Recognition of Two Overlapping CTL Epitopes in HIV-1 p17 by CTL from a Long-Term Nonprogressing HIV-1-Infected Individual

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J Immunol 1998; 161:4875-4881; ;
http://www.jimmunol.org/content/161/9/4875
Recognition of Two Overlapping CTL Epitopes in HIV-1 p17 by CTL from a Long-Term Nonprogressing HIV-1-Infected Individual

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HIV-1 infection has been shown to elicit strong CTL responses in some infected persons, but few data are available regarding the relationship between targeted epitopes and in vivo viral quasispecies. In this study, we examined the CTL response in a person infected for over 15 yr with a CD4 count persistently >500 cells/μl. The dominant in vivo activated CTL response was directed against two overlapping Gag CTL epitopes in an area of p17 known to be essential for viral replication. The 9-mer SLYNATVL (amino acids 77–85) was recognized in conjunction with HLA-A2, whereas the overlapping 8-mer TLVQR (amino acids 83–92) was recognized by HLA-A11-restricted CTL. Analysis of in vivo virus sequences both in PBMC and plasma revealed the existence of sequence variation in this region, which did not affect viral replication in vitro, but decreased recognition by the A11-restricted CTL response, with maintenance of the A2-restricted response. These results indicate that an essential region of the p17 protein can be simultaneously targeted by CTL through two different HLA molecules, and that immune escape from CTL recognition can occur without impairing viral replication. In addition, they demonstrate that Ag processing can allow for presentation of overlapping epitopes in the same infected cell, which can be affected quite differently by sequence variation. The Journal of Immunology, 1998, 161: 4875–4881.
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1.049 and 821/ml during the study period (14–16 yr after infection), and a CD4 count of 1.061/ml 23 mo after the first evaluation. Viral load analyses, performed as described below, ranged from 38,000 to 302,000 HIV-1 RNA copies/ml for the duration of study, and viral load was 110,000 copies/ml 7 mo later after the studies were completed. The patient was not receiving antiviral therapy.

HIV-1 quantitation

HIV-1 plasma viremia was quantitated using either quantitative competitive PCR (Amplicor HIV monitor test; Roche, Basel, Switzerland) or the branched DNA signal amplification assay (Quantiplex HIV-RNA assay; Chiron, Emeryville, CA).

Cell lines

EBV-transformed B-LCL were established and maintained as described previously (30). Allogeneic B-LCL were also obtained from American Society for Histocompatibility and Immunogenetics B cell line repository. The A11-transfected cell line T2-A11, expressing only HLA-A2 and A11, was generously provided by Dr. Masucci (Karolinska Institute, Stockholm, Sweden) (31).

HLA typing

HLA typing was performed using standard serologic techniques in the laboratory of Dr. Dean Mann at National Cancer Institute (Frederick, MD). The complete HLA type of subject 14279 is A 2,11; B 13,35; Cw 4 DR 3,11; DRW 52; DQ 27.

Recombinant vaccinia viruses

The recombinant vaccinia viruses vAbt141 expressing the full-length p55 gag protein, v228 expressing the p17 subunit, and v286 expressing the p24 subunit were kindly provided by Drs. Gail Mazzara and Dennis Panici (Thielor Biologics, Cambridge, MA) (32). Recombinant vaccinia viruses expressing the HIV-1-RT (VCF21), envelope (PE16), and the control lacZ (Vtlac) genes were provided by Dr. Bernard Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Synthetic HIV-1 peptides

Peptide p17.5 corresponding to the HIV-1 P22 sequence was synthesized by Multiple Peptide Systems (San Diego, CA) as a C-terminal amide, as described (33). Peptides p17.5 a, b, and c were synthesized by Cambridge Research Biochemicals (Cambridge, MA), and peptide p17.5c by Quality Controlled Biochemicals (Hopkington, MA), all as free acids. The sequences of the peptides are: p17.5, QTGSELRSYTVATLYCVHQ RIE (aa 69–93): p17.5a, ELRLSYNTV (aa 74–82); p17.5b, SYLNT VATL (aa 77–85); p17.5c, TLYCVHQRI (aa 84–92); and p17.5c, TLY CVHQKI (aa 84–92). Control peptides consisted of the HIV-1 (P22)-derived envelope peptide 116, IVTHSFCGEGFYCTNQLPSTW; the p24-specific peptide p24/124; the RT-derivative peptide RT50 (NPDI VIYQYMDDLLYGVSDIELGHR); or an HIV-1-RT peptide IV9, ILKPEHVGY (34). Additional peptides for fine mapping were synthesized as free acids with an automated peptide synthesizer (Applied Biosystems model 432A, Foster City, CA). Lyophilized peptides were reconstituted at 2 mg/ml in sterile distilled water with 10% dimethylsulfoxide with or without 1 mM DTT.

Generation of HIV-1-specific CTL lines by peptide stimulation

One million autologous B-LCL were incubated for 1 h with peptide at a concentration of 300 μg/ml in 200 μl R10 medium (rPMBM medium supplemented with antibiotics, HEPES, and 10% FCS). After washing with R10 medium, cells were irradiated with 60 Gy -irradiation and then cocultured in R10 without IL-2 with 10 million PBMC, which had been kept in R10 for 3 days without stimulation. On day 3, IL-2 was added to a final concentration of 20 U/ml, and increased to 100 U/ml after 1 wk.

Generation of HIV-1-specific CTL clones by cloning with CD3-specific Ab

CTL clones were isolated and maintained as already described (36), using the CD3-specific mAb 12F6 as stimulus for T cell proliferation (35). Developing clones were screened for CTL activity against autologous target cells expressing RT, Env, Gag, and Nef proteins, as well as the control β-galactosidase protein. Clones with sp. act. were then restimulated every 10 to 14 days with anti-CD3 and irradiated allogeneic PBMC.

Flow-cytometric analysis

Phenotypic analysis was performed using fluorescent probe-conjugated anti-CD8 mAb and phycoerythrin probe-conjugated anti-CD4 mAb, or similarly labeled control mAb (Coulter Electronics, Hialeah, FL), as described (33).

Cytotoxicity assay

B-LCL were either infected by recombinant vaccinia viruses or sensitized with synthetic peptides (10–100 μg/ml), as described, and tested in a 4-h chromium release assay (29). Supematant fluid was harvested and counted on an LKB ClinicGamma gamma counter (Pharmacia, Turku, Finland). Spontaneous release was less than 30% of maximum release, unless otherwise noted. For peptide titrations, chromium-labeled target cells were incubated with peptides on a 96-well plate for 1 h before adding effector cells. In some experiments, cold-target inhibition (cold:hot ratio 10:1) was used to lower background levels of presumed EBV-specific CTL.

Virus isolation and RNA preparation

Viral particles were isolated from supernatant after in vitro coculture (37) of PBMNs by centrifugation of 200 μl of supernatant for 1 h at 4°C and 22,000 rpm. The pellet was resuspended by vortexing in 0.8 ml of TRizol reagent and incubated 5 min at room temperature. Chloroform (0.2 ml) was added, followed by shaking and a second 5-min incubation at room temperature. Samples were spun for 15 min at 12,000 rpm at 4°C, and the aqueous phase was transferred to a polyallomer tube containing 10 μl of glycogen at a concentration of 2 μg/μl. After adding 0.5 ml of isopropanol, samples were incubated at room temperature for 10 min. RNA was precipitated by spinning for 10 min at 22,000 rpm at 4°C, followed by a 70% ethanol wash. Pellets were allowed to dry and then resuspended in 100 μl of H2O.

Reverse transcription and PCR amplification

A quantity amounting to 5 to 10 μl of RNA preparation was transcribed using random primers, as described (38), and Superscript reverse transcriptase (Life Technologies, St. Lawrence, MA). PCR was performed with Stratagene taq polymerase (Stratagene, La Jolla, CA) and with 40 cycles with amplification at 94°C for 30 s, annealing at 52.5°C for 30 s, and 72°C for 45 s. A 5′ primer ATCCGGAGATCCTGGAAAGGATCACCATC (position 919–935) and a 3′ primer GATCCGAGATCCTGTTAATTCCTCTATT (position 1081–1065) were used. Underlined sequences indicate BamHI tags added for cloning purposes.

Cloning and sequencing

PCR products were cloned using the TA Cloning System (Invitrogen, San Diego, CA). Clones containing the desired insert were sequenced using the M13 universal primer in the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems.

Construction of recombinant proviruses

A puC8 derivative, plin8Pr55 (39), including the complete gag/p24 region was used to generate the matrix mutations Q90E and R91Q (aa numbering according to HIV-1 LAI sequence). Site-directed mutagenesis was performed by applying the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) on the dsDNA template of plin8Pr55. The following oligonucleotides were used: Q90E (5′-C TCT TAT TGT GTG CAT GAA AGG ATA GAG ATA AAA GAC ACG AAG

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GAG GCT TTA GAC AAG ATA GAG G-3") and R91Q (5'-CTC TAT TGT GTG CAT CAA CAA ATA AAA GAC ACC AAG GAG GCT TTA GAC AAG ATA GAG G-3'). Underlined letters indicate the codon coding for the mutant clones. The presence of the desired mutations was confirmed by sequencing the complete gag reading frame by Taq cycle sequencing (Applied Biosystems, Weiterstadt, Germany). A 667-bp Giral Spe fragment of each mutant was subsequently cloned into the viral proviral HX10 DNA construct to generate pHX10Q90E and pHX10R91Q.

Cells and transfection

COS 7 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Transfections were performed by the CaPO4 procedure, as described elsewhere (40). CEM 4 cells were obtained from American Tissue Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium supplemented as described above. Cells were transfected with the mutant provirus constructs by the DEAE transfection procedure, essentially as described (41). Briefly, cells (5 x 106) were washed in 5 ml STBS (25 mM Tris/HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.7 mM CaCl2, and 0.5 mM MgCl2) and resuspended in a mixture of STBS transfection buffer containing 10 μl of sterile DEAE dextran (10 mg/ml) together with 5 μg of the viral DNA. After 30 min of incubation at 37°C, cells were washed twice in STBS, resuspended in 6 ml of complete RPMI 1640, and seeded in a 25-cm2 flask.

Monitoring of virus release and replication

COS 7 cells and cell culture supernatants containing released virus particles were harvested at day 2 and day 4 after transfection. Transfected COS 4 cells were split every 2 days at a ratio of 1:3 to maintain the cells in rapid growth. Aliquots of the supernatants were harvested at day 2 and day 4 after transfection. Transfected CEM 4 cells were split every 2 days at a ratio of 1:3 to maintain the cells in rapid growth. Aliquots of the supernatants were harvested each second day. Replicating cells were precleared by low speed centrifugation; viruses were then pelleted at 4°C with evidence of background EBV-specific lysis in the cultures tested for killing against the autologous peptide-sensitized B-LCL. Strong lysis of the p17.5-sensitized targets was observed along with the peptides p17.5a or control peptides (Fig. 2 C). Immune fluorescence analysis demonstrated a pure CD8+ phenotype of this cell line (not shown). Subsequent analysis of the HLA restriction showed that peptide p17.5b SLYNTVATL was recognized in association with HLA-A2, and thus represents a previously described A2-restricted epitope (34, 45). In contrast, peptide p17.5c was recognized in association with the laboratory strains HIV-1-MN, Env of HIV-1-MN, and the control lac.

Results

CTL activity of freshly isolated PBMC

Initial studies were performed to determine whether in vivo activated circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulating CTL could be detected in fresh PBMC from subject 14279. This subject had been infected for 14 yr, yet exhausted circulatingCTL activity.

FIGURE 1. HIV-1-specific bulk CTL activity. Fresh unstimulated PBMC from subject 14279 were tested against autologous B-LCL infected with recombinant vaccinia viruses expressing HIV-1 proteins in a 6-h chromium release assay. The E:T ratios were 100:1, 50:1, and 25:1. The recombinant vaccinia viruses expressed RT of HIV-1-MN, Gag of HIV-1-MN, Env of HIV-1-MN, and the control lac.

Having identified a CTL response to the peptide p17.5, which contains an A2-restricted CTL epitope, we next synthesized three 9-aa-long peptide truncations from peptide p17.5 fitting the proposed HLA-A2 binding motif for peptides (44) with a leucine at position 2 and a hydrophobic aa at position 9 (valine for p17.5a, leucine for p17.5b, and isoleucine for p17.5c). Using the same cell line as above, we observed specific killing of target cells sensitized with either p17.5a or p17.5c, but not of target cells sensitized with peptide p17.5a or control peptides (Fig. 2 C). Epitope specificity of the dominant CTL response

Having identified a dominant response in gag, we next determined the epitopes targeted by this response. Since an HLA-A11 allele was present (16, 43), using autologous B-LCL infected with recombinant vaccinia viruses, vigorous lysis of Gag- and Env-expressing target cells was observed (Fig. 1). The dominant target of these in vivo activated CTL was in the Gag protein, and this response was cross-reactive with the laboratory strains HIV-1-MN, HIV-1-IIB, and p17.5c.

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FIGURE 2. Recognition of two overlapping CTL epitopes. A and B, PBMC were stimulated either with peptide p17.5-pulsed autologous B-LCL (A) or with IL-2 alone (B). At day 17, expanding cells were tested at an E:T ratio of 25:1 in a 6-h chromium release assay against autologous B-LCL sensitized for 1 h with peptide p17.5 (QTVGEELRSLYNTV) (QCFLYQHLNIVQ), control peptide 9-valine (3-10), and with no peptide. C, Four days later, the p17.5-stimulated PBMC were tested in a 6-h chromium release assay at an E:T ratio of 20:1 against autologous target cells sensitized with the peptides p17.5a (QTVGEELRSLYNTV), p17.5b (QTVGEELRSLYNTV), and p17.5c (QTVGEELRSLYNTV).
the p17.5c sp. act. by restimulation of the cell line with peptide-pulsed HLA-A11 or HLA-A2-matched B-LCL, resulting in cell lines specific for the respective stimulating peptide, and loss of CTL specificity for the peptide not used in the stimulations (data not shown). Further evidence for the presence of two populations of CTL was obtained by limiting dilution cloning. Cells were stimulated at limiting dilution with the CD3-specific mAb 12F6, in the presence of irradiated allogeneic feeder cells. Applying this method, we identified a Gag-specific CTL clone, which also revealed an HLA-A11-restricted specificity against the peptide p17.5c, but not against p17.5b. The CTL activity against these two epitopes could be induced in the subject also at later time points (11 and 15 mo after the first evaluation) by in vitro stimulation of PBMC with both HIV-1 IIIB-superinfected (46) and peptide p17.5c-sensitized irradiated autologous CD4 cells (data not shown).

The HLA-A11-restricted epitope in p17 was further defined by smaller peptides. For HLA-A11, a putative HLA binding motif has been described with either M, L, F, Y, I, or A at position 2, and K or R as C-terminal anchor residues with interposition of 4 or 5 aa (47). This motif is fit by the 8-mer TLYCVHQK with a leucine at position 2 and a lysine at position 8 (Fig. 4A). The 9-mer ATLYCVHGK demonstrated higher specific lysis than the 8-mer TLYCVHQK, but the SD50 concentrations of both peptides for half-maximal lysis were similar with approximately 10 ng/ml. Together these data indicate that overlapping peptides can be processed and provided for CTL recognition in the same subject.

Analysis of autologous viral sequences

We next determined whether this targeted immune pressure was associated with any detectable sequence variation within the identified epitopes. Sequences corresponding to the CTL epitopes were amplified by PCR using plasma as well as virus culture supernatants, cloned, and sequenced. No mutations could be detected in the HLA-A2-restricted CTL epitope, either in plasma RNA or culture supernatants. Concerning the HLA-A11-restricted epitope, a

| Sequence analysis of autologous virus (aa 77–91) in subject 14279 |
|-------------------|------------------|
| **HIV-1 HXB2R**   | SLYNTVATLYCVHQK  |
| Autologous viral clones |                 |
| Culture supernatant |                  |
| 1 of 4 clones      | K                 |
| 3 of 4 clones      | Q                 |
| Plasma             |                  |
| 4 of 6 clones      | K                 |
| 2 of 6 clones      | E                 |

* Autologous viral sequence variation in PCR-amplified viral clones from plasma and virus culture supernatant. Four clones from culture supernatants and six clones from plasma were sequenced.
mixture of viral variants was found in the plasma with either a GLN or a GLU at position 91 and in the virus culture supernatant with either a LYS or a GLN at position 90 (Table I).

Effect of sequence variation in p17 CTL epitopes on recognition by specific CTL

In peptide titration experiments, the A2-restricted peptide SLYN TVATL could sensitize at concentrations as low as 1 ng/ml (Fig. 5). As sequencing of autologous viruses did not reveal any mutations in the A2-restricted epitope, we tested with synthetic peptides the influence of four different aa substitutions corresponding to the majority of viral variants, as published in the 1992 Los Alamos Database (48). All variants were recognized at high peptide concentrations, but there was up to a 3-log difference in the SD50 (concentration of peptide required for half-maximal lysis) for the various peptides. These data indicate that this CTL response has limited ability to recognize allowable variants within this region, and yet these variants did not dominate in vivo in this person.

Analysis of the viral sequences corresponding to the A11-restricted epitope demonstrated the presence of three viral variants showing either a lysine or a glutamine at position 91 and in the virus culture supernatant with either a lysine or a glutamine at position 90 (Table I).

Analysis of replication of HIV-1 variants. CEM 4 cells transfected with either HIV-HX10, the HIV-HX10Q90E mutant, or the HIV-HX10R91Q mutant were analyzed for HIV-1 p24 Ag production over time.

FIGURE 5. Recognition of sequence variation within the HLA-A2-restricted CTL epitope. p17.5b-specific cell lines from subject 14279 were tested against autologous target cells sensitized with the variant peptides at the indicated concentrations. E:T ratio was 20:1. Peptide p24/124 from HIV-1 p24 served as a control peptide.

FIGURE 6. Analysis of replication of HIV-1 variants. CEM 4 cells transfected with either HIV-HX10, the HIV-HX10Q90E mutant, or the HIV-HX10R91Q mutant were analyzed for HIV-1 p24 Ag production over time.

Discussion

In this long-term nonprogressing HIV-1-infected individual, we identified two overlapping CTL epitopes restricted by two different HLA Ags: HLA-A2 and HLA-A11. The A2-restricted CTL epitope SLYNTVATL, which is the immunodominant A*0201-restricted epitope (50), fits the HLA-A2 binding motif with leucines at the P2 and P9 anchor positions (44). As reported for other A2-restricted epitopes, an aromatic aa, tyrosine, is found at the P3 position and a valine at the P6 position (51). The characteristic anchor residues of the HLA-A11 binding motif are a leucine at position 2 and a lysine or an arginine at the C-terminal end at position P8 to P11 (52). The 8-mers TLYCVHQK/R fit this binding motif. Although the 9-mer ATLYCVHQK showed higher specific lysis at high peptide concentrations than the 8-mer TLYCVHQK, both demonstrated similar peptide concentration of 10 ng/ml for half-maximum lysis. Therefore, we would regard the 8-mer TLYCVHQK as the optimal peptide.

Peptides presented by HLA molecules are derived from the cytoplasm, where they are generated by enzymatic processing of endogenously synthesized proteins by the proteasome complex (53, 54). The precise mechanism of this processing and the involved enzymes are not yet resolved, but mutations both within and adjacent to CTL epitopes can significantly impair virus replication (49).
lysine at position 91 by the charged as glutamate, as found in the virus culture supernatant, and, even much more pronounced, the substitution of the glutamine at position 90 by the negatively charged glutamate, as found in plasma, caused a strong decrease of recognition.

Despite the presence of three viral variants in the HLA-A11 epitope, no variants were detected in the A2 epitope, although substitutions in this region have frequently been published (48, 58). This might be due to lack of selection by the immune system in this individual or to broad cross-recognition of possible variants.

Although HIV-1 is a virus with an enormous sequence variability, it is likely that there are constraints on the accumulation of mutations. By site-directed mutagenesis, it has been demonstrated that single aa mutations within these epitopes (aa 84–88) strongly inhibited or abrogated viral replication in vitro in CEM cells (49). In contrast, the CTL epitope mutations Q90E and R91Q, which we observed in subject 14279, had no negative effect on replication or cytopathic effects of HIV-1 on an HX10 background in CEM4 cells in our experiments. However, we have not studied potential influences of these mutations on replication and virulence of HIV-1 in other cell types. Interestingly, despite the high viral load, the patient preserved a normal CD4 count in the follow-up, suggesting an attenuated virulence of the patient’s autologous viral quasi species.

We could not detect aa substitutions in the HLA-A2-restricted CTL epitope, although mutations in this epitope frequently are found in the Los Alamos Database (48). In a recent study, CTL escape was found to be associated with an aa substitution at the P2 position of the A2 epitope (58). It only can be speculated whether the variation in the A11 epitope has put constraints on the variability in the A2 epitope. We hypothesize that targeting a localized area by two epitopes restricted by two different HLA molecules, as in this nonprogressing long-term seropositive person, may decrease the chances of the virus to evade the immunologic pressure by escape mutations in both epitopes without compromising its structure and function.

Based on mathematical models on antigenic oscillation and shifting immunodominance, it has been proposed that a CTL response against one or few epitopes should be more favorable than recognition of numerous epitopes (26). In this patient, we could define another unique CTL epitope in RT (59). Additional CTL clones against envelope and Nef were isolated, but not yet further analyzed. This indicates a polyclonal CTL response against at least five different epitopes in this patient. CTL clones against the two p17 epitopes could be recovered at several time points during the follow-up for more than 1 year, despite the presence of putative escape variants in the A11-restricted p17 epitope. This indicates that it is possible to maintain a polyclonal CTL response for prolonged periods of time. Whether the high viral load is due to partial escape from CTL responses remains speculative.

References


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