Limiting Dilution Analysis of Cytotoxic T Lymphocytes to Human Immunodeficiency Virus *gag* Antigens in Infected Persons: In Vitro Quantitation of Effector Cell Populations with p17 and p24 Specificities

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Summary

The presence of cytotoxic T lymphocytes (CTL) to the *gag* antigens of human immunodeficiency virus (HIV) has been described in infected populations. We found that the majority of this immune response as measured in bulk CTL assays of unstimulated peripheral blood mononuclear cells (PBMC) is directed against the p24 component of the p55 *gag* precursor protein. Using limiting dilution analysis of this effector cell population we confirm that the majority of activated *gag*-specific CTL circulating in the PBMC of infected hemophilic patients are directed at p24 determinants and are present at frequencies of 1/36,000 to 1/86,000 lymphocytes. By performing in vitro stimulation after limiting dilution, the precursor population of *gag*-specific CTL are characterized and quantitated. HIV *gag*-specific CTL precursors are identified at frequencies of 1/1700 to 1/17,000 lymphocytes and are made up of cells with both p17 and p24 specificities. No HIV *gag*-specific CTL precursor cells are identified in the PBMC of HIV-uninfected individuals. These studies demonstrate that CTL directed at both p17 and p24 determinants make up the cellular immune repertoire in HIV-infected individuals but that only the p24-specific CTL are routinely found in an activated state in the circulation.

The CTL response has been shown to be an important factor in both the clearance of acute viral infections (1, 2) and in the control of chronic viral infections (3). In recent years, the CTL response to HIV infection has been characterized by several groups of investigators (4–12). During the course of HIV infection a vigorous HIV-specific CTL response is generated which declines only with terminal progression to AIDS (13).

HIV-specific CTL responses directed against both structural and nonstructural proteins of HIV have been described (4–12). Among the structural proteins, activated CTL to HIV *gag* proteins are detectable in the PBMC of 25% to 85% of seropositive patients in the absence of in vitro stimulation (4, 12, 14), and represents one of the most consistent HIV CTL responses. This vigorous cellular immune response is atypical of other chronic viral infections. To more closely dissect this vigorous cellular immune response, HIV-specific CTL clones have been generated from PBMC of infected individuals (7, 9, 15). In some of these studies, nonspecific T cell stimuli such as PHA or antibody to CD3 have been used to expand and clone the HIV-specific CTL (9, 15). While these stimuli are capable of activating HIV CTL, the already activated (DR^+) $CD8^+$ population which contains the HIV-specific CTL activity measured in the absence of in vitro stimulation (16, 17), is resistant to expansion via this mechanism (16). This would suggest that there exist two distinct HIV-specific CTL populations within the PBMC of infected individuals: an activated population whose cytolytic activity can be measured in bulk CTL assays in the absence of in vitro stimulation, and a memory population which can be activated and expanded in response to non-specific stimulation. No studies have yet been performed however, to determine what portion of the total *gag*-specific response resides in either of these two CTL populations.

The contribution to the total *gag*-specific CTL response of the structural components of *gag* is also unknown. Studies to date of bulk CTL lysis have used vaccinia vectors which express full length *gag* protein, and do not discriminate p17,

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p24, and p15 specificities (4, 11, 12). Through epitope mapping methods, CTL epitopes within both p17 and p24 have been identified. The frequency of activated and memory CTL to specific linear epitopes within the p24 antigen of HIV *gag* have been described in certain individuals (14), however, these studies may underestimate the total CTL response of an individual since multiple CTL epitopes may be present in a single protein antigen (15, 18). In addition, the contribution of these and other epitopes to the total activated and memory *gag*-specific CTL repertoire have yet to be fully addressed (10, 14, 18, 19). A better understanding of the total cellular immune response to the components of *gag* may have important implications for HIV vaccine development.

In the studies described here, we have performed both bulk analysis and limiting dilution analysis on PBMC from HIV-infected hemophilic patients to quantitate the total activated and memory populations of HIV *gag*-specific CTL. In these studies, vaccinia vectors were specifically created to allow us to quantitate and characterize the CTL response to the major structural components (p17 and p24) as well as the precursor protein (p55) of the *gag* antigen. These studies allow a more comprehensive understanding of the HIV *gag*-specific CTL repertoire.

Materials and Methods

Patient Population. Patients were part of an ongoing study of immunoregulatory defects in hemophilia and were followed yearly at the New England Area Comprehensive Hemophilia Center, Medical Center of Central Massachusetts-Memorial (Worcester, MA) (20). Patients gave informed consent before entering study. Approximately 90% are positive by Western blot for antibodies to HIV with the majority having seroconverted before 1983.

Effector Cells. PBMC were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients (20). PBMC preparations contained 70% to 80% lymphocytes, 10% to 20% monocytes, and <20% polymorphonuclear leukocytes as determined by cell morphology. PBMC were used directly as effector cells in bulk CTL assays or subjected to limiting dilution analysis as described below.

Target Cell Lines. Autologous B-lymphoblastoid cell lines (B-LCL)[1] were created by incubation of PBMC with supernatant from the EBV-producing marmoset cell line B95.8 (NO. CRL-1612; American Type Culture Collection, Rockville, MD). Transformed cell lines were maintained in RPMI 1640 with 10% FCS.

Vaccinia Virus Vectors. Methods for the production, propagation and expression analysis of vaccinia virus (VV) vectors have been previously described (12, 21). All VV incorporate sequences from the BH10 strain of HIV (22). Recombinant VV used in these studies include vAbT 141 which contains the entire HIV *gag* coding sequence beginning at the translation initiation codon and extending approximately 200 bp beyond the *gag* translation termination codon. Recombinant vAbT 228 contains *gag* sequences from the initiation codon of *gag* through the PvuII site, 38 bp upstream of the end of p17 coding sequences. A synthetic linker was added to restore the terminal sequences of p17, along with a stop codon and a VV transcription termination sequence. Recombinant vAbT 286 contains the p24 coding sequence from nucleotide 508 through 1200 removed from the intact *gag* gene. The 5′ and 3′ ends were modified using synthetic linkers to insert a BamHI site and a translational start codon at the beginning, and a translation termination codon and a KpnI site at the end of the coding sequence. Recombinant sequences were inserted in the tk (vAbT 141) or Hind-M (vAbT 228, 286) regions of VV and were under the control of the 7.5K (vAbT 141) or 40K (vAbT 228, 286) VV promoters. The predicted molecular sequences of the recombinant viruses were confirmed by restriction endonuclease analysis and DNA hybridization of viral genomic DNA. Expression of appropriate recombinant proteins was confirmed by in situ ELISA and radioimmunoprecipitation using polyclonal human serum from HIV-seropositive VV-seronegative donors. VV strain NYCBH was used as the control virus in all CTL studies.

Radioimmunoprecipitation. Target cells were infected with vaccinia vectors at a multiplicity of infection (MOI) of 10 in the presence of 100 μCi of ^{3}H-leucine (Dupont NEN, Boston, MA). Harvesting and precipitation was performed as previously described (21) using HIV^{+} VV^{-} human serum.

Bulk Cytotoxic T Lymphocyte Assays. CTL assays using autologous B-LCL infected with recombinant VV were performed as previously described (12). All B-LCL were infected with VV at MOI of 10 for 16 h before assay. VV-infected autologous B-LCL were labelled with ^{51}Cr and used as targets in a standard ^{51}Cr-release assay in which effector cells were freshly isolated PBMC from HIV-infected hemophilic patients. Effector-to-target (E/T) ratios of 50, 25, and 12.5 to 1 were employed. Assays were performed in 96-well round-bottom microtiter plates containing 100 μl of effector cell suspension and 100 μl of target cell suspension. After 6 h of incubation at 37°C, 100 μl of supernatant was removed from each well and counted in a gamma counter. Spontaneous release wells in which media (RPMI 1640, 10% FCS) was used in place of effector cells were included in all assays. Assays were omitted from analysis if spontaneous release exceeded 30%. All tests were performed in triplicate wells. Maximal release was determined by counting the total cpm in a 50 μl volume of target cell suspension. Percentage of specific cytolysis was calculated by the formula: 100 × ([test cpm − spontaneous cpm]/[maximal cpm − spontaneous cpm]).

Effector Cell Depletions. Effector PBMC were depleted of cells expressing CD4 or CD8 surface antigens using mAbs OKT4 or OKT8 (Ortho Diagnostic Systems, Raritan, NJ) respectively, and rabbit complement (Cedarlane Laboratories, Ontario, Canada) by previously published techniques (12, 21). Effector cells were then resuspended in the original (predepletion) volume of medium so nondepleted cells would remain at predepletion concentrations. Adequacy of depletions was assessed by surface phenotype analysis using direct immunofluorescence with PE-conjugated Leu3a (Becton Dickinson and Co., Mountain View, CA) and fluorescein isothiocyanate-conjugated Leu2a (Becton Dickinson & Co.) using previously published techniques (20). Samples were analyzed on a FACScan (Becton Dickinson & Co.).

Limiting Dilution of Activated Cytotoxic T Lymphocytes. Relative frequencies of activated HIV *gag*-specific CTL were determined using standard methods of limiting dilution analysis (LDA) (23). Freshly isolated PBMC were assayed at 1,563 to 100,000 lymphocytes per well in 24 replicate wells for cytotoxicity on autologous B-LCL infected with NYCBH or recombinant VV expressing HIV *gag* proteins in a ^{51}Cr-release assay as described above. Fraction of nonresponding wells was taken as 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, divided by 24.

[1] *Abbreviations used in this paper:* B-LCL, B-lymphoblastoid cell line; LDA, limiting dilution analysis; MOI, multiplicity of infection; VV, vaccinia virus.

Activated cell frequency was estimated by single hit model Poisson distribution analysis (23).

Limiting Dilution Assays of Memory Cytotoxic T Lymphocytes. Precursor frequencies of HIV *gag*-specific CTL were estimated by performing limiting dilutions on freshly isolated PBMC followed by in vitro stimulation to allow expansion of CTL. PBMC were diluted at 250 to 16,000 lymphocytes per well in 24 replicate wells of 96-well microtiter plates. To each well was added 2.5×10^4 gamma-irradiated PHA blasts from an HIV-seronegative donor and either 2 μg/ml of PHA (HA-16; Wellcome Diagnostics, Research Triangle Park, NC) or 2 μg/ml of mouse monoclonal anti-human CD3 (OKT3; Ortho Diagnostic Systems). Cells were cultured at 37°C, 5% CO_2 in RPMI 1640, 10% FCS, 15% human IL-2 (Pharmacia Fine Chemicals) for 5 d. Wells were then split and assayed for cytotoxicity on ^{51}Cr-labeled autologous B-LCL infected with vaccinia-HIV expression vectors. Fraction of nonresponding wells and precursor frequencies were determined as described above.

Statistical Analysis. Simple linear regression analysis was performed on LDA using least mean squared method. Frequency was determined by solving the equation of the best fit line where the negative log of the fraction of nonresponding wells equaled one (fraction of nonresponding wells = 0.37). 95% confidence limits were calculated based upon the true mean value of the dependent variable, lymphocytes per well.

Results

Human Immunodeficiency Virus Gag Antigens Are Expressed by Vaccinia Vectors. Radioimmunoprecipitation was performed using HIV^+ VV^- human serum against cells infected with expression vectors vAbT 141, vAbT 228, and vAbT 286. Results are depicted in Fig. 1. Vector vAbT 141 produces a single band at p55 without evidence of processing to p24 and p17, and vectors vAbT 228 and vAbT 286 produce single bands at p17 and p24, respectively. All three vectors are strong expressers of HIV-1 *gag* proteins.

Human Immunodeficiency Virus Gag-specific Lysis Is Detectable in Bulk Assays. 43 bulk CTL assays using PBMC from HIV seropositive hemophilic patients were performed at E/T ratios of 50, 25, and 12.5 to 1 against *gag*-expressing targets. 16 assays included p17-expressing targets and 7 assays included p24-expressing targets in addition to the p55-expressing targets. Results of *gag*-specific lysis from bulk CTL assays at 50:1 E/T ratios are depicted in Fig. 2. Cytolysis directed against p55 and p24 were readily detectable in PBMC from the majority of seropositve hemophilic patients tested (86% against either p55 or p24). In contrast, detection of cytolysis to p17 was infrequent, occurring in 3 of 16 (19%) seropositive hemophilic patients. Cytolysis against p17 and p24 was always assayed in conjunction with p55 and in all cases where p17 or p24-specific lysis was observed, greater or equal cytolysis was observed against p55. Bulk cytolysis directed against HIV *gag* antigens was not detected in any of 8 seronegative subjects tested (data not shown). Vigorous cytolytic responses directed against p55 and p24 *gag* antigens are therefore detectable directly from PBMC of HIV-infected persons, while p17-specific responses are much less apparent.

Bulk Gag-specific Cytolysis Is Mediated by Major Histocompatibility Complex-restricted $CD8^+$ Cytotoxic T Lymphocytes. We have previously shown that bulk cytolysis directed against cells infected with vAbT 141 is blocked by antibodies to CD3, CD8, and class I antigens (12). To confirm that bulk cytolytic responses against p55, p17 and p24-expressing targets are CD8-mediated, effector cells from a rare individual with bulk cytolysis against all three *gag* antigens were depleted of $CD4^+$ or $CD8^+$ cells through complement-mediated, monoclonal antibody-directed lysis. As depicted in Fig. 3, CD4 depletion had little effect on *gag*-specific cytolysis, while CD8 depletion completely abrogated the *gag*-specific cytolytic response. In addition, HLA-mismatched targets expressing p55, p17 or p24 were not efficiently recognized by the bulk effector cells (Fig. 3). As such, the effector cells mediating bulk p55, p17 and p24-specific cytolysis are MHC-restricted $CD8^+$ CTL.

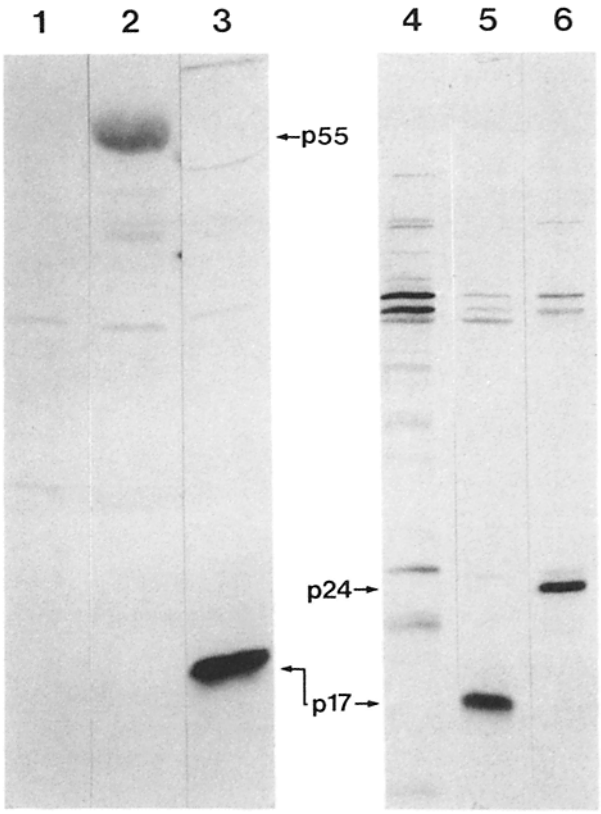

Figure 1. Expression analysis of vaccinia vectors. Radioimmunoprecipitations were performed on vector-infected cells using HIV^+, VV^- human serum. Composite of 2 separate gels are shown. Lanes *1* and *4*, NYCBH (VAC); lane *2*, p55 expression vector vAbT 141; lanes *3* and *5*, p17 expression vector vAbT 228; lane *6*, p24 expression vector vAbT 286.

Activated Gag-specific Cytotoxic T Lymphocytes Can Be Quantified by Limiting Dilution Analysis. Having shown that the circulating effector cells mediating *gag*-specific cytolysis are CTL, we attempted to quantitate the frequency of these activated cells in the circulation of infected hemophilic patients. To accomplish this, LDA was performed on fresh PBMC preparations. As shown in Fig. 4, no activated CTL to control vaccinia-infected B-LCL targets (i.e., vaccinia or EBV-specific CTL) were detected at up to 10^5 effector cells per well, indicating that frequency determinations based upon

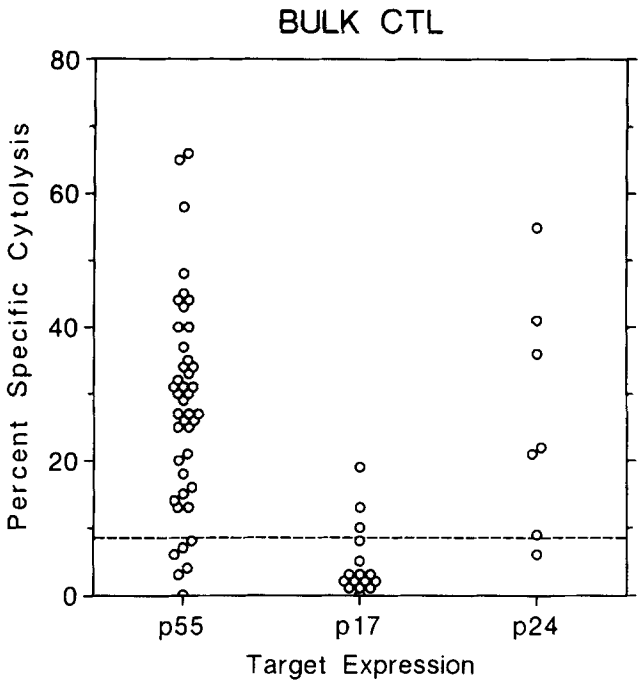

Figure 2. Bulk HIV *gag*-specific CTL responses. Vaccinia-specific cytolysis was subtracted from vaccinia/*gag*-specific cytolysis and is depicted for p55 (vAbT 141), p17 (vAbT 228) and p24 (vAbT 286)-expressing targets. Assays were performed at E/T of 50:1. Each point represents an individual assay on a specific HIV-seropositive hemophilic patient. Total *n* of assays is as follows: 43 (p55), 16 (p17), 7 (p24). The dashed line represents 2 SDs above the mean vaccinia-specific cytolysis in these assays.

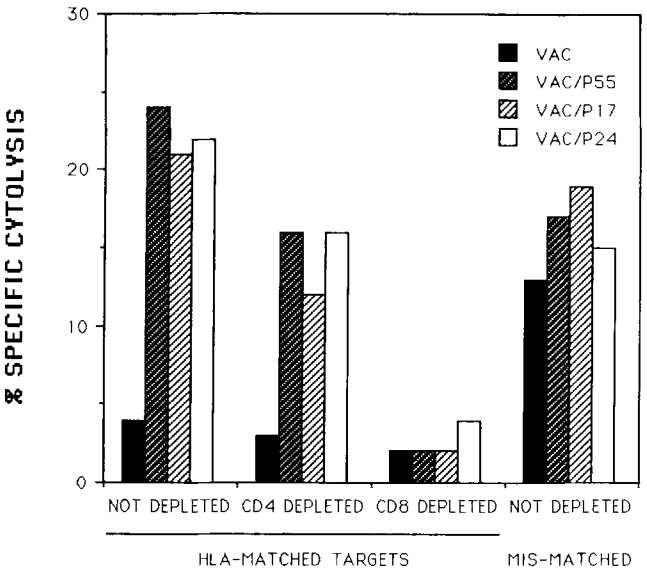

Figure 3. Characterization of bulk *gag*-specific cytolysis. Effector PBMC were either not depleted, or depleted of CD4 or CD8 cells using mAb-dependent complement-mediated lysis before testing in a bulk CTL assay at a 50:1 effector to target ratio. Depletions were >80% effective as determined by surface phenotype analysis. The HLA-matched targets were autologous B-LCL, the mismatched targets were allogeneic B-LCL which did not match at any of the A, B, or C loci.

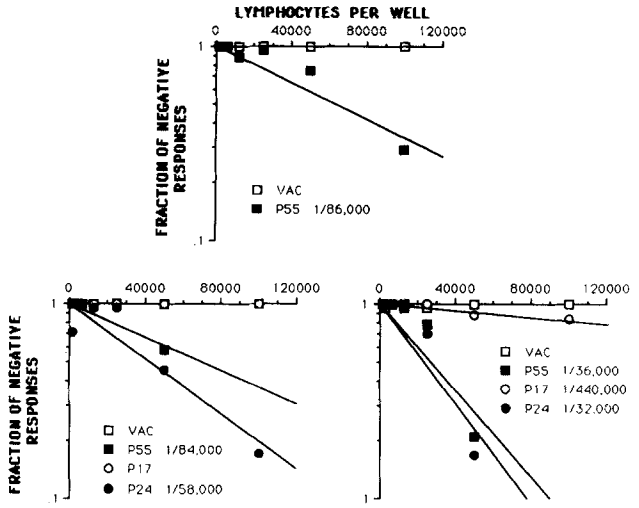

Figure 4. Quantitation of activated *gag*-specific CTL. Limiting dilution analysis was performed on freshly isolated PBMC from HIV-infected persons as described in the methods section. Targets were autologous B-LCL infected with NYCBH (VAC), vAbT 141 (P55), vAbT 228 (P17), or vAbT 286 (P24). Effector cell frequencies were calculated as reciprocal of x variable where y equals 0.37. Frequencies for each target are listed. Those targets for which no frequency is listed were not calculable as all wells failed to exhibit significant cytolysis even at the highest effector cell concentration.

cytolysis of targets infected with recombinant VV expression vectors reflect lysis by HIV *gag*-specific, and not vaccinia-specific CTL. Frequency determinations of p55-specific CTL for the three individuals depicted in Fig. 4 are 1/86,000, 1/36,000, and 1/84,000. In the two individuals whose frequency of activated CTL against p24 and p17 were also determined, p24-specific CTL frequencies were similar to p55 CTL frequencies, but p17 CTL frequencies were low to undetectable (Fig. 4, lower 2 graphs). The frequency of p24-specific CTL actually appears greater than the frequency of p55 CTL in these two individuals but the 95% confidence limits suggest no true difference in these frequencies. All three individuals had CTL killing directed against p55, and the individuals depicted in the lower two graphs also had CTL killing directed at p24 but not p17 in bulk assays at an E/T ratio of 50:1. This indicates that virtually all the activated HIV *gag*-specific CTL circulating in these individuals are directed towards p24 determinants and not p17 determinants. In addition, the lack of cytolytic activity against p17-expressing targets at low effector cell input argues against a suppressor cell population as the cause for deficient bulk p17 CTL activity.

Precursor (Memory) Gag-specific Lysis Can Be Quantified after In Vitro Stimulation. To determine if a population of resting (memory) *gag*-specific CTL precursors are also present at a given frequency in the PBMC of infected hemophilic patients, LDA followed by in vitro stimulation was performed. Stimulation with either PHA or anti-CD3 gave similar results in these assays and could be used interchangeably. The results of four separate precursor frequency determinations on four individuals are presented in Fig. 5. The precursor frequency of p55-specific CTL in these four individuals ranged from

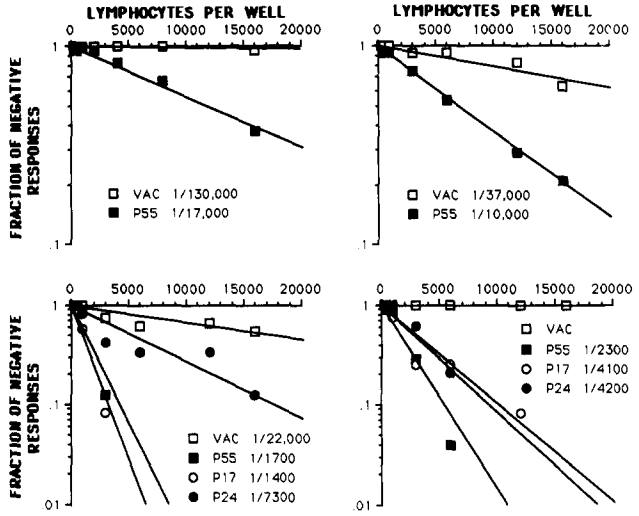

Figure 5. Quantitation of memory *gag*-specific CTL. Limiting dilution was performed on PBMC from HIV-infected persons followed by in vitro stimulation with PHA or anti-CD3 as described in the methods section. Targets were autologous B-LCL infected with NYCBH (VAC), vAbT 141 (P55), vAbT 228 (P17), or vAbT 286 (P24). Precursor frequencies were calculated as in Fig. 4. Results represent either PHA or anti-CD3 determinations based upon lowest background (VAC) precursor frequencies. Where no precursor frequency is listed, that frequency was not calculable as all wells failed to exhibit significant cytolysis even at the highest effector cell concentration.

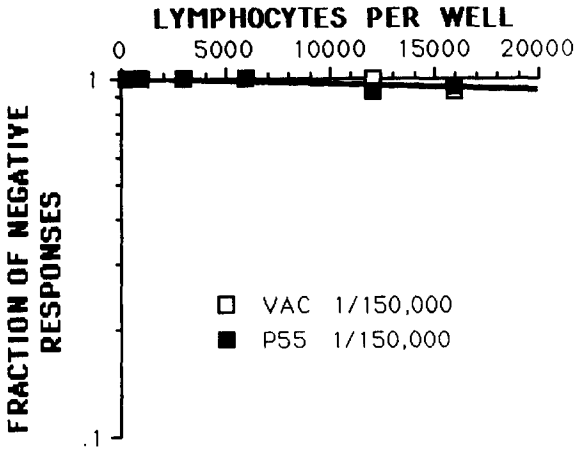

Figure 6. Limiting dilution followed by in vitro stimulation of PBMC from HIV uninfected donor. Targets and analysis performed as described in Fig. 5.

1/1,700 to 1/17,000. This frequency is an order of magnitude greater than the frequency of activated p55-specific CTL described in Fig. 4. The precursor frequency of p17 and p24-specific CTL was investigated in 2 of the 4 patients depicted in Fig. 5. Both patients had detectable bulk CTL activity against p55 and p24, but not p17. In both individuals, p17 and p24-specific CTL are contributing to the memory p55-specific CTL population. The p17-specific CTL are present at a precursor frequency of 1/1,400 to 1/4,100 PBMC while p24-specific CTL are present at 1/4,200 to 1/7,300 PBMC in these two individuals. This would indicate that while circulating activated p17-specific CTL are not routinely apparent in bulk CTL assays, memory CTL with this specificity are a part of the cellular anti-HIV repertoire.

Gag-specific Lysis in Stimulated Limiting Dilution Analysis Is Mediated by CD8⁺ Cells. While we have shown that the activated cells mediating *gag*-specific cytolysis are MHC-restricted CD8⁺ CTL, the process of in vitro stimulation and culture in the presence of IL-2 could stimulate nonspecific cytolytic effector mechanisms such as LAK cells (24). We therefore sought to determine the phenotype of the cells being measured in the stimulated LDA analyses. PBMC were depleted of CD4⁺ or CD8⁺ cells before LDA and in vitro

Table 1. *Surface Phenotype of Memory Cytotoxic T Lymphocyte*

Effector cells*	Not Depleted	CD4-depleted	CD8-depleted
Precursor frequency of CTL with specificity‡			
p55	1/4,980 (1/3,910–6,060)	1/6,550 (1/4620–8,470)	1/26,700 (1/17,600–35,800)
p17	1/19,600 (1/8,650–30,600)	1/28,600 (1/19,500–37,700)	1/124,000 (1/85,900–161,000)
p24	1/9,530 (1/5,780–13,300)	1/7,260 (1/4,410–10,100)	1/23,000 (1/14,100–31,900)
Surface phenotype§			
CD4	33%	<1%	74%
CD8	48%	80%	4%

* Effector cells were depleted of CD4 or CD8 cells using mAb-dependent, complement-mediated lysis. Cells were then subjected to LDA followed by 5-d in vitro stimulation with anti-CD3 and expansion with IL-2.
‡ Precursor frequencies of p55, p17, and p24-specific effector cells in the depleted and nondepleted PBMC populations were determined as described in the methods section. Values in parentheses represent 95% confidence limits of the calculated frequencies.
§ Efficiency of depletion was assessed by surface expression as determined by fluorescent mAb staining and FACS analysis.

stimulation. As shown in Table 1, memory cells capable of mediating lysis of p55, p17 and p24-expressing targets were removed through CD8, but not CD4 depletion. The precursor cells which are expanded and assayed for in the stimulated LDA analyses are therefore $CD8^+$ cells. Because we use a nonspecific in vitro stimulation method which will stimulate alloreactive CTL as well as virus-specific CTL, we were unable to demonstrate a MHC-restriction to the memory *gag*-specific cytolysis through use of MHC-mismatched targets because of the high precursor frequencies of alloreactive CTL (data not shown).

Gag-specific Cytotoxic T Lymphocytes Are not Present in Human Immunodeficiency Virus Seronegative Individuals. High precursor frequencies of *gag*-specific CTL should not be present in HIV-naive individuals. We therefore investigated the presence of *gag*-specific CTL in HIV-seronegative individuals. We have previously reported that bulk CTL to HIV *gag* antigens are not detected in seronegative hemophilic patients (12). In addition, we have been unable to detect activated *gag*-specific CTL by LDA in HIV-seronegative individuals (data not shown). As shown in Fig. 6, we were also unable to detect memory *gag*-specific CTL after in vitro stimulation in HIV-seronegative individuals, supporting the conclusion that the cytolytic mechanisms being measured in these assays reflect virus-specific CTL activity.

Discussion

We have used bulk, unstimulated CTL assays and limiting dilution analysis to define the HIV *gag*-specific CTL populations present in the PBMC of infected hemophilic patients. The data support the presence of an activated *gag*-specific CTL population whose activity can be measured directly in the PBMC of most infected individuals. These activated *gag*-specific CTL are directed principally at p24 determinants and not p17 determinants, though we cannot rule out a concomitant population of CTL with p15 specificity. Direct determination, through limiting dilution analysis, of the frequency of these activated *gag*-specific CTL in the PBMC of hemophilic patients indicates a frequency of 1/36,000 to 1/86,000. These determinations again indicate a paucity of activated p17-specific CTL in the circulation with virtually all p55-specific responses being attributable to p24 specificity. By using in vitro stimulation to expand all memory T cells (25), we have identified memory *gag*-specific CTL which exist at a frequency of 1/1,700 to 1/17,000 in the PBMC of seropositive patients. In contrast to the activated CTL population, this memory population is composed of cells directed against both p17 and p24 determinants of the p55 major protein precursor. As such, the vigorous HIV *gag*-specific CTL response which has been described in infected individuals represents the activity of only a small proportion of the total p24-specific CTL repertoire, and does not reflect the p17-specific CTL population which is present but not in an activated state.

All of the *gag*-specific effector cells measured in these studies have been characterized as $CD8^+$ cells. We and others have previously characterized the *gag*-specific effector cell population responsible for the cytolysis seen in bulk assays as MHC class I-restricted, $CD8^+$, $CD3^+$ CTL (11, 12, 14). No contribution of non-T cell or class II–restricted CTL have been identified for *gag*-specific cytolysis (11, 12). In addition, unlike HIV envelope-specific cytolysis, target cells expressing *gag* antigens through VV expression vectors are not lysed through antibody-dependent cell-mediated cytotoxic or other non-MHC-restricted mechanisms (21).

The fact that we were able to identify both p17 and p24-specific memory CTL populations within the PBMC of infected individuals is not surprising considering the fact that CTL epitopes within both p17 and p24 have been defined (10, 14, 18, 19). Our data suggest that CTL with both these specificities are part of the cellular immune response to HIV, but that the p24-specific response is more often activated in infected patients.

Several hypotheses can be ruled out as explanations for the lack of activated p17-specific CTL in these patients. It is unlikely that a suppressor cell population exists which is effecting the p17-specific CTL as this activity was not apparent on LDA. The lack of p17-specific CTL also does not represent a problem with expression of p17 by the VV expression vector since p17-specific cytolysis can be measured after in vitro stimulation and p17-specific CTL clones have been described which efficiently recognize and lyse autologous target cells infected with this VV expression vector (18). While loss of CD4 help in expanding virus-specific CTL clones has been described in HIV infection (26–28), it is unlikely that there is a specific defect in CD4 help which is hindering the expansion of p17-specific and not p24-specific CTL. One could also hypothesize that p17 does not contain CTL epitopes which are restricted by common HLA haplotypes, but the fact that p17-specific CTL are found after in vitro stimulation (18, 19) and that T cell epitopes have been identified within p17 by computer modeling (29) argues against this point.

It may be more appropriate to ask why the p24-specific CTL are so often found in an activated state in these patients, since this level of activated CTL has not been described for other chronic viral infections (3). However, more information regarding the number of potential CTL epitopes contained within the p24 molecule, and the role of infection of antigen presenting cells, such as monocytes/macrophages and dendritic cells, is required to adequately address the mechanisms underlying this high degree of specific CTL activation.

A recent report described precursor frequencies of 1/3,500 and 1/8,000 PMBC for p24 epitope-specific memory CTL in two infected individuals (14). These frequencies are similar to those reported here. In their report however, Gotch et al. (14) find a much higher frequency of activated p24 epitope-specific CTL (1/100–1/1,000 PBMC) than we report. This discrepancy probably relates to the different methods used to calculate the frequency of the activated CTL population. We use LDA analysis as performed by other investigators (13, 30) to calculate the frequency of activated CTL while Gotch et al. (14) derive their frequencies by comparing bulk p24-specific cytolysis with the cytolysis observed at varying E/T ratios for a CTL clone. In our analysis it is assumed that one CTL in an assay well is sufficient to mediate cytolysis greater than 3 SDs above the mean cytolysis, where in the analysis

employed by Gotch et al. (14) it is assumed that all the cloned CTL cells are capable of active cytolysis. Preliminary results from LDA of a p24-specific clone (kindly provided by R. Paul Johnson, Massachusetts General Hospital, Boston, MA) performed in our laboratory indicate that greater than 500 cloned CTL are required to lyse the approximately 150 target cells needed to observe >3 SDs of killing above the mean (data not shown). This would indicate that not all cells within a CTL clone are capable at any given time of efficiently lysing appropriate target cells, and could be one explanation for the discrepancy between our data and that of Gotch et al. (14).

Precursor frequencies of HIV envelope-specific CTL have been described (13). LDA of HIV envelope-specific CTL suggests a high precursor frequency even in seronegative subjects (13). Similar LDA studies in SIV-infected macaques found the effector cell population of envelope-specific killer cells to be composed of both MHC-restricted and nonrestricted cells (30) raising the possibility that the high HIV envelope CTL precursor frequency described on HIV-seronegative subjects may be mediated by nonspecific effector cell mechanisms and not CTL. Since a nonspecific effector cell mechanism for *gag*-specific cytolysis has not been described and *gag*-specific CTL precursors are not found in seronegative subjects, the concerns raised by the envelope CTL work do not apply to the *gag*-specific CTL precursor frequency determinations described here.

The studies described above have implications for the development of an HIV vaccine. HIV *gag* antigens are clearly recognized by the human immune system and a broad CTL response is generated to epitopes within both the p17 and p24 antigens. As such, these antigens may play an important role in protective immunity against this virus infection and should be not overlooked in the development of effective vaccine strategies.

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