GEFFY variant (derived from subject 18026 PBMC), although less well than the index epitope peptide. The FNC **R**GEFFY variant (from subject 08109) was recognized only at the highest peptide concentrations for each clone, and neither could recognize the doubly substituted variant from 08109 at any concentration.

DISCUSSION

We describe here four CTL clones, each from a different HIV-1-infected subject, which are restricted by different class I molecules and yet able to recognize epitopes contained within the same 10-aa conserved region of HIV-1 gp120. These data indicate that the same viral peptide can be recognized in the context of HLA A, B, and C molecules. Sequence analysis of in vivo virus revealed mutations within these epitopes, but the mutations present and the ability of clones to recognize the in vivo variants were quite different for each subject. The results indicate that the quasispecies which arise in vivo can differ significantly even in persons with CTL directed against the same envelope region, and that maintenance of CTL recognition despite autologous sequence variation can differ markedly among infected persons.

It has been suggested that sequence variation contributes to disease progression in HIV-infected individuals (24). HIV-1 has a high mutation rate due to the infidelity of the reverse transcriptase (2, 9), with envelope being the most highly variable of the HIV-1 proteins. For each of the four subjects described here, in vivo sequence variants through the envelope epitopes were identified by PCR amplification of proviral DNA, but the virus variants detected, as well as the ability of CTL clones to recognize them, distinctly differed for each person. This point is illustrated by comparing the variant recognition of the clones from subjects 08109 and 18026 which recognized the same A29-restricted CTL epitope (Fig. 5). The major autologous variant sequences amplified from proviral DNA from each subject differed only by 1 aa at position 4 of the epitope. Yet, clone 08109-37 failed to recognize the major viral variant sequence amplified from autologous proviral DNA, whereas clone 18026 recognized its major variant peptide, albeit less well than the index sequence. The demonstrated marked decrease in the ability of a CTL clone from subject 08109 to recognize the dominant amplified virus variant could theoretically be associated with survival advantage of the mutant virus in vivo, possibly leading to increased viral replication and the development of other resistant mutants. On the other hand, the CTL clone isolated from subject 18026 might more effectively inhibit replication of autologous virus variants in vivo.

The fact that T-cell clones in certain individuals (i.e., subjects 19142 and 035i, and 18026 to some extent) can recognize the dominant autologous viral variants detected by PCR amplification suggests that sequence variation within an epitope need not necessarily imply immune escape, although variation within epitopes has been shown to correlate with disease progression in some cases (33). There is evidence in other viral systems that single-amino-acid changes leading to abrogation of CTL recognition may lead to virus persistence in vivo (30) and that escape from even one or two CTL epitopes confers a survival advantage on the virus (22). Whether the in vitro patterns of variant sequence recognition by CTL clones described here have in vivo correlates (with respect to suppressing viral replication or altering disease progression) in HIV-1-infected individuals is yet to be determined. The analysis of proviral DNA does not distinguish between defective and replication-competent virus, and there is a possibility that some of the variant sequences amplified from these subjects might be less fit or replication incompetent. It is also possible that, under conditions of low viral load, resampling of proviral template might occur, leading to alterations in the relative variant frequencies observed (20). Shifts in variant frequencies should not affect the recognition patterns of the T-cell clones described and, in this qualitative study, would have little impact on the interpretation of our results. Further studies, however, including longitudinal analysis of viral variants and more-extensive sequencing of plasma-derived and tissue-derived virus will be necessary to more fully address the issue of immune escape.

The data presented here add to the limited number of CTL epitopes in the HIV-1 gp120 protein and provide another example of overlapping epitopes presented by multiple HLA alleles (12). CTL epitopes in gp120 presented by nine MHC class I molecules have been precisely defined (reviewed in reference 18). Three of these CTL epitopes restrict lysis in the region from aa 376 to 384 (A29, B15, Cw4), and four restrict lysis of overlapping epitopes in the region from aa 37 to 62 (A3, A24, B35, and B55). These data suggest that certain regions are preferentially processed for CTL recognition, but the factors involved in this selection remain to be defined.

Another point raised by these results is the possible competition for presentation of overlapping epitopes. Using direct T-cell cloning methods, we were unable to obtain clones reactive against the B15-restricted epitope SFNCGGEFF in the one subject who was both A29 and B15 positive, whereas a dominant CTL response to the adjacent A29-restricted epitope FNCGGEFFY was demonstrated in that subject. However, in

another HLA B15-positive subject, T-cell clones recognizing the SFNCGGEFF epitope in a B15-restricted manner were readily obtained by similar cloning methods. This clearly indicates that the latter epitope can be naturally presented by HLA B15. It could be postulated that a hierarchical pattern of presentation of these overlapping epitopes exists. This might result from competition at the level of binding to MHC, with preferential binding of peptide to HLA A29 leading to lower availability for binding to B15, or preferential processing of the A29-restricted epitope resulting in a subdominant B15-restricted epitope. This hypothesis may be supported by peptide titration data (Fig. 3) which shows that the A29-restricted peptide FNCGGEFFY sensitizes targets for recognition by cognate CTL at lower concentrations than the B15-restricted peptide, possibly suggesting differential binding affinities. Longitudinal studies in this $A29^{+}$ $B15^{+}$ subject will be needed to address whether autologous sequence variation through the A29 epitope leading to lack of A29 binding might uncover the B15-restricted epitope with subsequent generation of B15-restricted CTL.

These results provide further evidence that the HLA profile of a given individual significantly affects the specificity of the immune response to HIV-1. Population studies showing correlation between certain HLA class I and class II alleles and rapid or slow disease progression suggest a potentially important in vivo association (16). Although we have not yet performed longitudinal studies in all of these subjects to correlate CTL recognition of variants with viral load or disease progression, our data demonstrate that HIV-1 mutations in the same region of envelope may have different effects on T-cell recognition in different individuals. Understanding the effects of sequence variation on CTL recognition of epitopes in conserved regions of HIV-1 is likely to be important for several reasons. The evaluation of clonal T-cell responses to variant epitopes should improve our understanding of how the constraints placed on HIV-1 to maintain conserved sequences affect the ability of T cells from individuals with a given HLA type to control HIV replication. The Env epitopes described here are partly contained in the area of gp120 reported to be involved in binding to the CD4 molecule (25). Certain residues might therefore be involved directly in CD4 binding or be necessary for maintaining tertiary structure critical for binding and thus would tend to be conserved, whereas changes in other residues might not alter the replicative dynamics of the virus (3). Major differences were noted in the ability of T-cell clones targeting the same or a similar amino acid sequence to recognize autologous viral variants, and these differences could result in part from the type and location of mutations tolerated by the virus (i.e., which maintain function or structure) with respect to the position of such changes in the T-cell epitope. For instance, there is significant variation in laboratory strains of HIV-1 at Env aa 375 (position 1 of the SFNCGGEFF epitope) and Env aa 379 (position 5 for the SFNCGGEFF epitope and position 4 for the FNCGGEFFY epitope) (23). Mutations in these positions were also seen in the sequences derived from autologous clinical HIV-1 isolates. The effect of mutations occurring at these variable residues is likely to depend on the HLA molecule responsible for antigen presentation, as the interaction of a given amino acid in a processed viral peptide with the TCR and HLA complex will differ depending on the epitope. The ability of the TCR to broadly recognize variant peptides may also depend to some degree on the sequence which originally induced the CTL response. CTL responses to conserved immunodominant epitopes may offer an advantage to the host by more effectively suppressing viral replication. Thus, the identification and evaluation of T-cell reactivity against conserved epitopes should benefit the development of vaccines and immunotherapies for HIV infection.

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