Ex Vivo Expansion of HIV Type 1-Specific Cytolytic T Cells from HIV Type 1-Seropositive Subjects

JUDY LIEBERMAN, JESSICA A. FABRY, PREMLATA SHANKAR, LAUREL BECKETT, and PAUL R. SKOLNIK

ABSTRACT

Cytotoxic T lymphocytes that specifically lyse HIV-1-infected cells occur at uncommonly high frequency in the blood of infected individuals. The CTL response is dominated by the recognition of a small number of peptides encoded by HIV-1 structural and regulatory genes. These two facts have enabled us to develop potent HIV-specific CTL lines from the blood of infected patients without AIDS opportunistic infections by ex vivo culture of nonspecifically stimulated T cell lines with autologous antigen-presenting cells (APCs) preincubated with immunodominant HIV-1 peptides. After one selection, HIV-specific cytotoxicity is enhanced 1.4- to sixfold. Frequency analysis of the T cell line from 1 patient revealed that after exposure to peptide-incubated autologous B-LCLs, the frequency of CTLs specific for the gp160-expressing APCs was enhanced 6-fold and, after a second exposure, 11-fold compared to the nonselected T cell line. Because the APCs used for the frequency analysis were EBV-transformed B-LCLs, some of the specific CTLs in the culture recognized the EBV-expressing APCs. HIV-specific cytotoxicity is enhanced without augmentation of EBV-specific cytotoxicity when PBMCs are used as APCs. Because T cell lines enhanced for HIV-1 specificity are highly cytotoxic and can be expanded to approximately 10^9–10^10 cells/ml of blood, they may be useful for laboratory research or for immunotherapy.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS-specific cytotoxic T lymphocytes (CTLs) are unusually abundant in the blood of HIV-1-infected individuals before they develop opportunistic infections. In fact, HIV-1 and HTLV-1 are the only known human pathogens in which viral-specific cytolytic activity is detectable in freshly isolated peripheral blood mononuclear cells (PBMCs) without in vitro selection. Most asymptomatic infected patients have CTLs that recognize HIV-1 env-, gag-, and nef-encoded proteins, but other HIV-1 genes, including pol, vif, rev, and tat, have also been reported to encode CTL epitopes in some patients. Walker et al. mapped T cell epitopes recognized by CTL clones derived from the blood of HIV-infected patients using autologous B cell targets in a 51Cr release assay. The targets were first infected with nested truncated vaccinia-HIV recombinants to define epitopes to approximately 100-amino acid (aa) regions. Epitopes were further refined using as targets autologous B cells incubated with overlapping peptides spanning the region identified with the vaccinia truncations. Their method can be applied to characterize the dominant CTL epitopes recognized by nonspecifically stimulated CTL lines from the blood of HIV-infected patients. Immunodominant HIV-encoded CTL epitopes can also be mapped directly using overlapping peptides, bypassing the vaccinia step. We have found, using nonspecifically stimulated T cell lines generated from the PBMCs of HIV-1-seropositive subjects, that the CTL response to HIV-1 env, pol, gag, and nef gene products is dominated by the recognition of a small number of virally encoded peptide epitopes. Because of both the frequency of viral-specific CTLs in HIV-1 infection, which is unusually high compared with other latent viral infections, and the dominance of HIV-1 CTL recognition by a small number of peptide epitopes, we have been able to generate potent CTL lines by stimulation with immunodominant CTL peptides. In this article we present and com-

1Division of Hematology-Oncology, Department of Medicine, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111.
3Division of Geographic Medicine and Infectious Diseases, Department of Medicine, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts 02111.
pare alternate methods of generating HIV-specific CTL lines. Specific cell lines can be generated either from PBMCs or from cultured T cell lines generated by nonspecific stimulation. They can be selected either by directly adding recognized peptide or by presenting peptide with various antigen-presenting cells (APCs). Although naturally occurring CTL epitopes are 8–11 aa in length, longer, 15- to 22-aa peptides used to screen for peptide epitopes can be utilized for selective CTL expansion in serum-containing media.

T cell lines expanded by peptide–APC selection are more readily generated than HIV-specific clones and can be grown to $10^9$–$10^{10}$ CTLs/ml of blood. Therefore, this method of generating HIV-specific CTLs may prove useful for laboratory research. Moreover, because viral-specific CTLs may be important in controlling viral replication and HIV-1 disease progression in vivo, they may also be useful for immunotherapy. Polyclonal T cell lines have the further advantage over clones of representing the diversity of the T cell receptor (TcR) response to a viral antigen and are thus less likely to be the target of successful evasion by viral escape mutations. T cell lines, generated according to the methods described in this article, are currently being used to treat HIV-infected patients in a pilot trial supported by the National Institute of Allergy and Infectious Diseases (NIAID) Division of AIDS Treatment Research Initiative (DATRI).

MATERIALS AND METHODS

Subjects

Subjects were HIV-1-seropositive patients in the General Medical or Infectious Diseases Clinics of the New England Medical Center (Boston, MA). They were either asymptomatic or had generalized lymphadenopathy, thrush, or oral hairy leukoplakia as their only disease manifestation. Patients with AIDS-associated opportunistic infections were excluded. Only patients for whom we were able to characterize an immunodominant peptide CTL epitope were studied. The range of CD4 counts of the eight patients discussed in this study is 280–780/mm$^3$, with an average value of 434/mm$^3$. Informed consent was obtained from each patient. This study was approved by the New England Medical Center Human Investigation Review Committee.

Cell lines

T cell lines were generated by adding phytohemagglutinin P (PHA-P, 2 µg/ml; Difco, Detroit, MI) to PBMCs obtained by Ficoll-Hypaque density centrifugation from heparinized blood. Cells were incubated at $5 \times 10^6$ in T cell medium (RPMI 1640 supplemented with 15% fetal calf serum [JRH Biosciences, Lenexa, KS], recombinant human interleukin 2 [rhIL-2, 200 U/ml; Chiron, Emeryville, CA] 2 mM glutamine, 2 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES], penicillin [100 U/ml], streptomycin [100 µg/ml], and 50 µM 2-mercaptoethanol). Twice a week the cultures were adjusted to $5 \times 10^6$/ml with fresh T cell medium. The PBMCs for some of the experiments were thawed from samples previously frozen in liquid N$_2$ in 5% dimethylsulfoxide (DMSO), 95% fetal calf serum using a programmed cell freezer (Cryomed, New Baltimore, MI). Autologous B lymphoblastoid cell lines (B-LCLs) were generated for each subject, using B95-8 marmoset cell line supernatant and standard methods.28

Vaccinia vectors

Vaccinia vectors encoding lacZ (vSC8), gp160 of the BH8 isolate of HIV-1BH (vPE16), Gag of the HXB.2 subclone (vDK1), and all but the last 22 residues of HXB.2 reverse transcriptase (RT) (vCF21) were used to screen cell lines for specific cytotoxicity against gp160, Gag, and RT-expressing targets.29–31 A set of vaccinia vectors expressing nested truncations of the same env genomic sequence was used to localize dominant env CTL epitopes (vPE17, vPE18, vPE8, vPE20, vPE21, and vPE2231,32). The HIV-1 sequences were inserted downstream from the 7.5 early-late promoter of vaccinia. The vaccinia virus preparations were titered by plaque-forming assay on CV-1 cells.33 The expression of HIV-1 proteins by B cells infected with the HIV-vaccinia constructs was verified by radioimmunoprecipitation with sera from an HIV-1-infected subject (data not shown).

Peptides

Overlapping 22-mers spanning amino acids 748–851 (amino acid numbering as in Ref. 34) of the BH8 isolate of HIV-1BH with 8-amino acid overlaps were synthesized on a Milligen 9050 synthesizer (Biosearch, Marlboro, MA). The cleaved, deprotected peptide was dialyzed through an M, 1000 cutoff membrane (Spectra/Por #6, Scientific Products, McGaw Park, IL) against 5% acetic acid and lyophilized. Overlapping 20-mers with 10-amino acid overlaps spanning amino acids 37–851 of the HXB.2 subclone of LAI or of SF2 env and of SF2 p24ag were provided by the MRC AIDS Reagent Project. Overlapping 15-mers with 5-amino acid overlaps from SF2 pl5ag and p17ag were also provided by the MRC. Overlapping 20-mers with 10-amino acid overlaps for rev-, nef-, and tat-encoded proteins were synthesized by the European Vaccine against AIDS (EVA) Programme and provided by the MRC. All of the MRC peptides were analyzed by fast atom bombardment mass spectrometry and high-performance liquid chromatography (HPLC) and found to be of expected mass and greater than 80% purity. Peptide stock solutions (1 mg/ml in 10% DMSO, phosphate-buffered saline [PBS]) were diluted in medium for cytotoxicity assays. If peptide was not soluble at 1 mg/ml, a partially dissolved peptide slurry was vortexed before being added to the medium. All of the peptides were soluble at the concentrations (1–50 µg/ml) used for sensitization of APCs.

Chromium release assay

Cytotoxicity assays were performed against B-LCL target cells that were either untreated, preincubated with relevant peptides, or infected with vaccinia constructs encoding complete or truncated HIV-1 proteins. For infection, 2–10 plaque-forming units (PFU)/cell of vaccinia virus was added to $5 \times 10^5$ exponentially growing B cells in 500 µl in a 24-well plate. The plate was incubated at 37°C over CO$_2$ with rocking for 30 min. After 16 hr the cells were harvested and labeled with $^{51}$Cr. Infected or uninfected target cells were pelleted and resuspended in 200 µl of serum-containing medium to which 200 µCi of Na$^{51}$CrO$_4$ (NEN-Du Pont, Boston, MA) was added.
EX VIVO EXPANSION OF HIV-1 SPECIFIC CTLs

After incubation for 1 hr at 37°C with occasional mixing, the targets were washed three times and resuspended at 10⁵/ml. Labeled targets (10⁴) were added to triplicate wells of U-bottom microtiter plates. For peptide experiments, the radiolabeled targets were incubated with peptide at a final concentration of 50 µg/ml for 30 min at 37°C over CO₂ and unbound peptide was not removed before adding effector cells. Effector cells were suspended at various effector-to-target (E:T) ratios in 100 µl and added to target cells and the plates incubated at 37°C over CO₂ for 4 hr. For each target, spontaneous release (SR) was determined from wells to which 100 µl of medium was added and total release (TR) calculated from wells containing 100 µl of 1% Nonidet P-40 (NP-40). Supernatants (75 µl) from each well were counted on a γ counter after addition of 100 µl of 1% NP-40. Percent specific cytotoxicity was calculated from the average counts per minute (cpm) as [(average cpm – SR)/(TR – SR)] × 100. Spontaneous release was below 20% of total release. gp160, Gag, or RT-specific cytotoxicity was defined as the difference between the percent specific cytotoxicity against gp160-, Gag-, or RT-expressing targets and that against lacZ-expressing targets. Peptide-specific cytotoxicity was defined as the difference between the percent specific cytotoxicity in the presence or absence of peptide.

HIV-1 Isolation

To isolate HIV-1 from PBMCs or cell lines, 10 x 10⁶ cells to be tested were cocultivated with PHA blasts (5 x 10⁴, obtained 3–4 days earlier from an HIV-1-seropositive donor) and Polybrene (2 µg/ml) in 10 ml of T cell medium. The PHA-transformed blasts were generated in T cell medium at 2 x 10⁷/ml with PHA-P (10 µg/ml). Culture supernatant (5 ml) was removed, clarified, and divided into aliquots and fresh medium added to the cultures biweekly. Every 7 days for the first 2 weeks, 3 x 10⁶ PHA-transformed blasts from an HIV-1-seropositive donor were added to each flask. Cultures were maintained for 28 days. An aliquot of the supernatant (1 ml) was mixed with 111 µl of 5% Triton X-100 in enzyme-linked immunosorbent assay (ELISA) buffer for HIV-1 p24 Ag ELISA (NEN-Du Pont).

Flow cytometry analysis of T cell lines and clones

T cell lines (2–10 x 10⁶) of >90% viability were harvested and resuspended in 50 µl of cold fluorescence-activated cell sorting (FACS) medium (PBS with 1% bovine serum albumin [BSA] and 0.02% sodium azide) to which was added 5 µl of Leu2a-PE and 5 µl of Leu3a + 3b-FITC (Becton Dickinson, Mountain View, CA). After incubation for 20 min at 4°C, the cells were washed with 1 ml of FACS medium, pelleted, and washed again with 500 µl of FACS medium. Before two-color flow cytometry, the cells were fixed with 1% formaldehyde in FACS medium. Known CD4⁺ and CD8⁺ clones were stained simultaneously to establish gates and gain settings for FACS analysis on an EPICS cytofluorograph (Coulter, Hialeah, FL). For CD57 staining, 5 µl of Leu-7-FITC (Becton Dickinson) was added to the cells in 50 µl of FACS medium and stained as above.

Selection of HIV-1-specific T cell lines with peptide

The PBMCs or T cell lines (approximately 3–4 weeks after initiation) were harvested and resuspended in fresh T cell medium at 10⁸/ml. Cell lines were selected with either peptide alone (by adding an equal volume of peptide-containing medium to a final concentration of 1–50 µg/ml) or peptide presented by autologous antigen-presenting cells. For the latter, APCs were irradiated (5000 rad), resuspended at 10⁵/ml, and incubated with peptide at 1–50 µg/ml at 37°C with occasional mixing. After 2 hr, the incubated cells were washed and added to the T cell line (in a ratio of T cells to APCs of 3–10:1) in an equal volume of fresh T cell medium. Twice weekly, T cells were counted and fresh medium added to maintain a cellular concentration of 5 x 10⁶/ml. After 10–12 days treated T cell lines were tested for cytotoxicity against vaccinia-infected targets.

T cell clones

CD8⁺ CTL clone JL32, which is specific for the autologous Epstein–Barr virus (EBV)-transformed cell line JLEBV, was generated from PBMCs obtained from a normal volunteer and cloned at 20 cells/well in 200 µl of T cell medium in round-bottom microtiter plates in the presence of 10⁴ irradiated autologous PBMCs and 10⁶ irradiated JLEBV cells. Biweekly the plates were fed with fresh T cell medium and emerging wells screened for cytotoxicity against JLEBV. CD8⁺ CTL clone En15-204 was cloned at 5 cells/well from PBMCs from seropositive subject 204 stimulated with vPE16-infected autologous adherent PBMCs. These clones were verified to be clonal by the presence of a single rearranged band by Southern blot of restriction enzyme-digested DNA analyzed with a TcR β constant region probe (Oncor, Gaithersburg, MD).

Cytotoxic T lymphocyte frequency analysis

Varying numbers of effector T cells were added in 100 µl of T cell medium to 24 replicate microtiter wells containing 5000 ⁵¹Cr-labeled targets. For the gp160-specific T cell lines, targets were autologous B cells infected with either vPE16 or the control lacZ-vaccinia construct vSC8. For CTL clone En15-204, the radiolabeled B-CLLs were incubated with the specific peptide (SF2 gp160 peptide aa 49–68: VPVWKEATTTLFCASDAKY) at 10 µg/ml for 2 hr at 37°C and washed once before adding to microtiter wells. The assay was incubated for 16 hr and ⁵¹Cr released into the supernatant was counted. Wells with counts that were more than 3 standard deviations above the mean count of 24 wells containing no effector cells were considered positive. To calibrate the results, a frequency analysis was performed with clone JL32 as effector cell and its specific target, the autologous B cell line JLEBV, as target cell.

Statistical analysis

A probit analysis was used to estimate the ED₅₀ (50% effector lytic dose), or number of effector cells for which half of the wells would be expected to be positive, separately for each experimental condition. The SAS procedure PROC PROBIT was used to obtain maximum likelihood estimates of the ED₅₀, to produce 95% confidence intervals and to test the goodness of fit of the model using a chi-square test. 38
RESULTS

Cell growth and characterization of unselected T cell lines

T cell lines, stimulated from PBMCs with PHA and IL-2, grow vigorously for approximately 3 weeks without further stimulation, multiplying exponentially, after an initial lag phase, with a doubling time of about 2-3 days. From PBMCs, T cells multiply approximately 10^3 to 10^4-fold (Fig. 1). After 4 weeks in culture, most of the T cells (78 ± 9%) in five lines were CD8^+. (The selective outgrowth of CD8^+ cells is not specific for HIV infection because PBMCs from seronegative donors grown under these conditions are also mostly CD8^+.) Few of the cells (6 ± 4%) were CD4^+ and an additional 13 ± 6% were CD4^+CD8^-. These double-negative cells, as well as the CD4^+ cells, could be eliminated by binding to CD4 magnetic beads (data not shown) and therefore were likely to express low levels of CD4. A large proportion of the T cells (44 ± 8%) expressed the cell surface marker CD57, which has been postulated to identify suppressor cells.12

During culture the majority of the T cell lines develop cytotoxicity against HIV-1-expressing targets. For the purposes of this article we have defined significant HIV-specific cytotoxicity when the difference between the percent specific cyto-

toxicity against vaccinia-HIV-1 recombinant and against vaccinia-lacZ-infected autologous B cell targets is greater than 10% at an E:T ratio of 25:1. The choice of 10% is somewhat arbitrary, but was chosen to be well above the experimental error of less than 5% and is used by other investigators.17 In 42 asymptomatic patients, by this criterion the T cell lines of 50% recognize gp160_A1, 52% recognize Gag, and 29% recognize RT-expressing targets (J. Lieberman, unpublished observation). Because the HIV-vaccinia vectors encode genes from viral isolates that are likely to differ from those of the subjects, some of the T cell lines may recognize viral epitopes not detected by our assay system. The extent of HIV-1-specific cytolysis varies considerably between individuals. T cell lines, assayed after 20-40 days in culture, exhibit as much as 40% gp160-, RT-, or Gag-specific lysis at an E:T ratio of 12.5:1 in a 4-hr ^51Cr release assay. The patient T cell lines also lyse the control vaccinia-lacZ-infected autologous targets owing to a combination of NK-like killing, EBV-specific lysis of EBV-transformed cell lines, and occasionally vaccinia-specific lysis (data not shown). The NK-like lysis tends to decline with time in culture whereas the HIV-specific lysis was stable.

Despite the persistence of residual CD4-expressing T cells in the T cell lines, no HIV-1 could be cultured from 10 million cells from five of five T cell lines that demonstrated significant anti-gp160 or anti-RT CTL activity (>10% specific lysis above background at an E:T ratio of 25:1). The presence of replication-competent HIV-1 was assayed by p24 antigen capture ELISA of coculture supernatants. T cell lines maintained in culture for 3 weeks were cocultured for an additional 4 weeks with seronegative PHA blasts (see Materials and Methods). The eight coculture supernatants from each of the five cultures were negative for p24 Ag. Using identical methods, we have been able to culture HIV-1 from the PBMCs of 15 of 15 asymptomatic patients.

Selection of gp160-specific cytotoxic T lymphocytes with immunodominant peptide alone or presented by autologous B-lymphoblastoid cell line antigen-presenting cells

The T cell line 132, derived from a patient with generalized lymphadenopathy and a CD4 count of 300/mm^3, recognizes both an N-terminal and a C-terminal epitope in gp160 (Fig. 2). To investigate the possibility of selectively enhancing the growth of the HIV-1-specific CTLs in the nonspecifically generated T cell lines, the 3-week T cell line from this individual was incubated either directly with the C-terminal peptide that it recognizes (amino acids 802-823: YWWNLLQY-WSQELKNSAVNLLN) or with irradiated autologous B cells preincubated with peptide and washed to remove unbound peptide. In both instances, there was substantial enhancement of HIV-1-specific cytotoxicity. We can define the epitope-specific cytolysis as the difference in percent specific cytolysis of target cells infected with a recombinant vaccinia that encodes the epitope and with a vector that is lacking the epitope. C-Terminal epitope-specific cytotoxicity can be defined as the difference in percent lysis of targets infected with vPE16 and vPE17 and N-terminal specific lysis can be defined as the difference in lysis of vPE17- and vSC8-infected targets. In the selected lines, the epitope-specific lysis for the N- and C-terminal epitopes at an

![Graph](image-url)
FIG. 2. T cell line 132 recognized an N-terminal peptide (in the overlap region of peptides 49–68 and 59–78) and a C-terminal peptide (amino acids 802–823) in gp160. (A) \(^{51}Cr\) release assay of T cell line 132 against autologous B cells infected with vaccinia virus encoding nested truncations of gp160 (vPE16, vPE17, vPE8, vPE20, and vPE22) or lacZ (vSC 8). This T cell line recognized epitopes in the regions defined by vPE16–vPE17 (amino acids 748–851) and vPE22 (amino acids 1–204); (B) \(^{51}Cr\) release assay of T cell line 132 against autologous B cells incubated with a set of overlapping peptides spanning the region of gp160 encoded by vPE22. The percent specific release against B cells without peptide was subtracted to give peptide-specific cytotoxicity. Adjacent peptides were recognized and the cell line presumably recognized the overlapping region, amino acids 59–68. E:T ratio, 25:1; (C) \(^{51}Cr\) release assay against autologous targets incubated with overlapping peptides spanning the region defined by vPE16–vPE17. T cell line 132 recognized the peptide 802–823. E:T ratio, 50:1.

E:T ratio of 2.5:1 was greater than the epitope-specific lysis of the unselected line at an E:T ratio of 20:1 (Fig. 3). Peptide concentrations of 1 \(\mu\)g/ml (350 nM) and 50 \(\mu\)g/ml (1.75 \(\mu\)M) were equally effective (data not shown). The cell lines stimulated with peptide grew less well than untreated cells, possibly because of lysis of peptide-presenting T cells. The cell lines treated with peptide-presenting cells experienced a dramatic burst of cell growth and increased eightfold above untreated cells in 11 days (Fig. 4). They also, not surprisingly, developed significant low level cytotoxicity against the APC EBV-transformed autologous B cells. The increase in cell growth after exposure to peptide-incubated B-LCLs may be due to proliferation of peptide- or EBV-specific CTLs or both. T cell line 132 also recognized two adjacent gp160 N-terminal 20-mer peptides (amino acids 49–68, VPWKEAATTTLFCASDAKAY and amino acids 59–78, LFCASDAKYDTEVHNWVAT). The T cells of this patient could either recognize a single epitope in the 10-amino acid overlap region (LFCASDAKAY) or more than one peptide epitope. Autologous B-LCL APCs incubated with peptide(49–68) also selected gp160-specific T cells from the mixed T cell line although the enhancement was not as much as for the C-terminal peptide. Antigen-presenting cells alone or with an irrelevant peptide did not select for HIV-specific cytotoxicity (Fig. 3). Comparable enhancement of

FIG. 3. Enhancement of anti-gp160 cytotoxicity of T cell line 132 following stimulation with immunodominant gp160 peptides. (A) \(^{51}Cr\) release assay of unselected T cell line 132 against autologous B cells infected with vaccinia constructs containing both the N- and C-terminal peptide epitopes (vPE 16), only the N-terminal epitope (vPE 17), or neither (vSC 8). (B) \(^{51}Cr\) release assay 11 days after stimulation with peptide(802–823), at a concentration of 50 \(\mu\)g/ml, in the absence of added APCs. (C) \(^{51}Cr\) release assay 11 days following stimulation with peptide(802–823)–APCs. Antigen-presenting cells were autologous B-LCLs preincubated with peptide (50 \(\mu\)g/ml) and washed. (D) \(^{51}Cr\) release assay 11 days following stimulation with N-terminal peptide(49–68)-pulsed B-LCL APCs. Peptide concentration was 50 \(\mu\)g/ml. (E) \(^{51}Cr\) release assay 11 days following stimulation with B-LCL APCs in the absence of added peptide.
gp160-specific cytotoxicity was obtained with peptide–APC stimulation with T cell lines from subject 120 [peptide(219–238): PIPHYCAPAGFAILKCNK], subjects 146 and 4126 [peptide(844–863): YRAIRHIPRRIRQGLERILL] (subject 146, Fig. 5), and subject 138 (Fig. 6) [peptide(788–809): IVELLGRGWEALKYWWNLQLQY]. The CTL response to gp160 in subjects 120, 138, and 4126 was dominated by the recognition of a single epitope; T cells from subject 4126 also weakly recognized an epitope in the N-terminal region encoded by vPE22.21

**Frequency analysis of selected gp160-specific T cells**

To assess the frequency of gp160-specific CTLs in nonspecifically stimulated and peptide–APC-selected T cell lines, a frequency analysis was performed with T cell line 138. This T cell line from an asymptomatic subject recognized a single gp160 epitope (amino acids 788–809) restricted by HLA B27.21 After 1 round of peptide–APC selection, the percent specific cytotoxicity of selected T cells at an E:T ratio of 2.5:1 was comparable to that of unselected T cells at an E:T ratio of 20:1. Additional enhancement of gp160-specific cytotoxicity was

**FIG. 4.** Growth of selected and unselected T cell lines derived from the PBMCs of patient 132. After 3 weeks in culture, the nonspecifically stimulated T cell line was either continued in culture without any specific stimulation (●), stimulated with the Env C-terminal peptide(802–823) without APCs at a final peptide concentration of 1 (■) or 50 (●) μg/ml, or stimulated with peptide(802–823) presented by autologous B-LCL APCs. The APCs were preincubated with peptide at a concentration of 1 (■) or 50 (●) μg/ml for 2 hr, irradiated (5000 Gy), and then washed to remove unbound peptide before adding to the T cell line. The T cell line stimulated with peptide–APCs proliferated when compared with the unselected cell line, but the cell line stimulated with peptide alone grew less well. The specific cytolyis of the unselected (●), 50-μg/ml peptide only (■), and peptide (50 μg/ml)–APC (●) T cell lines is depicted in Fig. 3A–C, respectively. All selected cell lines demonstrated greater specific cytotoxicity than the unselected line.

**FIG. 5.** Enhanced anti-gp160 cytotoxicity by T cell line 146 after stimulation with the immunodominant peptide(844–863) presented by either autologous B cell or PBMC APCs. (A) Peptide-specific lysis of the unselected T cell line is compared with that of the cell lines selected 10 days earlier with peptide presented by either B-LCLs or PBMCs as APCs. The PBMCs were more effective APCs. The peptide-specific lysis is the difference in percent specific cytotoxicity in a 4-hr 51Cr release assay against B-LCL targets incubated with specific peptide or control media. (B) Background lysis of the EBV-transformed B-LCLs is enhanced when B-LCLs are used as APCs. The percent specific cytotoxicity of B-LCLs by the unselected and selected cell lines is compared by Cr release assay.
FIG. 6. Sequential enhancement of anti-gp160 cytotoxicity of T cell line 138 by stimulation with the immunodominant peptide(788–809) presented by autologous B cell APCs. (A) ⁵¹Cr release assay of unselected T cell line 138 against gp160-expressing autologous targets (vPE 16) or control targets (vSC8). (B) ⁵¹Cr release assay 12 days after stimulation with peptide–APCs. (C) Further enhanced cytotoxicity 12 days after a second stimulation with peptide–APCs. (D) The cytotoxicity curve for the CD8⁺ CTL clone JL32, which recognizes and lyses the autologous B-LCL JLEBV, is shown for comparison. This specific clone does not demonstrate NK-like lysis of the K652 target. The frequency analysis that compares this clone with the stepwise-selected T cell lines is shown in Tables 1–3.
seen following a second round of peptide selection (Fig. 6). Not surprisingly, with EBV-transformed B cells as APCs, these selected T cell lines also developed CTLs that were specific for autologous EBV-transformed targets. The specific lysis of the control vaccinia-lacZ-infected autologous B cell targets rose from 7.7% in the unselected T cell line to 17.7% after one round of selection to 21.6% after two rounds of peptide selection at an E:T ratio of 20:1. This increase in target B cell lysis, which does not require vaccinia infection and therefore is specific for EBV-transformed targets, has been seen with peptide–APC stimulation with EBV-transformed B cells as APCs in all subjects studied.

To quantitate the frequency of CTLs that lysed gp160-expressing B-LCL cells in this T cell line, before and after peptide selection, varying numbers of effectors were added to 5 × 10^3 vPE16 or vSC8-infected radiolabeled targets and incubated for 16 hr before counting the radioactivity in the supernatant. A well was defined as positive for responders if the cpm of the supernatant from that well was greater than 3 SD above the mean of the 24 wells with no added effectors. Because this assay can only detect wells with many CTL effectors, the assumptions of limiting dilution analysis, where the presumption is that a single effector cell can be detected, are not valid (Table 1). Nonetheless, the frequency of effector cells in the T cell lines could be analyzed using probit analysis. The basic assumption in the probit model is that a given well will be positive only if the number of effector cells in it exceeds a threshold level. The threshold levels are further assumed to follow a normal distribution, with the mean number of effector cells required to produce a positive response corresponding to the ED50. Half the wells would have a threshold lower than the ED50 and half would be higher. A probit analysis was performed for each experimental condition separately using the SAS procedure PROC PROBIT to estimate the ED50. The goodness of the fit for the model was evaluated by chi-square tests.37,38 A decrease in the ED50 should correspond to a comparable increase in the frequency of CTLs that recognize and lyse the target cells.

The first round of peptide selection resulted in a 6-fold decrease in the ED50 of CTLs lytic for gp160-bearing B-LCLs in the T cell line; after 2 rounds of peptide selection, the overall ED50 decreased 11-fold (Tables 2 and 3). The ED50 for the twice-selected T cell line was statistically indistinguishable from that of T cell clone JL32 against its specific target, the autologous EBV-transformed lymphoblastoid cell line JLEBV, but was approximately fourfold greater than for CTL clone En15-204 to lyse gp160-specific peptide-incubated targets. This result suggests that a significant fraction of the cells in the culture after one or two selection cycles is specific for the peptide–APC. The selection with EBV-transformed APCs also increased the frequency of EBV-specific CTLs by a comparable amount (10-fold) as measured by the ability of this cell line to lyse the lacZ-expressing EBV-transformed B cells.

An estimate of the absolute frequency of specific CTLs in a T cell line can be obtained by dividing the ED50 obtained for specific clones by the ED50 for the cell line. In this way we can measure how many T cells in a heterogeneous T cell line are equivalent to a single cloned T cell. This estimate is only as good as the assumption that different clones will have comparable ED50 values for lysing their respective targets. To estimate the variations in ED50 from clone to clone, we did a frequency analysis for two clones and found a 3.7-fold difference in the ED50. For clone En15-204, the ED50 is 281 with 95% confidence limits of 259–303. However, more JL32 cells/well are needed to attain lysis above background with an ED50 of 1036 (963–1118). Using the data from both clones, we can estimate roughly that 13–47 unselected T cell line 138 cells are equivalent at lysing gp160-presenting B-LCL targets to 1 specific cloned T cell; after 1 round of peptide–APC selection, 2–8 T cells and after 2 exposures to peptide–APC, 1–4 T cells are equivalent to a single specific cytolytic effector.

**Table 1. Data on Lysis by Cytotoxic T Lymphocyte Clones En15-204 and JL32 of Their Specific Targets gp160 Peptide (49–68)-Incubated Autologous B Lymphoblastoid Cell Lines and Autologous B Lymphoblastoid Cell Lines Respectively**

<table>
<thead>
<tr>
<th>En15-204 cells/well</th>
<th>Positive wells</th>
<th>JL32 cells/well</th>
<th>Positive wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/24</td>
<td>0</td>
<td>0/24</td>
</tr>
<tr>
<td>94</td>
<td>0/24</td>
<td>0</td>
<td>0/24</td>
</tr>
<tr>
<td>188</td>
<td>0/23</td>
<td>14.1</td>
<td>0/24</td>
</tr>
<tr>
<td>375</td>
<td>23/23</td>
<td>28.1</td>
<td>0/24</td>
</tr>
<tr>
<td>750</td>
<td>23/23</td>
<td>56.2</td>
<td>0/24</td>
</tr>
<tr>
<td>1,500</td>
<td>24/24</td>
<td>112</td>
<td>0/24</td>
</tr>
<tr>
<td>3,000</td>
<td>23/23</td>
<td>225</td>
<td>1/24</td>
</tr>
<tr>
<td>6,000</td>
<td>24/24</td>
<td>450</td>
<td>0/24</td>
</tr>
<tr>
<td></td>
<td>750</td>
<td>8/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>2/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>13/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,250</td>
<td>14/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,800</td>
<td>24/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,600</td>
<td>24/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7,200</td>
<td>24/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14,400</td>
<td>24/24</td>
<td></td>
</tr>
</tbody>
</table>

*For En15-204 targets, the 51Cr-labeled B-LCL was incubated for 2 hr with peptide (10 μg/ml) at 37°C and unbound peptide was removed by washing. Varying numbers of T cells were added to 24-replicate wells containing 5000 51Cr-labeled targets and after 16 hr supernatants were counted. Wells with counts that were more than 3 SD above the mean count of wells with no effectors were considered positive.*

Selection with peptide presented by autologous B-lymphoblastoid cell line or peripheral blood mononuclear cell-type antigen-presenting cells

Initial experiments of peptide selection used B-LCLs as APCs. In all cases, the peptide–APC-selected cell lines developed increased background recognition of the B-LCLs, presumably due to recognition of EBV-encoded epitopes expressed by the EBV-transformed APCs. To compare the effectiveness of B-LCLs and PBMCs as APCs, T cells from subject 146, which recognized gp160 peptide(844–863), were selected with peptide presented by either autologous PBMCs or B cell line as APCs (Fig. 5). Both PBMCs and B cells were able to present antigen to the extent that the peptide-specific cytotoxicity of the selected T cell lines at an E:T ratio of 3:1 was comparable to that of the unselected line at 25:1. However, the en-
**EX VIVO EXPANSION OF HIV-1 SPECIFIC CTLS**

**Table 2. Data on Frequency Analysis of gp160-Specific Cytotoxic T Lymphocytes in T Cell Line 138, before and after Selection with Immunodominant gp160 Cytotoxic T Lymphocyte Peptide**

<table>
<thead>
<tr>
<th>Effector T cell line</th>
<th>Results for gp160-expressing targets</th>
<th>Results for lacZ-expressing targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of effector cells/well</td>
<td>Number of positive wells/24</td>
</tr>
<tr>
<td>Unselected</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>2,000</td>
<td>1</td>
<td>2,000</td>
</tr>
<tr>
<td>8,000</td>
<td>2</td>
<td>10,000</td>
</tr>
<tr>
<td>10,000</td>
<td>6</td>
<td>20,000</td>
</tr>
<tr>
<td>10,000</td>
<td>12</td>
<td>30,000</td>
</tr>
<tr>
<td>12,000</td>
<td>6</td>
<td>40,000</td>
</tr>
<tr>
<td>14,000</td>
<td>16</td>
<td>50,000</td>
</tr>
<tr>
<td>Selected once</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>2,000</td>
<td>10</td>
<td>2,000</td>
</tr>
<tr>
<td>Selected twice</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,000</td>
<td>11</td>
<td>8,000</td>
</tr>
<tr>
<td>1,500</td>
<td>19</td>
<td>10,000</td>
</tr>
<tr>
<td>2,000</td>
<td>20</td>
<td>12,000</td>
</tr>
<tr>
<td>2,500</td>
<td>24</td>
<td>14,000</td>
</tr>
</tbody>
</table>

-a Varying numbers of T cells were added to 24-replicate wells containing 5000 51Cr-labeled autologous targets infected with gp160-vaccinia (vPE16) or lacZ-vaccinia (vSC8) and after 16 hr supernatants were counted. Wells with counts that were more than 3 SD above the mean count of wells with no effectors were considered positive.

-b For this entry, there were only 23 wells.

hanced lysis of EBV-transformed autologous targets seen with B cell APCs was not observed when PBMCs were used as APCs. The peptide selection was also more effective with PBMC APCs, probably because EBV-specific CTLs were not activated for proliferation at the same time.

**Selection with multiple peptides**

Using overlapping sets of peptides that span the coding sequences of the env, gag, nef, rev, and tat genes, we have been able to map the HIV-1 peptide CTL epitopes whose recognition dominates the CTL response to these proteins in infected individuals. Among 14 asymptomatic patients with CD4 counts between 100 and 400/mm3, 11 patients had CTLs that recognized 1–9 HIV-1 peptides each (mean number of HIV-1 peptides recognized = 3).26 Because these genes represent much of the viral open reading frames, the response to a small number of HIV-1 peptides is likely to dominate the CTL response to the virus. To determine the ability to enhance for CTLs that recognize multiple peptides at once and to extend our method to non-gp160 peptides, we examined our selection protocol for three cell lines (from patients 202, 204, and 214) that respond to at least three HIV-1 peptides, using peptide-incubated PBMCs as APCs (Figs. 7 and 8). The APCs were incubated for 2 hr in an equal mixture of the three peptides (total peptide concentration of 10 μg/ml), washed three or four times to remove unbound peptide, irradiated, and added at an effector:APC ratio of 10:1. After culture for 8 days, the selected and unselected T cell lines were compared for their ability to lyse peptide-presenting targets. The specific lysis of Nef, Env, and Gag peptide-presenting targets increased by 1.4- to fivefold after 8 days of selection and was comparable to the selective enhancement using a single gp160 peptide antigen.

**Comparison of selection directly from peripheral blood mononuclear cells with selection from T cell line**

For some purposes it may be advantageous to use PBMCs rather than T cell lines as the starting point for selection. For patient 204, we compared the selection of CTLs specific for three peptides from either PBMCs or a 3-week-old T cell line (Fig. 8). During the 3-week culture, there is some selection for HIV-specific CTLs even without adding specific peptide, which may be seen by the difference in peptide-specific cytolysis in the unselected T cell line compared with the unselected PBMCs. Although the starting CTL activity is greater in the T cell line, the relative enhancement of specific lysis is comparable for the two starting populations. It is likely that sequential stimulation of PBMCs will result in at least as potent a specific CTL line, although the absolute number of cells will be reduced.
**Table 3. Probit Analysis of Number of Effector Cytotoxic T Lymphocytes in T Cell Line 138 before and after Selection with an Immunodominant Epitope**

<table>
<thead>
<tr>
<th>Effector cell</th>
<th>Target specificity</th>
<th>( ED_{50} )</th>
<th>( 95% ) confidence level</th>
<th>Estimated absolute frequency of specific CTLs normalized with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower limit</td>
<td>Upper limit</td>
</tr>
<tr>
<td>En15-204</td>
<td>gp160</td>
<td>281</td>
<td>259</td>
<td>303</td>
</tr>
<tr>
<td>JL32</td>
<td>EBV</td>
<td>1,036</td>
<td>963</td>
<td>1,118</td>
</tr>
<tr>
<td>T cell line 138 unselected</td>
<td>gp160</td>
<td>13,093</td>
<td>12,044</td>
<td>15,092</td>
</tr>
<tr>
<td>T cell line 138 selected once</td>
<td>gp160</td>
<td>2,244</td>
<td>1,361</td>
<td>13,472</td>
</tr>
<tr>
<td>T cell line 138 selected twice</td>
<td>gp160</td>
<td>1,177</td>
<td>922</td>
<td>1,385</td>
</tr>
<tr>
<td>T cell line 138 unselected</td>
<td>EBV-vaccinia</td>
<td>47,546</td>
<td>42,390</td>
<td>55,027</td>
</tr>
<tr>
<td>T cell line 138 selected once</td>
<td>EBV-vaccinia</td>
<td>4,071</td>
<td>2,498</td>
<td>19,000</td>
</tr>
<tr>
<td>T cell line 138 selected twice</td>
<td>EBV-vaccinia</td>
<td>4,923</td>
<td>c</td>
<td>c</td>
</tr>
</tbody>
</table>

*Clones En15-204, derived from patient 204 (which recognizes a gp160 peptide contained in the 20-mer from aa 49-68 in the context of B8), and JL32, derived from a normal individual and specific for autologous B-LCLs, were used to determine the \( ED_{50} \) for CTL clones and to obtain an estimate of the absolute frequency of target-specific CTLs in the T cell lines before and after peptide selection.

\(^{2}\)T cell clone JL32 was tested against the autologous EBV-transformed B-LCL target. Clone En15-204 was tested against autologous B-LCLs preincubated for 2 h with the gp160 peptide (49-68), followed by washing of unbound peptide. T cell line 138 was tested for cytotoxicity against gp160 with vPE16-infected autologous B cell targets and for cytotoxicity against EBV and vaccinia with vSC8-infected autologous targets. Part of the lysis attributed to gp160 is actually directed against the EBV-transformed B cell line.

*There were insufficient data to validate the fit by the \( \chi^2 \) test for this cell line target pair.

**Discussion**

In previous work we have shown that the CTL response to HIV-1 gp160 and RT in T cell lines, generated from the peripheral blood lymphocytes (PBLs) of infected individuals by nonspecific stimulation with mitogen and IL-2, is dominated by the recognition of a small number of peptide epitopes.\(^{21}\) In this article we have shown that dominant HIV-1 peptides may be used to expand HIV-specific CTL \textit{ex vivo} from PBMCs or T cell lines. T cell lines, whose growth has slowed from the period of initial rapid exponential growth but has not stopped, are most responsive to peptide selection (data not shown). Repeated exposure to peptide–APC is able to select in a stepwise fashion for peptide-specific CTLs, until after a few selections a significant fraction of T cells in the culture recognizes the stimulating peptide–APC. Peptide-specific cytoxicity is enriched approximately 1.4- to sixfold by one selection, depending on the peptide and the starting cell line. The variability of enrichment for CTLs recognizing different peptides may be due to multiple factors. These might include the relative frequency or state of activation of peptide-specific CTLs in the heterogeneous cell lines, the efficiency of processing the 15- to 22-amino acid stimulating peptides into the natural 8- to 11-amino acid peptides that are presented by class I MHC molecules, the affinity of the HIV-1 peptide for MHC class I, or of the peptide–MHC complex for the TcR.

When free peptide was added to the T cell cultures without APCs, there was a relative enrichment of peptide-specific CTLs, although there was a dose-related decrease in the number of viable T cells compared with cultures to which no peptide was added. The decline in cell numbers suggests that peptide-sensitized T cells in the culture were lysed by peptide-specific CTLs. Two groups have shown that the addition of cognate peptide to CTLs results in dose-related destruction, probably by fratricide, the lysis of neighboring peptide-presenting CTLs.\(^{41-43}\) The enhancement of peptide-specific cytolysis after peptide addition suggests that at least at the doses of peptides used in these experiments (1–50 μg/ml), clonal expansion predominates over peptide-induced autolysis. However, the stimulation of heterogeneous T cell lines with peptide–APC is more efficient than stimulation with peptide alone—not only is the enrichment for peptide-specific lysis somewhat enhanced but the cultures treated with peptide–APC have a substantial proliferative advantage.

HIV-specific CTL lines may be useful for adoptive immunotherapy. There is a large body of circumstantial evidence that supports the hypothesis that viral-specific CTLs are important in controlling viral replication. This evidence includes \textit{in vitro} studies that demonstrate inhibition of viral replication by HIV-specific CTLs.\(^{44,45}\) Studies in acute infection that show that the appearance of viral-specific CTLs coincides with the resolution of viremia,\(^{46}\) evidence that viral-specific CTLs de-
FIG. 7. Recognition of multiple HIV-1 peptides enhanced simultaneously by stimulation with PBMC APCs incubated with a cocktail of three peptides. Autologous PBMCs used as APCs were incubated for 2 hr with an equal mixture of peptides at a total concentration of 10 μg/ml, washed, irradiated, and added at an APC: effector ratio of 1:4. (A) T cell line from patient 202 recognized three dominant peptides in HIV-1 Env, Gag, Nef, Rev, and Tat: Env 740-18 (amino acids 209–228: TQACPKVS-FEPIHIYCAPA), Nef 778-7 (amino acids 61–80: EEEVGPVPQVPLRPMTY), and Nef 778-17 (amino acids 161–180: TSLHPVSLHGMDDPEREL). (B) T cell line from patient 214 recognized 12 peptides, some of which were overlapping: Env 740-3 (amino acids 59–78), 740-4 (69–88), 740-9 (119–139), 740-17 (199–219), and 740-22 (249–268); Gag 788.3 (amino acids 153–172), 788.4 (163–182), and 788.8 (203–222); and Nef 778-11 (amino acids 101–120), 778-12 (111–132), 778-13 (121–140), and 778-18 (171–190). For this patient the three peptides used to expand the T cell line were Env 740-22 (VSTVQCTHIGR-PVVSTQLIL), Gag 788.4 (AFSPEVIPMPSALSEGATPQ), and Nef 778-12 (LWIYHTQGFDPWQNYTPPGV). The percent peptide-specific cytotoxicity is the difference of lysis of autologous B-LCLs in the presence and absence of peptide.

cline in the PBMCs in individual patients just prior to developing AIDS, and cohort studies that show that patients with Gag-specific CTLs are less likely to progress to AIDS. In murine models, adoptive transfer of viral-specific CTLs is effective at arresting the development of lymphomas induced by murine retroviruses and in preventing lethal lymphocytic choriomeningitis virus (LCMV), influenza, and other viral infections. The extension of this approach to treating HIV-in-
ected patients with HIV-specific CTLs merits consideration. The methods of generating HIV-specific CTL lines described here result in T cell lines that meet the necessary criteria for use in treating patients. The nonspecific T cell lines multiply by approximately 10^4-fold from PBMCs and become free of detectable replicating virus if they have HIV-specific cytolytic activity, providing a source of large numbers of infusible CTLs. The cytolytic potency of the cell lines in lysing HIV-presenting targets is not substantially less than that of viral-specific clones. Although it would be possible to use HIV-specific T cell clones for such therapy, the labor, skill, and expense involved in developing and screening such clones and the difficulties often encountered in expanding human CD8+ clones makes their use for clinical trials feasible only in the setting of small pilot trials. Moreover, infusion with HIV-specific CTL clones recognizing a single HIV-1 peptide is more likely to induce viral escape mutants than infusion with oligoclonal cell lines that represent the diversity of the natural HIV-specific immune response. Indeed, the infusion of a Nef-specific CTL clone in a patient with advanced disease by Koenig et al. resulted in the death of the treated patient of viral mutants with deletions of the recognized nef epitope.52 The method of ex vivo expansion we present here is currently being used for immunotherapy for HIV-infected patients in a phase I trial supported by the Division of AIDS Treatment Research Initiative. For this trial we are using the selection with multiple peptides protocol with PBMCs as APCs. The data in Figs. 7 and 8 (right) represents the cytototoxicity of T cells infused into three trial patients. To obtain Food and Drug Administration approval for this trial, PBMCs, as opposed to B-LCLs, were required to be used as APCs.

Our method can also be generalized to generate T cell lines directed against other viruses; however, the significantly lower frequency of viral-specific CTLs in the blood for other infections may limit the ability to extend this method to generate specific T cell lines for other infections. One likely exception is the EBV virus; using EBV-transformed APCs we have been able to select EBV-specific CTLs.17 Other chronic persistent infections, such as cytomegalovirus (CMV) or hepatitis B or C, may be amenable to a similar approach. When autologous EBV-transformed B cells were used as APCs, the T cell lines from the five subjects whose cell lines were selected with peptide-B-LCLs, not surprisingly, also developed significant lytic activity against autologous EBV-transformed B cells infected with the lacZ-vaccinia construct. This lysis, which was comparable in amount to the lysis of uninfected EBV-transformed B cells, represents recognition of the EBV-transformed phenotype (data not shown). No such enhancement of EBV-specific lysis was observed when PBMCs were used as APCs. Because an increasing number of HIV-1-infected patients develop B cell lymphomas, some of which are EBV-related, the concurrent generation of CTLs specific for EBV-transformed B cells might be useful. Development of CTL immunotherapy for AIDS lymphoma, which is difficult to treat by conventional chemotherapy, might be possible using the methods developed here.

Our method for generating viral-specific CTLs can be applied, however, only when there exists some detectable characteristic peptide-specific CTLs in the beginning T cell line. In the clinical trial that we have initiated for HIV-infected patients with no AIDS-related opportunistic infections and CD4 counts of 100–400/mm^3, we have been able to characterize at least 1 peptide epitope in 12 of 17 individual T cell lines. The mean CD4 count of individuals for whom CTL epitopes were identified was significantly higher (299 ± 92/mm^3 vs. 198 ± 82/mm^3; p < 0.04).53 Alternate approaches for developing potent viral-specific CTL lines using virus-infected APCs or recombiant pathogens, such as vaccinia or fowlpox engineered to express HIV genes, are being developed by us and others and may bypass the need to identify immunodominant peptide epitopes.18,54-56 The safety of infusing HIV-infected patients with T cell lines stimulated with live viral vectors will, however, need to be demonstrated.

Limiting dilution analysis has been used to determine the frequency in polyclonal populations of immunocompetent cells with a range of functional capacities. Varying numbers of mixed effector cells are added to replicate wells of microtiter plates and a functional assay that detects an all-or-nothing response is performed. The statistical analysis of the data is based on the assumption that the distribution of effector cells in the individual wells is described by a Poisson distribution and that the assay is able to detect a single effector cell. The mathematical analysis for limiting dilution analysis cannot be extended to cover situations in which the assay can only detect multiple effector cells because the mathematical sums cannot be solved in closed form.39,40 To use limiting dilution analysis for CTLs, one approach has been to measure the frequency of CTL precursors (pCTLs) by allowing pCTLs seeded at various dilutions in the presence of stimulator and feeder cells to multiply in each microtiter well until there are enough progeny to detect lysis. While this approach will give a lower limit for the pCTL frequency, its accuracy is limited by the indeterminate CTL cloning efficacy. For human CD8+ T cells this cloning efficiency is often low, varies significantly with the cloning technique and reagents, and may be substantially altered in different disease states. In particular, in patients who are infected with HIV-1, the cloning efficiency of T cells from PBMCs stimulated nonspecifically with PHA and IL-2 in the presence of autologous PBMC feeder cells was as low as 2.1% for an asymptomatic subject and 0.2% for a subject with AIDS (data not shown).57

In this article we describe an alternate statistical approach, based on the assumptions that (1) lysis will be detected in a particular well if the number of effector cells exceeds the well threshold and (2) the distribution of threshold dosages of CTLs is normal. The statistical appropriateness of the model can be evaluated by chi-square tests. Although this method was developed for enumerating CTLs, it could be applied to other assays of immunological function in which the sensitivity of the assay does not enable the detection of a single cell. In this article we have used this method to estimate the frequency of HIV-specific CTLs in a nonspecifically stimulated T cell line from a seropositive subject and analyzed the changes in frequency after selection with an immunodominant peptide.

Except for one of the experimental control conditions (lysis of vaccinia-lacZ-infected targets by the twice-selected T cell line) in which there was a paucity of relevant data points (dilutions for which some but not all of the wells demonstrate statistically significant lysis), a chi-square analysis verified the consistency of the model. As in limiting dilution analysis, the statistical accuracy of the method depends on the ability to es-


EX VIVO EXPANSION OF HIV-1 SPECIFIC CTLs

While investigating the clonal expansions of T cells that will make some, but not all, wells positive. For the twice-selected T cell line against the lacZ-expressing targets, the choice of dilutions was suboptimal and should have included wells with fewer number of effector cells. Using a titration analysis, the relative enhancement of T cells lytic for gp160-expressing B-LCL autologous targets in a heterogeneous T cell population after a single selection with peptide–APC was sixfold. After 2 exposures to antigen–APC, the enhancement was 11-fold and the number of T cells needed per well for detectable cytotoxicity was indistinguishable from the number of T cell clones needed to lyse its specific target.

This method is ideally suitable for determining the frequency of specific effector cells. It could be used to compare the frequency of specific CTLs in different samples, including changes with disease course or after therapeutic intervention either in in vitro experiments, in animal models, or in clinical trials. To get an estimate of the absolute frequency of specific effector cells, the ED50 (the number of effector cells needed to detect lysis in half the wells) for the cell line to be analyzed is compared to the ED50 of specific clones. The accuracy of our determination of the absolute frequency will depend on the possible variations in the ED50 from clone to clone.

We would expect the same sort of variations in ED50 from clone to clone that are seen in percent specific cytotoxicity curves—specifically, the lowest E:T ratio at which there is detectable killing is lower than others; moreover the plateau of maximum achievable percent specific cytotoxicity will also vary between clones. Looking at two clones in this study, we found a substantial variation in ED50. This difference may reflect either variations in cytotoxic potency from clone to clone (which are evident if percent specific cytotoxicity values for different E:T ratios are compared for different clones) or the sensitivity to lysis of the targets. For one clone we used naturally infected cells as targets, and for the other we used targets incubated with saturating concentrations of specific peptide antigen. It is possible that the peptide-incubated cells present higher concentrations of antigen on their cell surface and make better targets. However, the variation in ED50 between clones is unlikely to be as great as the errors attributable to cloning inefficiency for the use of limiting dilution analyses in the pCTL assay.

Others have used standard limiting dilution analysis to estimate the number of HIV-specific CTLs and pCTLs in the blood and alveolar lavage fluid of seropositive and seronegative subjects. The precursor frequency studies were done after the effector cells were allowed to proliferate in culture for approximately 14 days to provide enough CTL effectors to detect sufficient target cell lysis. The analyses for CTL frequency, however, assumed that the lysis by a single CTL in a well with 5000–10,000 51Cr-labeled targets could be detected. An analysis of the raw data from one of our experiments will demonstrate that this is implausible. The standard deviation from 24 replicate wells without added CTLs was 7.6% of the average number of counts; 3 standard deviations were 23%. For 1 CTL to lyse more than 23% of the targets in a well, it would have had to lyse more than 2000 targets in 16 hr or more than 100 targets in an hour. Our data with an EBV-specific CTL clone, with effective killing of its target cell line (Table 1 and Fig. 6), demonstrates that this is indeed improbable. Standard limiting dilution analysis with this clone (confirmed to be a clone by the presence of a single rearranged band in the TcR β gene by Southern blot; data not shown) would have given a CTL frequency of 1/1200. Our results on CTL cloning efficiency in HIV-infected patients and on the number of specific CTLs needed to give a positive result in a limiting dilution analysis assay suggest that many published estimates of Env- and Gag-specific pCTL and CTL frequency may actually be underestimated by as much as 100- to 1000-fold. Because published estimates of HIV-specific pCTL and CTL frequency run as high as 500 and 30 per million, respectively, the actual frequency may be high enough to be measured in percents. Our finding is consistent with that of McMichael, who reported that in two patients the frequency in the PBMCs of T cells bearing a particular TcR identified with the recognition of an HIV peptide–MHC pair was as high as 1 in 100–200.

The application of this method to determining the frequency of HIV-specific CTLs reveals an unusually high frequency of gp160-specific T cells in a nonspecifically stimulated T cell line. The ability of PBMCs to lyse HIV-expressing targets is usually somewhat less than that of the T cell lines (Fig. 8, and data not shown). Nonetheless, our estimate that as many as 1 in 13 or 1 in 47 (or 2–8%) of the T cells in the unselected T cell line is gp160-specific suggests that the frequency of HIV-specific pCTLs in PBMCs may be much higher than current estimates. The statistical method of frequency analysis presented here could be used to reexamine the frequency of viral-specific CTLs in blood and lymph nodes of patients at various disease stages.

ACKNOWLEDGMENTS

We thank J. Mazzullo, J. Noble, and J. Moore for providing clinical subjects, B. Moss for HIV-vaccinia constructs, the AIDS Reagent and Reference Program for the gag-vaccinia recombinant vDK1 (contributed by D. Kouritzkes) M. Kuo, the MRC AIDS Reagent Project, and the EVA Programme for peptides.

This work was supported in part by a Pew Biomedical Scholar Award, NIH K08-CA01449, R01-AI30926, and N01-AI15123 (J.L.), NIH K08-A101046, R01-AI33290, and a Carter Wallace Research Grant (P.R.S.), and NIH-NIAID Contract N01-AI95030 (L.B.).

REFERENCES


Address reprint requests to:
Judy Lieberman
Division of Hematology-Oncology
P.O. Box 245
New England Medical Center Hospital
NEMCH# 245
750 Washington Street
Boston, Massachusetts 02111