Longitudinal Analysis of T Cell Receptor (TCR) Gene Usage by Human Immunodeficiency Virus 1 Envelope-specific Cytotoxic T Lymphocyte Clones Reveals a Limited TCR Repertoire

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Summary

Human immunodeficiency virus 1 (HIV-1) infection is associated with a vigorous cellular immune response that allows detection of cytotoxic T lymphocyte (CTL) activity using freshly isolated peripheral blood mononuclear cells (PBMC). Although restricting class I antigens and epitopes recognized by HIV-1–specific CTL have been defined, the effector cells mediating this vigorous response have been characterized less well. Specifically, no studies have addressed the breadth and duration of response to a defined epitope. In the present study, a longitudinal analysis of T cell receptor (TCR) gene usage by CTL clones was performed in a seropositive person using TCR gene sequences as a means of tracking responses to a well-defined epitope in the glycoprotein 41 transmembrane protein. 10 CTL clones specific for this human histocompatibility leukocyte antigen–B14–restricted epitope were isolated at multiple time points over a 31-mo period. All clones were derived from a single asymptomatic HIV-1–infected individual with a vigorous response to this epitope that was detectable using unstimulated PBMC. Polymerase chain reaction amplification using $V\alpha$ and $V\beta$ family–specific primers was performed on each clone, followed by DNA sequencing of the V-D-J regions. All 10 clones utilized $V\alpha14$ and $V\beta4$ genes. Sequence analysis of the TCR revealed the first nine clones isolated to also be identical at the nucleotide level. The TCR-α junctional region sequence of the tenth clone was identical to the junctional region sequences of the other nine, but this clone utilized distinct $D\beta$ and $J\beta$ gene segments. This study provides evidence that the observed high degree of HIV-1–specific CTL activity may be due to monoclonal or oligoclonal expansion of specific effector cells, and that progeny of a particular CTL clone may persist for prolonged periods in vivo in the presence of a chronic productive viral infection. The observed limited TCR diversity against an immunodominant epitope may limit recognition of virus variants with mutations in regions interacting with the TCR, thereby facilitating immune escape.

H IV-1 is a retrovirus that causes a persistent productive infection in humans (for a review see reference 1). Despite the progressive and ultimately profound immunosuppression characteristically induced by this virus, infection is initially associated with a cellular immune response of unprecedented magnitude. The extent of this immune response is such that HLA class I–restricted, HIV-1–specific CTL activity can be detected in freshly isolated PBMC from infected persons without the need for in vitro stimulation and expansion that is required to isolate CTL in other viral infections (2–7). A potential functional role for these cells as a host defense is supported by the finding that CD8+ T lymphocytes from infected persons, with characteristics of HIV-1–specific

CTL, can inhibit virus replication in vitro (8–10). These observations support the hypothesis that HIV-1–specific CTL play an important role in the course of HIV-1 infection and contribute to the prolonged asymptomatic phase typical of this disease.

CTL recognize infected cells through a specific interaction involving the TCR and MHC–antigen complex on the target cell surface. In general, CTL of the CD8 phenotype recognize viral peptides of 8–10 amino acids in length that are processed endogenously within infected cells and presented to the TCR as a trimolecular complex involving class I molecule and β_2-microglobulin (11–13). CTL of the CD4 phenotype recognize longer peptides, often 13–17 amino acids

in length, which are processed exogenously through phago-lysosomes and presented to the CTL as a complex with a class II molecule (14, 15). The specificity of CTL for this antigen-HLA complex is imparted by the TCR, a heterodimer consisting of variable α and β chains (16, 17) that are noncovalently associated with five invariant molecules comprising the CD3 complex. In a manner analogous to Ig gene rearrangements, TCR diversity is generated by the somatic rearrangement of noncontiguous V, D, and J regions. Diversity is further increased by the addition of nongermline encoded nucleotides at junctions of these rearranged segments (N region diversity). This allows for an enormous potential repertoire of $>10^{15}$ distinct TCR (17). It is the highly variable CDR3 region that is thought to interact with antigenic peptides bound to the MHC cleft, whereas the constant proximal domains function to secure the TCR complex in the cell membrane (18-20).

Studies of a number of viral infections have attempted to define the extent of TCR diversity among CTL clones specific for a particular epitope, and thereby address the structure-function relationship of the TCR and provide insights into the host effector response to infection. The majority of these studies have been in inbred murine model systems and have analyzed TCR usage by immune effector cells at a single point in time. These murine studies have often shown limited heterogeneity in Vα and Vβ gene usage among CTL clones specific for the same epitope, but V-D-J diversity has usually been observed (21-23), and similar results were recently reported in a rhesus monkey infected with simian immunodeficiency virus (SIV) (24). The study of TCR usage against viral epitopes recognized by human CTL has been limited and largely confined to acute viral infections in which the immune response is able to effectively eradicate infection (25, 26). There have been no studies of TCR gene usage by virus-specific, class I-restricted CTL responses in humans over the course of a chronic viral infection.

The precise characterization of HIV-1-specific CTL responses in infected persons, including the definition of restricting HLA antigens and optimal CTL epitopes, now offers the unique opportunity to study TCR gene usage in a chronic human viral infection, and to determine the duration of a given effector response by using TCR sequence analysis as a means of identifying specific clonal responses. In the present study, we have examined the TCR usage by CTL clones specific for a dominant nine amino acid epitope in the transmembrane glycoprotein (gp)[1] of HIV-1 (gp41/584-592). Over a 31-mo period, multiple CTL clones were derived from an infected individual by limiting dilution directly from the peripheral blood, using a CD3-specific mAb or PHA as a stimulus for T cell proliferation. All of the HIV-1 envelope-specific CTL clones were found to utilize genes from the identical Vα and Vβ TCR gene families. The first nine clones isolated over a 27-mo period had identical TCR

sequences, even at the nucleotide level, indicating an unprecedented degree of TCR homology in the response to a viral CTL epitope, and providing evidence for a sustained clonal effector response to a CTL epitope in an ongoing chronic viral infection.

Materials and Methods

Subjects. Subject 010-115i has been previously shown to have significant envelope-specific CTL activity (27, 28). During the period of study from February 1990 to January 1993, this subject was asymptomatic with a CD4$^+$ lymphocyte count ranging from 600 to 800 cells/mm^3. This subject gave written informed consent and the study was approved by the Massachusetts General Hospital Human Studies Committee.

Cell Lines. EBV-transformed B lymphoblastoid cell lines (B-LCL) were established and maintained as described previously (2, 4) in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 20% (vol/vol) heat-inactivated FCS (Sigma Chemical Co.). RPMI 1640, used for all cell lines, was supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and Hepes (10 mM). All B-LCL were free of mycoplasma infection by standard culture techniques.

HLA Typing. HLA typing was performed by the Massachusetts General Hospital Tissue Typing Laboratory using standard serological techniques. The complete HLA type of subject 010-115i is A2,28; B14,52; Cw8; DR1,2; DQ1.

Synthetic Peptides. Synthetic HIV-1 envelope peptides 25 amino acids in length and adjacent peptides that overlapped by eight amino acids were synthesized by Multiple Peptide Systems (San Diego, CA) according to the PV22 sequence (29), as was peptide RT/648-672. Smaller peptides for fine mapping studies were synthesized by Cambridge Research Biochemicals (Wilmington, DE). The nine amino acid peptide gp41/584-592 was synthesized as a COOH-terminal acid according to the HIV-1 HXB2 sequence. The sequences of peptides containing CTL epitopes in the single letter amino acid code described in this study were as follows: gp41/584-592: ERYLKDQQL; RT/648-672:AIYLALQDSGLEVNIVTD-SQYALGI.

Isolation of HIV-1 Envelope-specific CTL Clones. CTL clones were isolated and maintained as described previously (27, 28, 30). Briefly, PBMC obtained by separation on Ficoll-sodium diatrizoate (Histopaque 1077; Sigma Chemical Co.) were incubated at 50 or 25 cells per well in 96-well plates with 200 μl of feeder cell solution containing 10^6/ml irradiated allogeneic PBMC from HIV-1-seronegative subjects in RPMI 1640 with 10% heat-inactivated FCS (R10) supplemented with 100 U/ml of human rIL-2 (Hoffman-La Roche, Nutley, NJ). After 2-3 wk, the percentage of wells exhibiting growth typically was 40-60% of wells plated at 50 cells/well, and 15-35% at 25 cells/well (27, 30, 31). As a stimulus to T cell proliferation, either the CD3-specific mAb 12F6 (32) was added at 0.1 μg/ml or PHA was added at 0.25 μg/ml (Nurex Diagnostics Inc., Atlanta, GA). After 2-3 wk, cells from wells demonstrating growth were then transferred to 24-well plates and restimulated by adding 1 ml of rIL-2-containing medium with irradiated allogeneic PBMC (10^6/ml) and anti-CD3 (0.1 μg/ml) or PHA (0.25 μg/ml). Approximately 2 wk later, clones were screened for CTL activity against autologous targets infected with recombinant vaccinia virus expressing the HIV-1 envelope glycoprotein of the BH8 isolate of HIV-1 (33). Vaccinia virus expressing other HIV-1 gene products (27) or the *Escherichia coli* β-galactosidase gene (VSC8) were used as controls. Clones exhibiting envelope-specific CTL activity were

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[1] *Abbreviations used in this paper:* B-LCL, B lymphoblastoid cell line; gp, glycoprotein; LCMV, lymphocytic choriomeningitis virus.

then restimulated every 10–14 d with anti-CD3 mAb or PHA and irradiated allogeneic PBMC. Selected CTL clones were subcloned by limiting dilution at 30, 10, 3, and 1 cell per well in the presence of irradiated feeder cells and PHA. Fine mapping of epitopes recognized by these CTL clones was performed using autologous B-LCL incubated with overlapping synthetic HIV-1 peptides as described (27, 28).

Cytotoxicity Assay. Target cells consisted of B-LCL infected with vaccinia–HIV-1 expression vectors, vaccinia control, or B-LCL incubated with 100 μg/ml of the relevant peptide for 60 min during ^{51}Cr labeling. Cytolytic activity was determined in a standard ^{51}Cr-release assay (34) using U-bottomed microtiter plates containing 10^4 targets per well. Plates were incubated in a humidified incubator at 37°C for 4 h. All assays were performed in duplicate. Supernatant fluids were then harvested and counted on a Cobra gamma counter (Packard Instrument Co., Inc., Downers Grove, IL), and percent lysis was determined from the formula: $100 \times$ [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was determined by lysis of targets in detergent (1% Triton X-100; Sigma Chemical Co.). Spontaneous release was <30% of maximal release for all reported assays.

Limiting Dilution Assays of Memory CTL. Precursor frequencies of HIV envelope–specific CTL were estimated by performing limiting dilutions on freshly isolated PBMC followed by in vitro stimulation with the CD3-specific mAb 12F6 (35). PBMC were cultured at 250–16,000 lymphocytes per well in 24 replicate wells of 96-well microtiter plates. To each well was added 2.5×10^4 γ-irradiated PBMC from an HIV-1–seronegative donor and 0.1 μg/ml of 12F6. Wells were then split and assayed for cytotoxicity on ^{51}Cr-labeled autologous B-LCL infected with a vaccinia gp160 expression vector, or incubated with synthetic envelope peptide gp41/584-592 as well as control target cells infected with VSC8, or without peptide. The fraction of nonresponding wells was the number of wells in which ^{51}Cr release did not exceed the mean plus three standard deviations of the spontaneous release of the 24 control wells over the number of assayed wells (35, 36). Activated cell frequency was estimated by the maximum likelihood method (37, 38).

RNA Isolation and cDNA Synthesis. Cells were grown for 14–16 d after restimulation and separated from cell debris by Ficoll gradient centrifugation in order to minimize RNA contamination from residual feeder cells. Total RNA was isolated from ~5 × 10^6 cells by extraction with RNAzol (Cinna/Biotecx, Laboratories International Inc., Friendswood, TX) as described (39). CTL clones isolated at different time points underwent RNA extraction at different times in order to minimize the possibility of cross contamination. Total RNA (1 μg) was converted into first strand cDNA using an oligo dT primer and avian myeloblastosis virus reverse transcriptase according to the manufacturer's specifications (Promega Corp., Madison, WI). For specimens that were also analyzed by anchored PCR, the C region oligonucleotides 3'Cα 599-578 (Table 1) and 3'Cβ 529-510 (Table 2) were used to prime cDNA synthesis.

PCR Amplification. PCR was performed with a 5' primer from each variable α (Table 1) or β (Table 2) region and a 3' primer from the respective C α or β region. Each reaction was carried out in a total volume of 50 μl containing 1 U of Amplitaq, 200 μM of each dNTP, 1.5 mM MgCl₂, and 20 pmol of each primer in buffer supplied by the manufacturer (Perkin-Elmer Cetus Instruments, Norwalk, CT). The PCR conditions consisted of 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C for 30 cycles in the case of the TCR α chain, and 25 cycles for the TCR β chain. In all experiments, two negative controls were included: a reagent control, and an aliquot of the

dissolved total RNA. The RNA control was included to ensure that amplification products were derived from cDNA and not genomic DNA carried over from the RNA extraction process. In all cases, no PCR product was visible in the negative control lanes as visualized by ethidium bromide–stained agarose gels or Southern blot analysis.

Anchored PCR was performed by a modification of the procedure of Loh et al. (40). After total RNA extraction and cDNA synthesis, cDNA was spun through a column (chromaspin 400; Clontech Laboratories, Inc., Palo Alto, CA) to remove unused primer that can interfere with the subsequent tailing reaction (41). The cDNA was then concentrated with a microcon-100 concentrator (Amicon, Inc., Beverly, MA) and incubated with 10 μl 5× terminal deoxynucleotidyltransferase (TdT) buffer, 1 mM dGTP, and 15 U TdT (GIBCO BRL, Gaithersburg, MD) in a total reaction volume of 50 μl at 37°C for 30 min. PCR was performed with an anchor 5' poly C primer (CUACUACUACUACCCCCCCCC-CCCCC) and the original 3' primer that was used to prime cDNA synthesis for 30 cycles. The first five cycles of amplification were annealed at 53°C with subsequent cycles annealed at 58°C. The denaturation step was 94°C for 1 min, the annealing step was for 1 min, and the extension was at 72°C for 1 min. PCR products were then reamplified with the 5' anchor primer and an internal "nested" 3' primer (3'Cα 470-447 in the case of the α chain and 3'Cβ 491-468 in the case of the β chain) for 25 cycles with identical conditions to the final 25 cycles of the primary amplification. After gel purification, the resulting product was cloned directly into the pAMP1 vector using the Cloneamp system (GIBCO BRL) (42). This method relies on the incorporation of dUMP residues in place of dTMP into the 5' end of each amplification primer. After amplification, the PCR products contain the dUMP containing sequence at their 5' termini. Treatment with uracil DNA glycosylase removes uracil residues from the DNA strands derived from the amplification primers. The resulting 3' protruding termini allow the fragment to anneal to complementary pAMP1 vector ends. This system eliminates the need for restriction endonuclease digestion and ligation and improves the efficiency of cloning PCR products (42). After transformation of DH5α cells, plasmid DNA was sequenced to determine the TCR gene sequences of HIV-1–specific CTL.

Characterization of the Amplified PCR Products. The PCR products were size fractionated on a 1% agarose gel after 25 or 30 cycles of PCR. After electrophoresis, the products were transferred to nylon membranes (Micron Separations Inc., Westboro, MA). Specific DNA sequences were identified by hybridization with ^{32}P-labeled Cα and Cβ probes at a concentration of 1–5 × 10^6 cpm/ml in a mixture containing 6× SSC, 5× Denhardt's solution, and 0.5% SDS. Hybridization was carried out at 65°C overnight. The membranes were washed using a high stringency protocol consisting of sequential washes with 5× SSC, 0.5% SDS at room temperature, 1× SSC, 1.0% SDS at 37°C, and 0.1% SSC, 1.0% SDS at 65°C. Specific DNA sequences were visualized by autoradiography using Kodak XAR-films.

Sequencing. PCR products were sequenced directly with Sequenase (United States Biochemical Corp., Cleveland, OH), using conditions optimized for direct sequencing of double stranded PCR products (43). Double stranded plasmid DNA was sequenced after alkaline hydrolysis (44).

Results

Recognition of a gp41 Epitope by Unstimulated PBMC. Prior analysis of the HIV-1 envelope–specific CTL response in sub-

Table 1. *TCR α Primers*

Variable region primers*

Vα1	TTGCCCTGAGAGATGCCAGAG
Vα2	GTGTTCCAGAGGGAGCCATTGCC
Vα3	GGTGAACAGTCAACAGGGAGA
Vα4	AAGACAGAAAGTCCAGTACCTTGATCCTGC
Vα5	GGCCCTGAACATTCAGGA
Vα6	GTCACTTTCTAGCCTGCTGA
Vα7	AGGAGCCATTGTCCAGATAAA
Vα8	GGAGAGAATGTGGAGCAGCATC
Vα9	ATCTCAGTGCTTGTGATAATA
Vα10	AATTCTCCGTGTCCATTCTTTGGA
Vα11	AGAAAGCAAGGACCAAGTGTT
Vα12	CAGAAGGTAACTCAAGCGCAGACT
Vα13	TGCTGTGTGAGAGGGAATACAAGTG
Vα14	GATCTCCACCTGTCTTGAATTTAG
Vα15	CAGAGTCTTTTCCTGAGTGTCCGAG
Vα16	GAGTGGGCTGAGAGCTCAGTCAGTG
Vα17	GCTTATGAGAACACTGCGT
Vα18	GCAGCTTCCCTTCCAGCAAT
Vα19	AGAACCTGACTGCCCAGGAA
Vα20	CATCTCCATGGACTCATATGA
Vα21	GACTATACTAACAGCATGT
Vα22	ATGTCAGGCAATGACAAGGGAAGC
Vα23	CAGGAGGTGACACAGATTCC
Vα24	GATCATCCTGGAGGGAAAGA
Vα25	GGTCAACAGCTGAATCAGCC
Vα26	TCAGTCCTTGATCGTCCAAG
Vα27	TCTGTTCCTGAGCATGCAGG
Vα28	TCTATCTCTGGTTGTCCACG
Vα29	TCAAGCCGTGATCCTCCGAG

Constant region primers‡

5'Cα	9–31	GAACCCTGACCCTGCCGTGTACC
5'Cα	26–49	TGTACCAGCTGAGAGACTCTAAAT
3'Cα	599–578	ATCATAAATTCGGGTAGGATCC
3'Cα	470–447	GAGGAAGGAGCGAGGGAGCACAGG

All sequences listed 5' to 3'.
* The oligonucleotides for Vα2, 3, 5-9, 11, 12, and 17-21 are according to Panzara et al. (61). The oligonucleotides for Vα23-29 were derived from published sequences (62).
‡ The 3'Cα 599-578 and 5'Cα 9-31 oligonucleotides have been described by Choi et al. (63). The numbering for the C region primers corresponds to the nucleotide position of the C region exon (64).

Table 2. *TCR β Primers*

Variable region primers*

Vβ1	AAGAGAGAGCAAAAGGAAACATTCTTGAAC
Vβ2	GCTCCAAGGCCACATACGAGCAAGGCGTCG
Vβ3	AAAATGAAAGAAAAAGGAGATATTCCTGAG
Vβ4	CTGAGGCCACATATGAGAGTGGATTTGTCA
Vβ5	CAGAGAAACAAAGGAAACTTCCCTGGTCGA
Vβ5b	TTCCCTAACTATAGCTCTGAGCTG
Vβ6	GGGTGCGGCAGATGACTCAGGGCTGCCCAA
Vβ7	ATAAATGAAAGTGTGCCAAGTCGCTTCTCA
Vβ8	AACGTTCCGATAGATGATTCAGGGATGCCC
Vβ9	CATTATAAATGAAACAGTTCCAAATCGCTT
Vβ10	CTTATTCAGAAAGCAGAAATAATCAATGAG
Vβ11	TCCACAGAGAAGGGAGATCTTTCCTCTGAG
Vβ12	CTGAGATGTCACCAGACTGAGAACCACCGC
Vβ13	CAAGGAGAAGTCCCCAAT
Vβ14	GTGACTGATAAGGGAGATGTTCCTGAAGGG
Vβ15	GATATAAACAAAGGAGAGATCTCTGATGGA
Vβ16	CATGATAATCTTTATCGACGTGTTATGGGA
Vβ17	GCACAAGAAGCGATTCTCATCTCAATGCCC
Vβ18	CATCTGTCTTCTGGGGGCAGGTCTCTCAAA
Vβ19	ATAGCTGAAGGGTACAGCGTCTCTCGGGAG
Vβ20	TCTAATATTCATCAATGGCCAGCGACCCT
Vβ21	GCAGTAGACGATTCACAGTT
Vβ22	ATGCAGAGCGATAAAGGAAG
Vβ23	ATCTCAGAGAAGTCTGAAAT
Vβ24	GATTTTAACAATGAAGCAGA

Constant region primers‡

5'Cβ	26–49	CCGAGGTCGCTGTGTTTGAGCCAT
3'Cβ	529–510	AATCCTTTCTCTTGACCATG
3'Cβ	491–468	CTGACCAGCAC I GCATACA I GGTG

* The oligonucleotides Vβ1-11, and Vβ13-17 have been described by Wucherpfennig et al. (65). The oligonucleotides Vβ21-24 were derived from published sequences (66, 67).
‡ The numbering for the C region primers correspond to nucleotide position of the C region exon (68). Primer 3'Cβ 491-468 has inosine residues to allow amplification of Cβ1 and Cβ2 sequences.

ject 010-115i showed that fresh unstimulated PBMC were able to lyse autologous target cells expressing the HIV-1 envelope protein (27). In addition, characterization of CTL clones obtained by limiting dilution after in vitro stimulation with

a CD3-specific mAb indicated that a nine amino acid epitope in the transmembrane protein gp41 (gp41/584-592) was the target recognized by these CTL clones (28). Recognition of this nonamer occurs at concentrations as low as 1 ng/ml (data not shown). To determine the relative magnitude of the envelope-specific response directed at this gp41 epitope, unstimulated PBMC from subject 010-115i were tested directly for recognition of autologous target cells sensitized with this epitope. PBMC were isolated after Ficoll-sodium diatrizoate separation and incubated either with vaccinia-infected autologous B-LCL expressing gp160, or incubated with HIV-1

peptides. Lysis of gp41/584-592-sensitized B-LCL using un-stimulated PBMC was as high as 53% at an E/T ratio of 100:1 (Fig. 1). The magnitude of the response to the gp41 epitope was similar to that observed with the vaccinia-expressed gp160, suggesting that this epitope was the dominant target of the activated envelope-specific CTL response in this person.

Precursor Frequency Analysis of gp41/584-592-specific CTL. To further quantify the CTL response directed at the gp41 epitope, a formal precursor frequency analysis was performed as described (35). Varying concentrations of cells were stimulated in vitro with a CD3-specific mAb as a polyclonal stimulus for T cell proliferation, and tested for lysis of autologous B-LCL either infected with the recombinant vaccinia–HIV-1 enve-lope vector or sensitized with HIV-1 peptides. As shown in Fig. 2, both envelope-expressing B-LCL as well as B-LCL sensitized with peptide gp41/584-592 were recognized, whereas the control target cells were not. The single hit Poisson model was fulfilled for all assays as demonstrated by the χ^2 value for goodness of fit (45). The calculated precursor fre-quency of CTL directed at the gp41 peptide was 1/1016 (95% confidence interval 1/678–1/1,523). This value was consis-tent with the value of 1/2,041 (95% confidence interval 1/1,290–1/3,229) obtained using the vaccinia vector expressing the entire envelope protein, indicating that gp41/584-592 is the dominant if not the only detectable envelope CTL epi-tope recognized by this individual using target cells expressing the HIV-1 IIIB strain of HIV-1.

Isolation of Envelope-specific CTL Clones. Having demon-strated a dominant CTL response to a single epitope in gp41 using both unstimulated PBMC and CTL clones, we wished to determine the TCR repertoire as well as the longevity of effector cells specific for this epitope. Limiting dilution cloning was performed at multiple time points over a 31-mo period using a CD3-specific mAb or PHA as a polyclonal stimulus to proliferation, and clones were screened for recognition of vaccinia-expressed envelope protein. During this period, 10
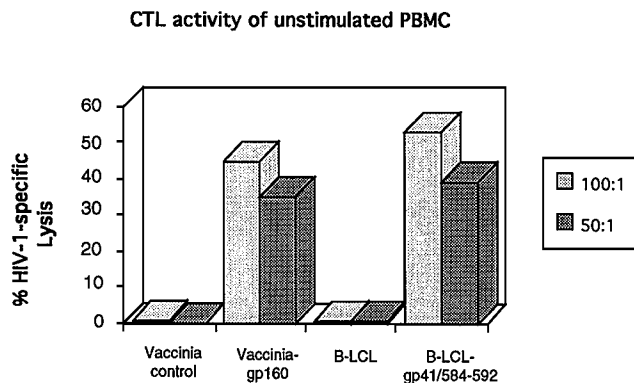
HLA class I-restricted, envelope-specific CTL clones were analyzed, all of which were shown to be HLA-B14 restricted and all of which were specific for the epitope gp41/584-592 (Table 3). Results were similar whether PHA or anti-CD3 were used as the stimulus for T cell proliferation, indicating that the results were not dependent upon the method of poly-clonal stimulation. Despite screening more than 1,000 clones over the period of this study, no clones with specificities for other envelope epitopes were identified. The percentage of screened clones that were specific for this epitope was 0.5–1% (data not shown). As the limiting dilution cloning of HIV-1-specific CTL from this patient has been reproducible using 10–25 PBMC per well, this would correspond to a calcu-lated frequency of gp41-specific CTL clones of ~0.1% of seeded PBMC, which is similar to the value derived from formal precursor frequency analysis. These data again indi-cate that the envelope-specific response in this person is nar-rowly directed, and that the gp41 epitope remains the major detectable target for the envelope-specific CTL response in this patient.

CTL Clones Specific for gp41/584-592 in Subject 010-115i Utilize TCR Vα14 and Vβ4 Genes. Envelope-specific CTL clones recognizing gp41/584-592 were isolated from PBMC obtained in February of 1990, August and September of 1991, and April and September of 1992. To further ensure clonality, representative clones from different time points were subcloned at limiting dilution. RNA was extracted from active clones, and reverse transcription was performed using an oligo dT primer. TCR gene utilization was determined by PCR of cDNA using specific TCR gene V region primers followed
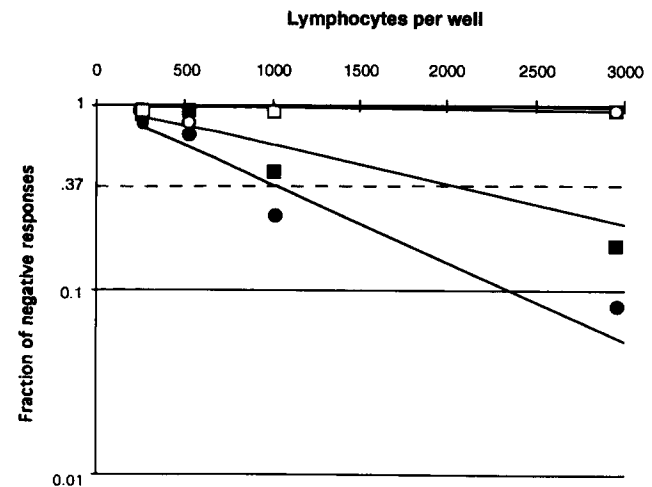

Figure 2. Quantitation of envelope specific CTL. Limiting dilution anal-ysis was performed on freshly isolated PBMC from HIV-1-infected sub-ject 010-115i. Targets were (□) autologous B-LCL infected with control vaccinia (VSC8), (■) autologous B-LCL infected with gp160-expressing vaccinia (VPE16), (○) autologous B-LCL alone, or (●) autologous B-LCL incubated with peptide gp41/584-192. Regression lines were calculated by the method of maximum likelihood (37, 38). Data are shown for PBMC obtained in January 1993. A similar precursor frequency of CTL specific for gp41/584-592 was obtained using cryopreserved cells from September 1991 (data not shown).

Figure 1. Lysis of autologous B-LCL infected with vaccinia expressing gp160 or incubated with peptide gp41/584-592. Fresh PBMC from sub-ject 010-115i were isolated over a Ficoll gradient and incubated with 10^4 target cells at E/T ratios of 100:1 and 50:1. Target cells were B-LCL in-fected with a control vaccinia virus, a vaccinia-gp160 vector, or incubated with peptide gp41/584-592 or no peptide. Lysis of target cells incubated with irrelevant peptide was always <5%.

Table 3. *TCR Vα and Vβ Gene Usage of HLA-B14–restricted HIV-1–specific CTL Clones*

| Clone | Isolation date | Percent HIV-1 specific lysis* | | | Epitope specificity | TCR Vα | TCR Vβ |
		Autologous	HLA B14 match	HLA B14 mismatch			
115 H10‡	2/90	53	53	0	gp 41/584-592	Vα14	Vβ4
115 N10‡	2/90	50	17	0	gp 41/584-592	Vα14	Vβ4
115 J7‡	2/90	53	51	0	gp 41/584-592	Vα14	Vβ4
115 G1	8/91	88	85	1	gp 41/584-592	Vα14	Vβ4
115 I26	9/91	94	99	9	gp 41/584-592	Vα14	Vβ4
115 K4	9/91	99	99	12	gp 41/584-592	Vα14	Vβ4
115 M19	9/91	99	99	9	gp 41/584-592	Vα14	Vβ4
115 M21	9/91	99	99	9	gp 41/584-592	Vα14	Vβ4
115 A7	5/92	76	52	0	gp 41/584-592	Vα14	Vβ4
115 E15	9/92	64	64	2	gp 41/584-592	Vα14	Vβ4
115 D7	5/92	85	74	1	RT/648-672	Vα21	Vβ14

* Target cells consist of B-LCL incubated with synthetic peptide. E/T ratios are 10:1. Spontaneous lysis of target cells was always <30%.
‡ The epitope specificity and HLA restriction of clones 115 H10, 115 N10, and 115 J7 have been previously reported (28).

by Southern blot and hybridization with radiolabeled probe. In all cases, a single dominant Vα and Vβ band was visualized by ethidium bromide–stained gels and Southern blot analysis. A representative Southern blot is shown in Fig. 3. All envelope-specific CTL analyzed from this patient utilize the Vα14 and Vβ4 TCR genes (Table 3).

To further assess the similarity among the envelope-specific clones, the α and β TCR genes were sequenced. PCR amplified products were isolated and sequenced directly in order to minimize the effects of Taq-generated errors. The TCR Vα-Jα and Vβ-Dβ-Jβ sequences of the first nine clones isolated were identical at the nucleotide level, suggesting that these clones, which were obtained over a 27-mo period, were all progeny of a single progenitor clone (Fig. 4). The TCR Vα-Jα region sequence of a clone isolated at the final time

point, 115 E15, was also identical to the nine clones previously isolated. Although this clone shared Vα14 and Vβ4 gene usage with the other clones, sequencing of the β chain revealed distinct Dβ and Jβ gene segments. To confirm identical Vα-Jα usage and distinct Dβ-Jβ usage by clone 115 E15, additional studies were performed. cDNA from both the parent clone and a subclone was subjected to PCR amplification using the Vα14 primer, followed by direct sequencing. RNA was extracted from the clone and subclone at separate times, and cDNA synthesis was performed separately as well. PCR amplifications of the different cDNA preparations were not performed concurrently with previously isolated envelope-specific CTL clones. Results were identical for both clones, confirming the presence of Vα14-Jα sequences identical to the nine clones previously isolated. As in all other experi-
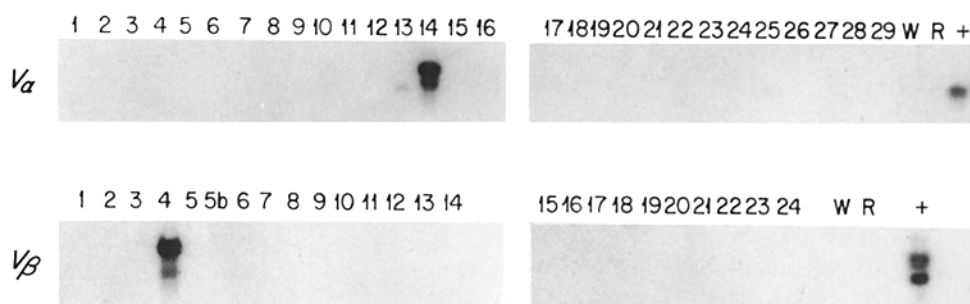

Figure 3. Representative Southern blot of a gp41/584-592–specific CTL clone using family-specific primers for the Vα and Vβ regions. The PCR products generated from clone 115M21 were separated on a 1% agarose gel and blotted onto nylon membranes. Southern blot analysis was performed with a random labeled C region fragment. (W) Reagent control, (R) RNA control. (A) Vα1-29, probed with Cα fragment generated from primers 5'Cα 26-49 and 3'Cα 470-477. Positive control is Cα region amplified with 5'Cα 9-31 and 3'Cα 599-578 primers. The faint lower molecular weight band seen in lane Vα13 was sequenced and found to be a fragment of C region DNA without a Vα13 sequence. (B) Vβ1-24, probed with Cβ fragment generated from primers 5'Cβ 26-49 and 3'Cβ 491-468. Positive control is Cβ region amplified with 5'Cβ 26-49 and 3'Cβ 529-510 primers.

A

Vα14

A	A	M	Y	F	C	A
gcc	gcg	atg	tat	ttc	tgt	gct

N

Y	R	T
tat	agg	aca

Jα 9.3

S	G	G	S	N	Y	K	L	T	F	G	K	G	T	L	L	T	V	N	P
agt	gga	ggt	agc	aac	tat	aaa	ctg	aca	ttt	gga	aaa	gga	act	ctc	tta	acc	gtg	aat	cca

Cα

N	I	Q
aat	atc	cag

```
115 H10   (sequences above)
115 N10   --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 J7    --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 G1    --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 I26   --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 K4    --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 M19   --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 M21   --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 A7    --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
115 E15   --- --- --- --- --- --- ---    --- --- ---    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---    --- --- ---
```

B

Vβ4

I	Y	L	C	S
ata	tat	ctc	tgc	agc

N - Dβ - N

V	K	D		G	A
gtg	aag	gac		ggg	gct

Jβ

G	Y	T	F	G	S	G	T	R	L	T	V	V	Jβ1.2
ggc	tac	acc	ttc	ggt	tcg	ggg	acc	agg	tta	acc	gtt	gta	

Cβ

E	D	L
gag	gac	ctg

```
115 H10   (sequences above)                                                                          Jβ1.2
115 N10   --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 J7    --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 G1    --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 I26   --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 K4    --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 M19   --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 M21   --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
115 A7    --- --- --- --- ---    --- --- ---    --- ---    --- --- --- --- --- --- --- --- --- --- --- --- ---          --- --- ---
```

V	E	D	W	G	G	A	T		S	Y	N	E	Q	F	F	G	P	G	T	R	L	T	V	L	Jβ2.1
gtt	gaa	gat	tgg	ggc	gga	gca	acc		tcc	tac	aat	gag	cag	ttc	ttc	ggg	cca	ggg	aca	cgg	ctc	acc	gtg	cta	

```
115 E15   --- --- --- --- ---    (sequences above)                                                                     --- --- ---
```

Figure 4. (A) TCR sequences of Vα-Jα region of CTL clones from subject 010-115i. The nomenclature of the Jα segment is according to Moss et al. (69). (B) TCR Vβ-Dβ-Jβ sequences of CTL clones from subject 010-115i. Non germline insertions of α and β TCR sequences are underlined. These sequence data are available from EMBL/Genbank/DDBJ under accession numbers Z29579, Z29580, Z29581, Z29582, and Z29614.

ments, reagent controls were negative, indicating that template contamination of the Vα14 primer had not occurred. In addition, attempts to amplify cDNA from clones isolated from other individuals with the Vα14 primer were unsuccessful, again ruling out contamination of reagents.

Further analysis of Vα and Vβ usage by clone 115 E15 was also undertaken. Anchored PCR and sequencing of a subclone of 115 E15, as well as a subclone of a representative clone from an earlier time point, 115 M21, was performed in order to determine whether Vα14 and Vβ4 usage by these clones might represent different subfamilies. Results revealed the use of identical Vβ4 genes. Each subclone had only one TCR β chain rearrangement and the sequences of the Dβ and Jβ regions were identical to those derived from direct sequencing using the family-specific Vβ4 primer. Anchored PCR of the α sequences of these two clones confirmed the Vα-Jα sequences derived using the family-specific primers. However, different subfamilies of Vα14 were utilized by each clone, Vα14.2 in the case of CTL clone 115 M21 and Vα14.1 in the case of CTL clone 115 E15 (data not shown).

Sequence comparison between the D region of 115 E15

and the previously isolated clones reveal four shared amino acid residues (Fig. 4). In addition, this clone from the September 1992 time point utilizes the Jβ 2.1 gene instead of the Jβ 1.2 gene common to the other nine clones. These data suggest that the earlier CTL response to the dominant gp41 epitope was mediated predominantly by clonal expansion of a single effector cell population that persisted over a prolonged period of time in the setting of a persistent infection. The finding of a distinct clone with identical epitope specificity at a later time point with an identical TCR Vα-Jα sequence and shared Vβ4 usage implies selection for certain TCR elements in the envelope-specific response of this patient.

Vα14 and Vβ4 Usage Is Not a Feature of all HLA-B14–restricted Clones. Although the use of Vα14 and Vβ4 was a common feature of all HLA-B14–restricted envelope-specific CTL clones from subject 010-115i, the use of these genes was not a feature of other HLA-B14–restricted clones. An HLA-B14–restricted CTL clone from this subject that was specific for a reverse transcriptase (RT) epitope utilized the Vα21 and Vβ14 genes (Table 3). In addition, preliminary data generated from a CTL clone from a second subject that is also

specific for the gp41/584-592 epitope and restricted by HLA-B14 indicates that the TCR utilizes the $V\alpha22$ and $V\beta1$ genes (data not shown). These data indicate that structural constraints imposed by the HLA-B14 molecule or the HLA-B14–peptide complex do not limit recognition to CTL expressing $V\alpha14$ and $V\beta4$ genes.

Discussion

In this study we have investigated the CTL response to a defined immunodominant HIV-1 epitope, using TCR analysis as a means of identifying and following clonal CTL responses. To characterize the breadth and duration of a response that was of sufficient magnitude to be detected using freshly isolated PBMC, this study was performed on an asymptomatic seropositive person with a vigorous CTL response to a nine amino acid epitope in the transmembrane protein gp41. This HIV-1 epitope is contained in a region that has been shown to be a target for both class I (28, 46)– and class II–restricted CTL (47), and also contains a major B cell epitope (48). Two different methods were used to obtain clones in order to protect against possible selection bias. Both anti-CD3 mAb and PHA as stimuli for T cell proliferation yielded clones directed against this epitope. The high frequency with which these clones were obtained by limiting dilution was similar to the calculated frequency of these cells in bulk PBMC, providing supportive evidence that these clones are reflective of the circulating CTL population. Our data indicate a striking homogeneity in this response in that all clones isolated over the first 27 mo of study utilized $V\alpha14$ and $V\beta4$ genes, and were sequence identical. This indicates that the detectable response during the initial period of study was mediated by a clonal expansion of a single progenitor cell. A single gp41-specific clone isolated at the 31-mo time point also utilized $V\alpha14$ and $V\beta4$, with an identical $V\alpha$-$J\alpha$ sequence, but with sequence changes occurring in the D-J segments of the β chain, suggesting structural constraints on epitope recognition.

The analysis of CTL in animal models of viral infections has shown limited TCR heterogeneity among CTL specific for defined epitopes, but the degree of homogeneity observed in our study, as well as the durability of the response, has not been previously reported. Each of the previous studies has had important distinctions compared with our study. In the murine lymphocytic choriomeningitis virus (LCMV) model, recognition of a dominant H-2D^b–restricted envelope epitope, GP-2, was shown to be mediated by CTL that utilized the $V\alpha4$ and $V\beta10$ gene segments. Southern blot analysis indicated that each clone had a distinct TCR rearrangement. However, in that study, the DNA sequencing was performed on a single clone so the degree of similarity between the D and J regions of the α and β chains could not be determined (21). Another study analyzed CTL clones specific for this same epitope and although four of four clones utilized $V\alpha4$, three different $V\beta$ genes were utilized. No patterns were evident in the $V\alpha$-$J\alpha$ regions or the $V\beta$-$D\beta$-$J\beta$ regions in these clones (22). The restricted V gene usage was not dependent on MHC restriction in this system as CTL clones specific for an H-2D^b–restricted GP-1 epitope did not

share $V\alpha4$ gene segment usage (23). In a primate model of SIV infection, Chen et al. (24) have described limited TCR $V\beta$ and $J\beta$ gene usage by SIV gag-specific CTL. In that study, six CTL clones with identical epitope specificity and MHC class I restriction were isolated at a single time point from a single monkey. Five out of six of these clones utilized the $V\beta23$ gene segment and four out of six utilized the $J\beta$ 1.2 gene segment. Two pairs of clones differed in the $D\beta$ region by only one amino acid but despite this similarity, all α and β chains were distinct, indicating a polyclonal response to this epitope. This response was analyzed at only a single point in time, therefore this study could not determine whether this pattern of TCR gene usage remained stable over the course of the disease.

The analysis of TCR gene usage by human virus–specific class I–restricted CTL to date has been limited to influenza virus infection. In a study of HLA-A2–restricted CTL specific for an influenza matrix epitope, PBMC were stimulated with influenza virus and antigenic peptide and subcloned on autologous B-LCL pulsed with antigenic peptide (25). That study demonstrated shared $V\alpha10$ and $V\beta17$ gene usage among three CTL lines from two subjects. Two of the influenza-specific CTL lines from one patient had identical TCR α chains at the nucleotide level and shared $V\beta$ gene usage, yet had distinct $D\beta$ and $J\beta$ gene segments, similar to our finding of an invariant TCR $V\alpha$-$J\alpha$ sequence among gp41/584-592–specific CTL clones from subject 010-115i. This phenomenon of invariant α or β TCR chain usage has also previously been described in murine T cell clones specific for 2,4,6-TNP (49), pigeon cytochrome c (50), Ig $\lambda2$ L chain (51), λ repressor (52), and myoglobin peptides (53). Conserved TCR gene usage among HLA-B27–restricted influenza A virus–specific CTL clones has recently been described as well (26). Whereas the findings of limited TCR usage by a CTL response in an individual were similar, the protocols for the generation of CTL were different. We cloned T cells directly from peripheral blood and did not rely on in vitro stimulation with viral antigen in hopes of obtaining a more representative sample of circulating CTL. We also avoided the potential problem of generating primary in vitro CTL responses (54). Because of the chronic nature of HIV-1 infection, we were able to follow this TCR usage over an extended period of time and we provide evidence that in certain instances the high level of CTL activity may be due to clonal expansions of specific CTL.

In addition to demonstrating a homogeneous response to a viral CTL epitope, our study also provides important information regarding the duration of a specific clonal CTL response. The persistence of activated HIV-1–specific human CTL has not previously been evaluated. A previous study assessing the longevity of virus-specific CTL has involved the adoptive transfer of LCMV-specific CTL in the murine model. In these studies it was shown that donor CD8$^+$ CTL could be recovered up to 9 mo after transfer and still maintain their activity against LCMV in vitro and in vivo (55). These CTL persist despite the absence of antigen in the recipient mice. Our study has allowed us to evaluate the persistence of CTL in a chronic viral infection. The ability of bulk PBMC to

recognize target cells sensitized with gp41/584-592 indicates ongoing in vivo activation of these CTL.

The homogeneous TCR response to an immunodominant epitope may be significant for a number of reasons. It has been suggested that clonal expansion followed by clonal exhaustion due to the continuous stimulation of effector cells may be involved in the diminution of specific CTL responses, as recently demonstrated in a murine model of persistent viral infection (56). Our data provide support for the hypothesis that continuous in vivo stimulation by a chronic viral infection can result in clonal expansion of effector cells. Continued longitudinal study of the TCR repertoire of HIV-1-specific CTL will allow us to address the ultimate fate of these cells. Limited clonal responses to dominant epitopes may also facilitate immune escape, which has been postulated to play a role in disease pathogenesis (57). The homogeneous TCR response directed against this immunodominant epitope may imply that antigenic variation in this region of the virus can more readily lead to escape from immune recognition. Escape from a homogeneous CTL response has been described in LCMV-infected mice transgenic for a single TCR (58), and a later study showed that LCMV-specific CTL clones can select for

escape mutants in vitro (59). Two of the envelope-specific CTL described in this report have been evaluated for their ability to recognize peptides with amino acid substitutions representing those found in naturally occurring HIV-1 isolates. Several of these substitutions resulted in partial to complete loss of recognition of this epitope (27). It has also been shown that T cells with distinct TCR, all of which recognize the same epitope, can differ in their fine specificity toward amino acid substitutions (60). It is possible that a broader TCR repertoire for specific epitopes may allow for continued recognition of virus by a subpopulation of CTL despite mutations in CTL epitopes. Studies are in progress to analyze HIV-1 sequence variation in areas of CTL epitopes to address the issue of immune escape from CTL recognition.

In summary, this study provides evidence of a persistent clonal response to a CTL epitope in a chronic viral infection and indicates that the vigorous effector response is mediated by an extremely restricted TCR repertoire. Expanded studies should facilitate the analysis of structural constraints on epitope recognition and, by tracking TCR usage by HIV-1-specific CTL, help to determine whether clonal CTL deletion is a factor in disease pathogenesis.

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