

Fine-specificity of cytotoxic T lymphocytes which recognize conserved epitopes of the Gag protein of human immunodeficiency virus type 1

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Human immunodeficiency virus type 1 (HIV-1) Gag-specific cytotoxic T lymphocyte (CTL) responses were studied in seven seropositive long-term asymptomatic individuals (CDC A1) with stable CD4 counts for more than 8 years. Using a set of partially overlapping peptides covering the whole Gag, five 15–20-mer peptides were found to contain CTL epitopes. Further characterization of these epitopes revealed a new HLA-A25-restricted CTL epitope in p24, p24_{203–212} ETINEEAAEW. This region of Gag is

highly conserved in clades B and D of HIV-1. Naturally occurring amino acid sequences, containing p24₂₀₃D (consensus HIV-1 clades A, C, F, G and H) or p24₂₀₄I (HIV-2_{ROD}) were not recognized by CTL recognizing the index peptide. No virus variants with mutations in this sequence were found in peripheral blood mononuclear cells from the HIV-1-infected individual concerned during the 8 year observation period, indicating that the virus had not escaped from the observed CTL response.

Introduction

A number of observations suggest a role for cytotoxic T lymphocytes (CTL) in mediating protection against human immunodeficiency virus type 1 (HIV-1) infection. HIV-1-specific CTL have been detected well before neutralizing antibodies (Koup *et al.*, 1994). In seronegative prostitutes who had been exposed to HIV-1 infection, and in seronegative children from HIV-1-infected mothers, the presence of HIV-1-specific CTL may be an indication of their contribution to protective immunity (Rowland-Jones *et al.*, 1993, 1995; Cheyner *et al.*, 1992). In a simian immunodeficiency virus (SIV)–macaque model we showed that vaccine-induced protective immunity may correlate with the presence of a major histocompatibility complex (MHC) class I haplotype and associated CTL responses (Heeney *et al.*, 1994). During the

asymptomatic phase following the acute stage of infection, HIV-1-specific CTL can be detected in most individuals, with Gag being the most commonly recognized protein (Riviere *et al.*, 1994; Johnson & Walker, 1994).

In a recent study by Klein *et al.* (1995) we showed that frequencies of CTL detected early after infection, using HIV-1_{LAI} Gag as whole protein antigen, do not have a predictive value for the time that elapses before AIDS is diagnosed. Besides quantitative aspects, qualitative differences in CTL response may be important (Johnson & Walker, 1994; Van der Burg *et al.*, 1995). CTL directed against epitopes located within structurally constrained and thus conserved regions may be most effective in limiting virus replication and spread (Riviere *et al.*, 1994; Johnson & Walker, 1994).

In this paper we present data on the identification of HLA class I-restricted CTL epitopes in conserved parts of Gag, using peripheral blood mononuclear cells (PBMC) from long-term asymptomatic (LTA) individuals and a set of 48 partially overlapping peptides, which together span the entire protein.

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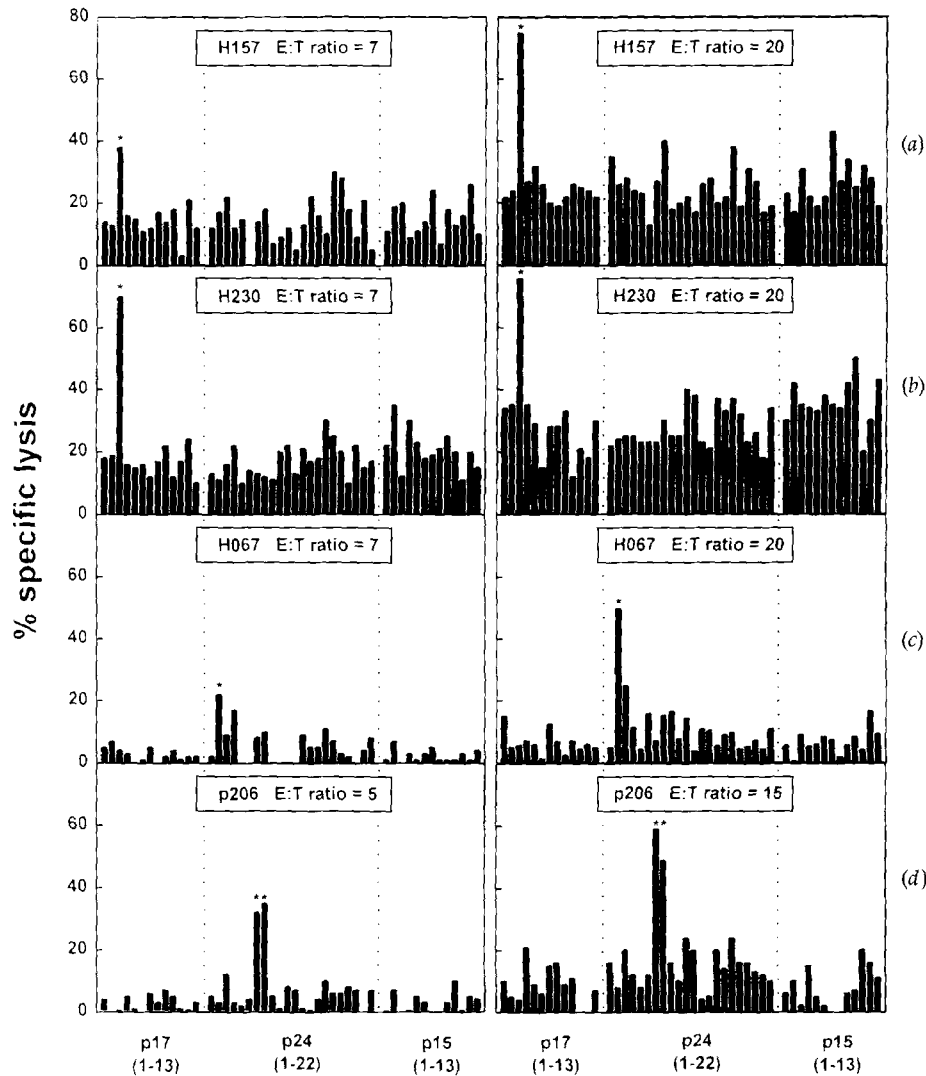


Fig. 1. CTL lines generated from the PBMC of LTA HIV-1 seropositive individuals H157 (a), H230 (b), H067 (c) and p206 (d) recognize one or two peptides from a set spanning the entire Gag. CTL lines were tested for lysis of 48 different target cell populations pulsed with one of 13 15-mer peptides (five residue overlap) spanning p17, or one of 22 20-mer peptides (ten residue overlap) spanning p24 or one of 13 15-mer peptides (five residue overlap) spanning p15. Preparation of target cells was as follows: autologous B-LCL were incubated with 100 μCi $\text{Na}_2^{51}\text{CrO}_4$ in 100 μl of medium for 1 h at 37 $^\circ\text{C}$. Cells were washed twice and incubated with 20 μM -peptide in 100 μl of medium for 1 h. Subsequently, 900 μl of medium supplemented with 5% fetal bovine serum was added and cells were incubated overnight. Target cells were washed twice and suspended in 96-well plates at 5×10^3 cells per well. Results are expressed as percentage-specific ^{51}Cr -release (average of duplicates). Effector:target (E:T) ratios are indicated in a box in each graph. * indicates CTL responses directed at peptides that were selected for further characterization.

LTA may represent the best candidates for studies on the identification and characterization of potentially protective CTL and their corresponding epitopes. It may be expected that if indeed CTL are involved in protective immunity, their prevalence will be most pronounced in this group of individuals. Besides the identification of five regions of Gag that have been shown to contain CTL epitopes previously, a novel HLA-A25-restricted and conserved epitope recognized by CD8^+ CTL was identified and characterized. Comparison of sequences of the epitope region in samples collected from the

LTA individuals concerned over an 8 year observation period did not reveal escape from CTL activity.

Methods

Study population. A total of seven HIV-1-infected individuals who remained asymptomatic with stable CD4 counts [Centers for Disease Control category A1 (Centers for Disease Control, 1992)] for more than 8 years, and who participate in the Amsterdam Cohort Studies on AIDS (de Wolf *et al.*, 1987), were studied. Their clinical characteristics and laboratory markers were recently described (Keet *et al.*, 1994; Klein

et al., 1995). HLA class I typing was performed at the Department of Transplantation Immunology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Their types were as follows: H008, HLA-A2, -A26, -B27, -B44, -Cw1; H067, HLA-A26, -A28, -B7, -B57; H157, HLA-A3, -A28, -B13, -B44, -Cw7; p206, HLA-A3, -A25, -B18, -B51; H230, HLA-A3, -B7, -B51, -Cw7; H233, HLA-A3, -A24, -B35, -B60, -Cw3, -Cw4; H358, HLA-A2, -A29, -B18, -Cw5, -Cw7. Individual p206 was monitored at the Academic Medical Centre in Amsterdam.

■ **Peptides.** Sets of overlapping peptides spanning the entire Gag of HIV-1 (strain SF2) included 13 15-mer peptides with a five residue overlap spanning p17 (ADP704), 22 20-mer peptides with a ten residue overlap spanning p24 (ADP788) and 13 15-mer peptides with a five residue overlap spanning p15 (ADP703). These peptides were kindly provided by H. Holmes (Medical Research Council AIDS Directed Programme, Potters Bar, South Mimms, UK). The 9-mer and 10-mer peptides spanning p24₂₀₃₋₂₂₂ with a nine amino acid overlap were kindly provided by R. Melen (Central Veterinary Institute, Lelystad, The Netherlands). The variant p24₂₀₃₋₂₁₂ peptides with sequences that correspond to related lentiviruses were kindly provided by R. van Herwijnen (European Veterinary Laboratory, Woerden, The Netherlands). Amino acid (aa) numbers are according to the consensus sequence of HIV-1 Gag clade B (Myers *et al.*, 1994).

■ **Recombinant vaccinia viruses.** Recombinant vaccinia viruses (rVV) TG1144, containing the pr56^{gag} gene from HIV-1_{LA1} (Rautmann *et al.*, 1989; Myers *et al.*, 1994), TG2112, containing the pr56^{gag} gene from HIV-2_{ROD}, and rVV 186-poly, containing a polycloning site without insert, were kindly provided by M.-P. Kieny (Transgene SA, Strasbourg, France). The rVV containing the pr56^{gag} gene from SIV_{MM32H} was kindly provided by A. McMichael (Oxford, UK).

■ **Preparation of effector cells.** PBMC were isolated by Lymphoprep (Nycomed) gradient centrifugation and cryopreserved. Stimulation of PBMC *in vitro* and maintenance of cell lines was performed as previously described (Van Baalen *et al.*, 1993). Briefly, PBMC were cocultivated with a paraformaldehyde-fixed autologous Epstein-Barr virus-transformed B-lymphocytic cell line (B-LCL) expressing HIV-1 Gag from infection with TG1144 for 14 to 21 days and subsequently used as effector cells in standard ⁵¹Cr-release assays. Gag-specific cell lines were subcloned under limiting dilution conditions by stimulation with 1 µg/ml (PHA)-L (Boehringer Mannheim) and irradiated feeder cells as described (Van de Griend *et al.*, 1984). The phenotype of resulting clones was determined by FACScan analysis according to the manufacturer's instructions (Becton Dickinson). Clones were maintained by stimulation every 7 to 14 days alternately with PHA-L (Van de Griend *et al.*, 1984) and by cocultivation with autologous stimulator cells as described (Van Baalen *et al.*, 1993).

■ **⁵¹Cr-release assays.** Cytotoxicity was determined in standard 4 h ⁵¹Cr-release assays as described previously (Van Baalen *et al.*, 1993). Target cells were pulsed with peptides as described in the legends to the figures or infected overnight with rVV TG1144 or 186-poly and subsequently labelled with Na₂⁵¹CrO₄ (Amersham). Effector cells were added at indicated E:T ratios. Supernatants were harvested with the Skatron harvesting system and counted on a gamma counter (LKB Wallac). Percentage specific lysis was determined from the formula: 100 × [(experimental release - spontaneous release)/(maximum release - spontaneous release)]. Maximum release was determined by lysis of target cells in 3% Triton X-100. Spontaneous release was less than 30% of maximum release.

■ **Virus sequencing.** Virus isolation procedures were performed with PBMC samples from p206 collected at seven regular intervals during

follow-up. Generation of biological clones and sequence analysis of the p24₁₉₃₋₂₂₂ region and of the gp120 V1, V2 and V3 regions of the biological clones was performed as described (Schuitemaker *et al.*, 1992).

Results

Mapping of the Gag-specific CTL response

PBMC from six LTA individuals (H157, H230, H067, p206, H233 and H385) were screened for HIV-1 Gag-specific CTL activity. Polyclonal T cell lines established by *in vitro* antigen-specific stimulation of the PBMC were tested for cytolytic activity against 48 different target cell populations pulsed with single partially overlapping peptides, spanning the entire Gag. Target cells pulsed with the third peptide of p17 (p17₂₁₋₃₅), and with the second (p24₁₄₃₋₁₆₂), the seventh (p24₁₉₃₋₂₁₂) and eighth (p24₂₀₃₋₂₂₂) peptides of p24 (Fig. 1*a-d*) were recognized by cells from individuals H157, H230, H067 and p206. Cultures from individuals H233 and H385 exhibited no specific lysis of the peptide-pulsed target cells (data not shown).

Clones from individuals H157 and H067 specific for p17₂₁₋₃₅ and p24₁₄₃₋₁₆₂ also recognized endogenously expressed HIV-1 Gag as evidenced by lysis of autologous cells expressing Gag upon rVV infection (data not shown). Some clones from H157 recognized HIV-1 Gag but not p17₂₁₋₃₅ (data not shown), indicating that Gag-specific CTL with fine-specificities other than the dominantly recognized p17₂₁₋₃₅ were present. Using essentially the same approach it was found that effector cells from a seventh individual, H008, recognized p24₂₆₃₋₂₈₂. The CD8⁺ fraction of this culture recognized endogenously synthesized Gag and lysed p24₂₆₃₋₂₈₂-pulsed autologous and heterologous HLA-B27-matched target cells but not peptide-pulsed target cell lines matched for the other HLA class I alleles (data not shown).

Identification of p24₂₀₃₋₂₁₂ ETINEEAAEW as an HLA-A25-restricted epitope

As shown in Fig. 1, the CTL response observed with cells from p206 was directed against p24₁₉₃₋₂₁₂ and p24₂₀₃₋₂₂₂. CTL with this specificity were found at all the five time-points tested during the 45 to 93 month follow-up (data not shown). To date, no epitopes located in the region p24₂₀₃₋₂₂₂ and restricted by one of the HLA-A or -B alleles of p206 (HLA-A3, -A25, -B18, -B51) have been described. A T cell line, p206/E2, containing 95% CD8⁺ T cells (data not shown) recognized both 20-mer peptides p24₁₉₃₋₂₁₂ and p24₂₀₃₋₂₂₂ as well as the 10-mer sequence p24₂₀₃₋₂₁₂ ETINEEAAEW, which is shared by both 20-mers (Fig. 2*a*). Neither the 9-mer peptide ETINEEAAE nor the 10-mer peptide TINEEAAEWD were recognized (Fig. 2*a*), indicating that both the C-terminal residue p24₂₁₂W and the N-terminal residue p24₂₀₃E are essential for T cell recognition. Therefore, it was concluded that p24₂₀₃₋₂₁₂ is the minimal epitope recognized. This peptide was recognized in association with autologous and HLA-A25-

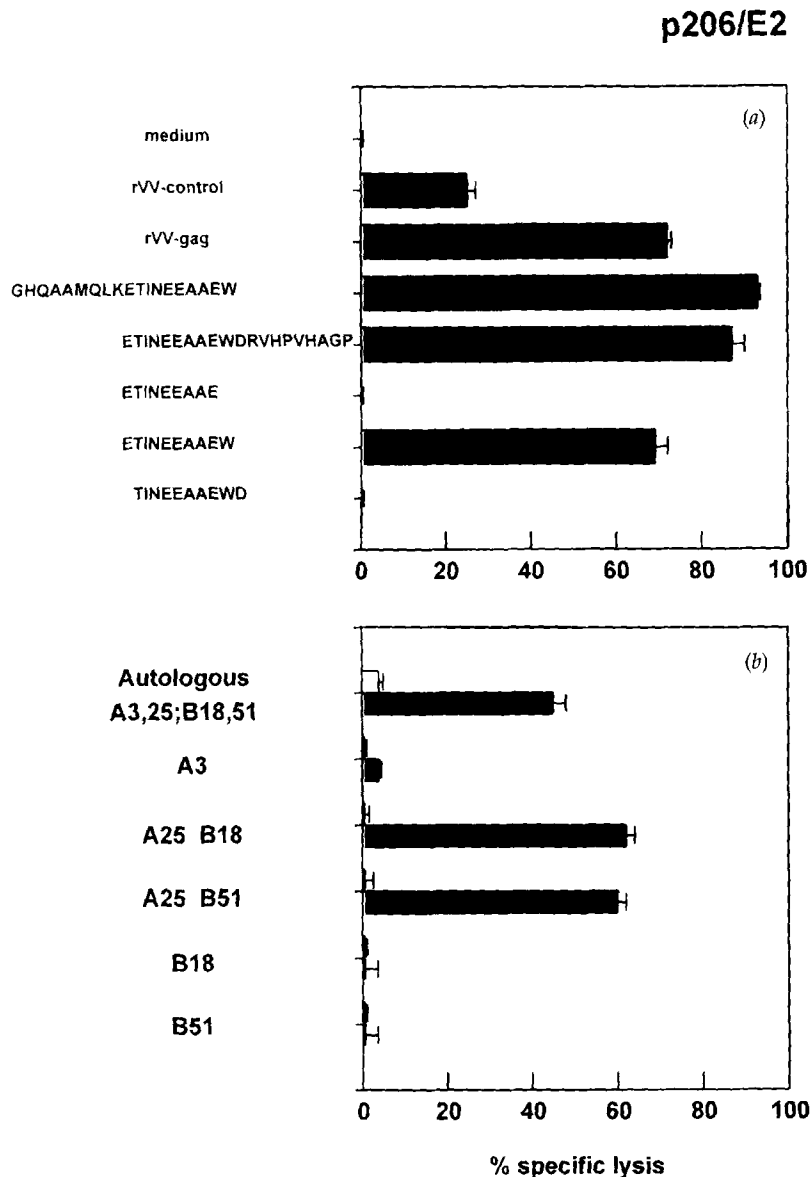


Fig. 2. Characterization of the p24₁₉₃₋₂₂₂-specific CTL from p206. (a) The CD4⁻ CD8⁺ Gag-specific CTL line p206/E2 was tested for lysis of autologous target cells infected with rVV-gag or pulsed with the peptides indicated on the vertical axis. (b) A panel of partially HLA class I-matched B-LCL was pulsed with the peptide ETINEEAAEW and tested for lysis by CTL line p206/E2 (black bars). B-LCL without peptide were tested as negative controls (open bars). Results are expressed as percentage specific lysis (average of triplicates \pm 1 sd). Incubation with 20-mer peptides was as described in legend to Fig. 1. The 9- and 10-mer peptides were added to ⁵¹Cr-labelled target cells at a final concentration of 1 μ M 1 h prior to the addition of effector cells.

matched target cells (Fig. 2b). In contrast, peptide-pulsed B-LCL matched for HLA-A3, -B18 or -B51 alone were not lysed (Fig. 2b). These data indicate that ETINEEAAEW is recognized in the context of HLA-A25.

CTL epitope p24₂₀₃₋₂₁₂ is restricted to HIV-1 clades B and D

Amino acid sequence p24₂₀₃₋₂₁₂ ETINEEAAEW is highly conserved amongst HIV-1 clade B and D sequences (Myers *et al.*, 1994) (Fig. 3). The corresponding regions of the consensus sequences of the other HIV-1 clades differ from those in clade B and D only by an aspartic acid at position p24₂₀₃. Similarly, the corresponding HIV-2_{ROD} sequence differs only by an isoleucine at position p24₂₀₄. The homologous region of SIV_{MM32H} differs at three positions from the HIV-1 B and D

consensus sequences: aspartic acid residues at positions p24₂₀₃ and p24₂₁₁, and isoleucine at position p24₂₀₄. Autologous target cells pulsed with variant 10-mer peptides corresponding to these regions were used in a CTL assay with ETINEEAAEW-specific CD8⁺ CTL clones from p206 as effector cells. None of these variant peptide sequences were recognized (Fig. 4). Furthermore, rVV-infected target cells expressing Gag of clade B strain HIV-1_{LAI} were lysed, whereas target cells expressing HIV-2_{ROD} Gag or SIV_{MM32H} Gag were not (Fig. 4).

No escape variants detectable in PBMC of p206

Since Gag sequences containing either aspartic acid at position p24₂₀₃ or isoleucine at position p24₂₀₄ were not recognized by ETINEEAAEW-specific CTL from p206, but do occur in related lentiviruses, it was speculated that virus escape

Clade	No. of isolates	Amino acid sequence
A-H consensus		DTINEEAAEW
A (31) ¹	22	-----
	1	----G----
	4	E-----
	1	N-----
	2	-----D-
	1	-A-----G-
B (26)	25	E-----
	1	-----
C (6)	6	-----
D (10)	8	E-----
	1	E---D----
	1	E--I-----
F (4)	4	-----
G (3)	3	-----
H (2)	2	-----
HIV-2 _{ROD}		-I-----
SIV _{MM32H}		-I-----D-

Fig. 3. Sequence variability in the minimum epitope p24₂₀₃₋₂₁₂ in viruses from HIV-1 clades A to H, HIV-2_{ROD} and SIV_{MM32H}. Numbers in parentheses are the number of sequenced isolates within each clade. Figure adapted from Myers *et al.* (1994).

mutations at these positions could occur in p206. Virus isolation procedures carried out with PBMC collected after the baseline visit were successful at 84 and 90 months of follow-up (Klein *et al.*, 1995). Twelve and seven biological clones were generated from these two time-points, respectively. Sequence analyses of a 30 aa region containing the epitope of each of the

biologically cloned viruses revealed conservation of the original clade B and D consensus sequence in each of the 19 virus clones tested (Fig. 5). In contrast, the variable regions of gp120, V1, V2 or V3 proved to show aa variability when mutually compared (Fig. 5), showing that the 19 virus clones analysed originated from different viruses.

Discussion

Fine-specificities of HIV-1 Gag-specific CTL were studied in seven seropositive LTA individuals with stable CD4 counts for more than 8 years. Using PBMC from five of them and a panel of overlapping peptides spanning the entire Gag of HIV-1, at least five peptides were identified as areas containing CTL epitopes. These peptides overlap with sequences which have previously been shown to contain CTL epitopes. One of these, p17_{21-35'}, overlaps with an HLA-A3-restricted epitope, p17₁₈₋₃₁ (Jassoy *et al.*, 1992). Indeed, the two individuals showing p17_{21-35'}-specific CTL activity, H157 and H230, expressed HLA-A3. Similarly, peptides p24₁₄₃₋₁₆₂ and p24₂₆₃₋₂₈₂ overlap with regions containing HLA-B57- and HLA-B27-restricted epitopes, respectively (Johnson *et al.*, 1991; Nixon *et al.*, 1988; Buseyne *et al.*, 1993). The individuals recognizing these two peptides, H067 and H008, were positive for HLA-B57 and HLA-B27, respectively. It was indeed confirmed that peptide p24₂₆₃₋₂₈₂ was recognized by HLA-B27-restricted CD8⁺ CTL (data not shown).

No Gag peptide-specific CTL were detected in PBMC from H233 and H385. This may have been due to the absence of Gag-specific CTL, or to the presence of Gag-specific CTL that recognize epitopes which are not shared with sequences from the laboratory strains of HIV-1 used in this study. Furthermore, not all epitopes may have been generated from the 15- and 20-mer peptides. The latter explanation is in agreement with the observation that a CTL clone from H157 recognized Gag but

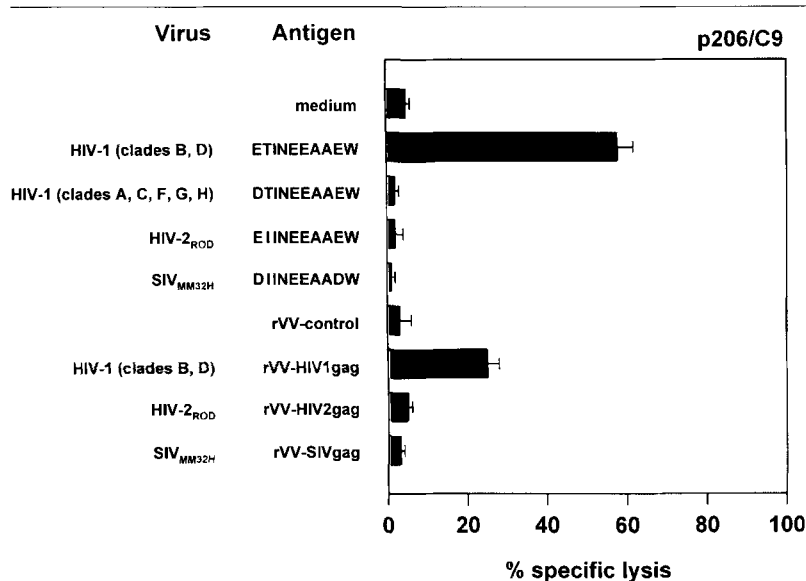


Fig. 4. CTL clones from p206 specific for p24₂₀₃₋₂₁₂ recognize the sequence from HIV-1 clades B and D, but not the corresponding sequences from related lentiviruses. The peptide sequences indicated on the vertical axis were tested for recognition in a ⁵¹Cr-release assay using as effector cells four ETINEEAAEW-specific CTL clones from p206. Target cells expressing Gag of HIV-1_{LAI}, HIV-2_{ROD} or SIV_{MM32H} from rVV-infection were also tested. Each clone exhibited a similar recognition pattern and the result of a representative clone, p206/C9, is shown. Peptides were added at a final concentration of 1 μM 1 h prior to the addition of effector cells. Bars represent the percentage specific lysis (average of triplicate measurements ± 1 SD).

Virus clone	p24 (193-222)	SP120-V1	SP120-V2	SP120-V3
Consensus B	GEQAARQLK ETINEEAAEW DLRVRVRAQ	CVTLNCTD...NAT...NTT...NST.....MEKGEIKN	CSFNITTSIRKQVQREYALFKLDVVPID.NDWTLS.....YLLIISC	CTSPNNITRESHIGFGRAFYATGDIIDGIRQAH
17-06-92 /01/
17-06-92 /02/
17-06-92 /03/
17-06-92 /04/
17-06-92 /06/
17-06-92 /07/
17-06-92 /08/
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07-04-93 /19/

Fig. 5. Sequences of the p24₁₉₃₋₂₂₂ region and the gp120 V1, V2 and V3 regions of biological virus clones isolated at 84 and 90 months of follow-up compared with the consensus sequence of HIV-1 clade B. Dashes represent aa identity with the clade B consensus. Dots represent gaps. (*) Sequence not determined. The minimal HLA-A25-restricted epitope is underlined.

not the peptide p17₂₁₋₃₅, which was dominantly recognized by the parental T cell line.

A novel CTL epitope was minimally defined to the 10 aa sequence p24₂₀₃₋₂₁₂ ETINEEAAEW, and proved to be recognized by CD8⁺ CTL in the context of HLA-A25. Target cells expressing Gag from rVV infection were also lysed by CD8⁺ ETINEEAAEW-specific CTL clones, showing that the epitope can be generated from endogenously synthesized Gag. To our knowledge, this is the first record of an HLA-A25-restricted epitope.

Corresponding sequences from closely related lentiviruses, HIV-1 strains of clades A, C, F, G and H, HIV-2_{ROD} and SIV_{MM32H} were not recognized by p24₂₀₃₋₂₁₂-specific CTL clones from p206. A change from aa p24₂₀₃E to D and from aa p24₂₀₄T to I were each sufficient to abolish recognition, indicating that residues p24₂₀₃E and p24₂₀₄T are essential for functional presentation. Loss of recognition may indicate that the variant peptides have a lower HLA-A25 binding affinity or that complexes of HLA-A25 and the variant peptides can no longer interact with the T cell receptor of the CTL recognizing the index peptide. The observation that *in vitro* recognition of the epitope was abrogated by single mutations at positions p24₂₀₃ or p24₂₀₄ to residues that occur in replicating viruses prompted us to look for virus variants with mutations at these aa positions in PBMC from p206. None of the 19 different biological virus clones tested showed sequence variation in the region p24₁₉₃₋₂₂₂, indicating that the HLA-A25-restricted epitope was conserved within the virus quasispecies of p206. Although it cannot be excluded that potential negative selection conditions for escape variants may have been present during *in vivo* culture, it is most likely that they were not generated *in vivo*. This may be due to structural constraints that limit the ability of the virus to mutate in the epitope region. Consistent with this hypothesis is the observation that 35 out of 36 known clade B and D Gag sequences contain aa p24₂₀₃E and 82 out of 85 known HIV-1 Gag sequences contain aa p24₂₀₄T (Fig. 3) (Myers *et al.*, 1994), indicating that variation at these positions is limited. It may be speculated that CTL, like those specific for ETINEEAAEW, have been instrumental in limiting virus replication in p206 without possibilities for the virus to escape, thereby delaying disease progression. The CTL specific for conserved epitopes in HIV-1 Gag described here originated from long-term asymptomatic individuals. We are presently analysing the CTL responses from individuals who progress to AIDS more rapidly, to determine whether CTL responses against epitopes like those found in this study may play a role in the prolonged survival after HIV infection.

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