Quantitative Analysis of the Human Immunodeficiency Virus Type 1 (HIV-1)-specific Cytotoxic T Lymphocyte (CTL) Response at Different Stages of HIV-1 Infection: Differential CTL Responses to HIV-1 and Epstein-Barr Virus in Late Disease

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Summary

Major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL) are part of the cellular immune response to human persistent virus infections. Measurements of the frequency and specificity of human immunodeficiency virus type 1 (HIV-1)-specific CTL and their variation with time may indicate their relative importance in modulating the progression of HIV-1 infection. We have used limiting dilution analysis (LDA) to derive quantitative estimates of the frequency of HIV-1-specific CTL precursors in a cross-sectional study of 23 patients at different clinical stages of HIV-1 infection and to compare these with the frequency of CTL precursors specific for another persistent virus (Epstein-Barr virus [EBV]) in the same patients. Peripheral blood mononuclear cells (PBMC) were stimulated in vitro with autologous HIV-1-infected lymphoblasts and assayed for cytotoxicity in ^{51}Cr release assays against autologous and MHC-mismatched lymphoblastoid B cells infected with recombinant vaccinia viruses expressing the three HIV-1 structural gene products. The frequency of MHC-restricted precursors was high in asymptomatic HIV-1-infected patients (env-specific CTL precursors up to $73/10^6$ PBMC; gag-specific CTL precursors up to $488/10^6$ PBMC), although the relative frequency against the different structural gene products varied from patient to patient. The HIV-1-specific CTL precursor frequency was reduced in patients who had more severe ($<400/\mu l$) $CD4^+$ lymphocyte depletion, while in the majority of such patients the frequency of CTL precursors against EBV was maintained at levels observed in healthy controls. Direct CTL activity in unstimulated PBMC was observed in three of nine patients but no correlation was found between the presence of an activated CTL response and the magnitude of the CTL response detected after stimulation in LDA. Thus, CTL precursors were detected against all three HIV-1 structural gene products in patients with $CD4^+$ lymphocyte counts $>400/\mu l$, at frequencies that are high compared with those reported for other persistent viruses. A CTL response directed against multiple protein antigens of HIV-1 may protect the patient against epitope variation. The fact that the EBV-specific CTL precursor frequencies were maintained in advanced HIV-1 infection suggests that there may be selective impairment of the HIV-1-specific CTL response associated with disease progression.

The immune response to HIV infection has been extensively studied and the antibody response against the virus has been characterized in detail. Because of the capacity of the virus to spread in cells, there has been increasing interest in the role of cell-mediated immunity, and in particular MHC-restricted CTL in HIV-1 infection (1). These are effector T cells that are able to kill virus-infected cells through recognition of viral protein antigens that have been processed within the infected cell into short peptide fragments and transported to the cell surface and presented in association with MHC molecules. Virus-specific CTL have been shown to play a role in the clearance of acute infections and in the control of persistent virus infections in animal models (2, 3) and in humans (4, 5).

HIV-1-specific CTL have previously been detected in HIV-1-infected patients at different clinical stages of infection and at different sites, including peripheral blood, cerebrospinal fluid (6), and bronchoalveolar lavage fluid (7). In such studies, HIV-1-specific CTL have been shown to recognize antigens derived from the major viral structural gene products (env, gag, pol) and also from some of the nonstructural regulatory gene products (nef, vif, rev) (1). In a proportion of asymp-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/02/0249/08 $2.00
Volume 177 February 1993 249-256

tomatic HIV-1-infected patients, activated HIV-1-specific CTL can be detected directly in unstimulated PBMC without secondary in vitro stimulation. This is unusual since detection of virus-specific CTL in other asymptomatic persistent virus infections normally requires in vitro stimulation with antigen. The fine antigen specificity and MHC-restricting alleles for different HIV-1-specific CTL clones have been defined (1). CD8-positive T lymphocytes have also been shown to inhibit the replication of HIV-1 in vitro by a mechanism independent of direct cellular cytotoxicity (8).

The relative importance of the HIV-1-specific CTL response compared with other components of the immune response in restraining viral replication during asymptomatic HIV-1 infection and the subsequent progression to AIDS is unclear. Quantitative information regarding the HIV-1-specific CTL response might help to define its contribution to the immune response; knowledge of the relative frequency of CTL against different HIV-1 gene products and their fine antigen specificity would assist the rational development of vaccines capable of inducing an effective CTL response. However in contrast to the antibody response against HIV-1, to date little quantitative information is available regarding the HIV-1-specific CTL response. This paucity of information reflects the considerable technical difficulty in measuring the number of antigen-specific effector T cells; the principal technique that has been used is limiting dilution analysis (LDA)[1] (7, 8, 10, 11). Hoffenbach et al. (7) studied the env-specific CTL response in LDA, using as target cells the mouse plasmacytoma cell line P815 stably transfected with both human MHC class I A2 and the env gene of HIV-BRU. In four asymptomatic HIV-1-infected patients, the env-specific CTL precursor frequency was between 270 and 2,000/10^6 PBMC. In one patient there was a decline in CTL precursor frequency over a 6-mo period before the patient developed AIDS. Gotch et al. (10) studied gag-specific CTL in three asymptomatic HIV-1-infected hemophiliacs, and defined the CTL epitope within gag p24 with synthetic peptides. The frequency of peptide-specific CTL precursors was between 120 and 280/10^6 PBMC.

In our previous studies applying LDA to studies of human herpes virus–specific CTL (12, 13), we developed automated methods (14) to quantify virus-specific CTL precursors in larger numbers of patients than has previously been possible. Here we report our quantitative analysis of the CTL response directed against each of the HIV-1 structural gene products, env, gag, and pol, compared with the CTL response against another persistent virus, EBV, in a cross-sectional study of 23 patients at different stages of HIV-1 infection.

Materials and Methods

Patients. 23 adult patients (19 males, 14 females) were recruited at different clinical stages of HIV-1 infection as assessed by clinical features and $CD4^+$ lymphocyte counts. 18 were Center for Disease Control (CDC) stage II, including six who had $CD4^+$ lymphocyte counts in the normal range (530–2,200/μl), one patient was CDC stage IVa, three were CDC stage IVcl, and one was CDC stage IVd. HIV-1 infection was acquired through intravenous drug use in eight, male homosexuality in 11, heterosexuality in three, and through blood products in one patient. All were seropositive for HIV-1 by both ELISAs (Serodia; Fujirebio Inc., Tokyo, Japan) and peptide blotting (Liatek; Organon, Teknika, Holland), and positive for anti-EBV IgG by indirect immunofluorescence on acetone-fixed EBV-infected HR1K cells. EBV-transformed lymphoblastoid B cell lines from each subject were established by standard methods using B95.8 virus (type 1 isolate); all were free of mycoplasma contamination by DNA staining. MHC class I tissue typing was performed using serological lymphocytotoxicity plates (Lymphotype ABC-120; Biotest, Frankfurt, Germany). All subjects underwent clinical and hematological assessment every 3–6 mo. In addition, seven healthy laboratory workers (three males, four females) at low risk of HIV-1 infection were studied as controls.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses expressing specific HIV-1 gene products were grown on Vero cells that were harvested, sonicated, and plaqued on Vero cells (titer, 2.0×10^7 to 10^8 50% tissue culture infectious doses [$TCID_{50}$]/ml). Cell-associated viruses were stored in 1-ml aliquots at −70°C and sonicated before use. The recombinant vaccinia viruses used were vac env (expressing env gp160 5515–8375 of HIV-IIIB; M. Mackett, Manchester, UK), vac gag (expressing gag p55 1–1871 of HIV-IIIB; M. Mackett), vac pol (expressing pol p105 of HIV-BRU; M. Kieny, Transgene, Strasbourg, France), and, as a negative control expressing an irrelevant protein, vac T7 (expressing bacteriophage T7 RNA polymerase; B. Moss, Bethesda, MD), all made available through the MRC AIDS Directed Programme Reagent Project.

Limiting Dilution Analysis. LDA is an in vitro technique by which the CTL response can be analyzed at the level of the individual responding cell. By growing a large number of short-term CTL clones, it is possible to derive quantitative estimates of antigen-specific CTL precursors (9). PBMC were prepared from fresh heparinized venous blood samples by Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway) density gradient centrifugation. Replicate microcultures (n = 27) of fresh PBMC were set up in 96-well round-bottomed plates (Linbro; Flow Laboratories Ltd., Irvine, Scotland) in which the number of PBMC per well was progressively reduced over an appropriate range of dilutions in RPMI 1640 (Flow Laboratories Ltd.) supplemented with 10% (vol/vol) myoclone plus FCS (Gibco Laboratories, Grand Island, NY), 2.0 mM L-glutamine, 10^5 IU/liter of penicillin, and 100 mg/liter of streptomycin (Flow Laboratories).

HIV-1-specific CTL Precursors. Fresh autologous PBMC were superinfected with HIV-IIIB (MOI, 1:100) for 4 h at 37°C in medium supplemented with 4 μg/ml PHA (unpurified PHA-P; Sigma Chemical Co., Poole, UK), washed three times in PBS, and irradiated (2,400 rad). 2×10^4 of these autologous lymphoblasts were added to each well as APC/feeder cells, with human rIL-2 (MRC AIDS Directed Programme) in medium at a final concentration of 5 IU/ml and a final volume of 100 μl. The cultures were incubated at 37°C in 5% CO_2 and were refed with medium supplemented with 5 IU/ml IL-2 on days 5 and 9. On day 15, the cells in each individual well were resuspended and divided into four aliquots that were assayed for cytotoxicity in 6-h ^{51}Cr release assays against 4×10^3 autologous or MHC-mismatched target cells. These comprised lymphoblastoid B cell lines that had been infected for 12 h with different recombinant vaccinia viruses (10 $TCID_{50}$/cell), incubated with radioactive chromium ^{51}Cr (Amersham Corp., Arlington Heights, IL) (100 μCi/2×10^6 cells) for 1 h at 37°C, and washed four times in medium. On each plate six wells with target cells only were incubated with aqueous 2% triton-X (Merck Ltd., Poole, UK)

[1] *Abbreviation used in this paper:* LDA, limiting dilution analysis.

to determine maximum ^{51}Cr release, and target cells were also added to six wells on each plate containing irradiated feeder cells previously cultured without responder cells to determine spontaneous release. Spontaneous release was <30% of maximum release in all experiments. After incubation for 6 h at 37°C, culture supernatants were harvested and the radioactivity counted using a gamma counter (Cobra 5010; Hewlett-Packard Co., Meriden, CT). The assays were automated using a computer-controlled robotic processor (Biomek 1000 Automated Laboratory Workstation; Beckman Instruments, Inc., Palo Alto, CA) (14).

EBV-specific CTL Precursors. Replicate dilutions of fresh PBMC were set up as described above from the same blood samples. As APC/feeder cells, 5×10^3 autologous irradiated (2,400 rad) lymphoblastoid B cells were added to each well in medium supplemented with 5 IU/ml IL-2 final concentration in a final volume of 100 μl. The cultures were refed on days 5 and 9 as above. On day 14 the cells in each well were resuspended and divided into four aliquots that were assayed for cytotoxicity against 4×10^3 ^{51}Cr-labeled autologous or MHC-mismatched lymphoblastoid B cells in a 6-h ^{51}Cr release assay.

Analysis. Each individual well was regarded as positive for cytotoxicity when the ^{51}Cr release exceeded a defined threshold. In previous studies using LDA, a threshold for each target cell type equal to the mean plus 3 SD of radioactivity release in control wells (containing irradiated feeder cells only) has been used (7, 11, 15). However, the threshold calculated this way varies from one target cell type to another over a range equivalent to 3–13% of specific ^{51}Cr release, which alters the sensitivity of the assay between autologous and mismatched targets and between experiments, and which can adversely affect the linearity of the LDA plots. In our experiments, the distribution of MHC-restricted ^{51}Cr release from limiting dilution cultures was biomodal, as expected from wells that contain either no precursors or at least one precursor (16); from this bimodal distribution the threshold that consistently distinguished between wells lacking a precursor or containing a precursor was 10% specific lysis (Carmichael et al., manuscript in preparation). Therefore, all experiments were analyzed using a threshold of 10% specific lysis, i.e., spontaneous release + 0.1 (maximum release − spontaneous release) for that target cell type, which had the advantage of standardizing the sensitivity in all experiments.

Because we used split-well analysis for each target cell type in every experiment, it was possible to identify wells with non-MHC-restricted cytotoxicity (killing of both autologous and MHC-mismatched target cells) that were excluded from further analysis. Limiting dilution plots for MHC-restricted killing were produced by plotting the proportion of negative wells against the initial responder cell number per well on a semilogarithmic plot. Provided the single-hit Poisson model (9) was fulfilled (a straight line relationship demonstrated by the χ^2 value for goodness of fit), the regression line was calculated by the maximum likelihood method (17). From the single-hit Poisson model, the frequency of antigen-specific MHC-restricted CTL precursors was estimated from the initial responder cell number at which 37% of the wells were negative for cytotoxicity. Calculations were performed using Statistical Package for Social Sciences/Personal Computer + (SPSS/PC) + V2.0 (SPSS Inc., Chicago, IL) and GLIM 3.77 (Royal Statistical Society, London, UK), and the estimates of CTL precursor frequency and 95% confidence intervals were expressed per 10^6 PBMC.

Direct Cytotoxicity Assays. Unstimulated fresh PBMC, from the same samples used to determine the CTL precursor frequency, were plated on 96-well round-bottomed plates at five E/T ratios (from 1.6:1 to 100:1) in triplicate, and assayed for cytotoxicity in 6-h ^{51}Cr release assays against 4×10^3 autologous and MHC-mismatched target cells infected with recombinant vaccinia viruses expressing specific HIV-1 gene products as described above. Spontaneous release was determined from six replicate wells containing medium only; maximum release was determined from three replicate wells

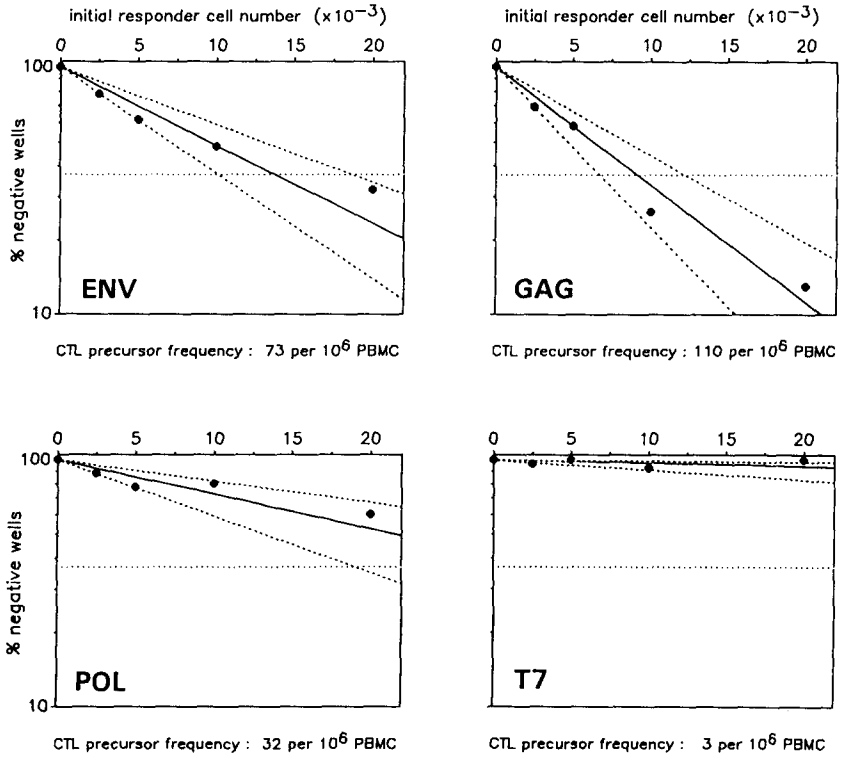

Figure 1. Simultaneous determination of the frequency of CTL precursors against env, gag, and pol using LDA. Fresh PBMC from an asymptomatic HIV-1-infected patient were cocultured with autologous irradiated HIV-IIIB-infected lymphoblasts and IL-2 as described. On day 15, the cultures were assayed for cytotoxicity against autologous and MHC-mismatched lymphoblastoid B cell lines infected with recombinant vaccinia viruses expressing individual structural gene products of HIV-IIIB and negative control T7 RNA polymerase. Limiting dilution plots of MHC-restricted killing are only plotted with the regression line and its 95% confidence intervals (calculated by the method of maximum likelihood). The CTL precursor frequency is estimated from the intercept of the regression line at 37% negative wells: env-specific CTLp ($73/10^6$ PBMC), gag-specific CTLp ($225/10^6$ PBMC), pol-specific CTLp ($36/10^6$ PBMC), negative control ($3/10^6$ PBMC).

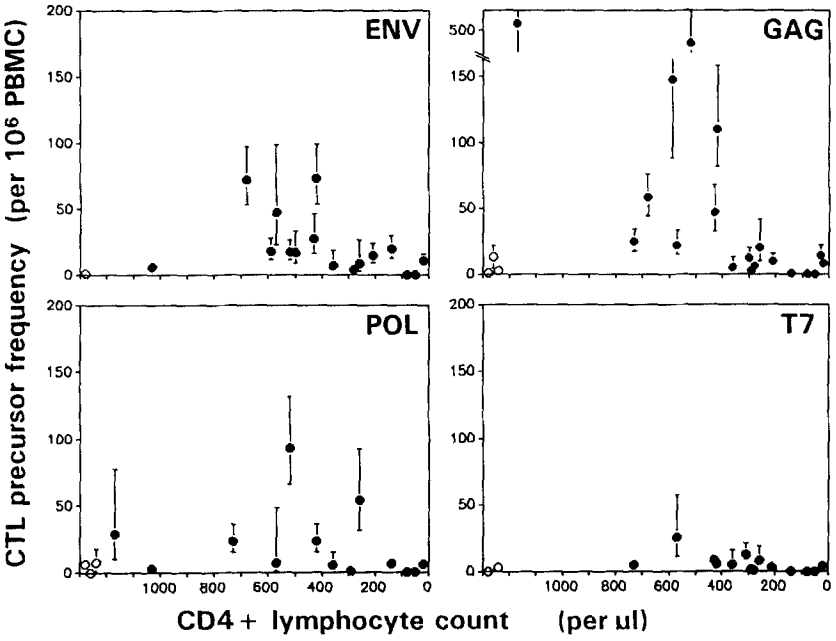

Figure 2. In the cross-sectional analysis, the frequency of HIV-1 specific CTL precursors was reduced in HIV-1-infected patients who had more severe $CD4^+$ lymphocyte depletion. For each patient, the CTL precursor frequencies (with 95% confidence intervals) against target cells expressing env, gag, pol, and negative control T7 are plotted against the patient's $CD4^+$ lymphocyte count decreasing from left to right as an index of the progression of HIV-1 infection. *(Filled symbols)* HIV-1-infected patients. *(Open symbols)* Healthy controls.

to which aqueous 2% triton-X was added. The percent specific lysis was calculated as follows: 100× (test release − spontaneous release)/(maximum release − spontaneous release). The difference between the percent specific lysis of autologous and mismatched target cells at an E/T ratio of 50:1 was assessed by student's t test; differences where $p < 0.05$ were considered significant.

Results

Frequency of HIV-1-specific CTL Precursors. 62 of the 74 experiments yielded linear LDA plots (Fig. 1). The frequency of env-specific CTL precursors was 0–73/10^6 PBMC, for gag-specific CTL precursors, 0–488/10^6 PBMC, and for pol-specific CTL precursors, 0–93/10^6 PBMC (Fig. 2, *filled symbols*). After in vitro stimulation of PBMC in LDA, three healthy HIV-1-uninfected controls showed only strong non-MHC-restricted cytotoxic responses against all target cell types. Three other controls showed low responses that were not HIV-1 specific (Fig. 2, *open symbols*).

Frequency of EBV-specific CTL Precursors. The EBV-specific CTL response was studied in LDA in 20 patients. In seven experiments the frequency of EBV-specific CTL precursors could not be estimated either because of very high levels of non-MHC-restricted killing (which made it impossible to identify any underlying MHC-restricted CTL activity) or because the LDA plot was nonlinear. In the remaining patients, the EBV-specific CTL precursor frequency was between 2 and 579/10^6 PBMC.

Relation between CTL Precursor Frequency and Stage of HIV-1 Infection. In the cross-sectional analysis of the CTL precursor frequency according to the stage of HIV-1 infection in the patient cohort, the CTL precursor frequency was reduced in patients who had more severe (<400/μl) $CD4^+$ lymphocyte depletion. In contrast, in six of eight patients whose $CD4^+$ lymphocyte count was <400/μl, the frequency of

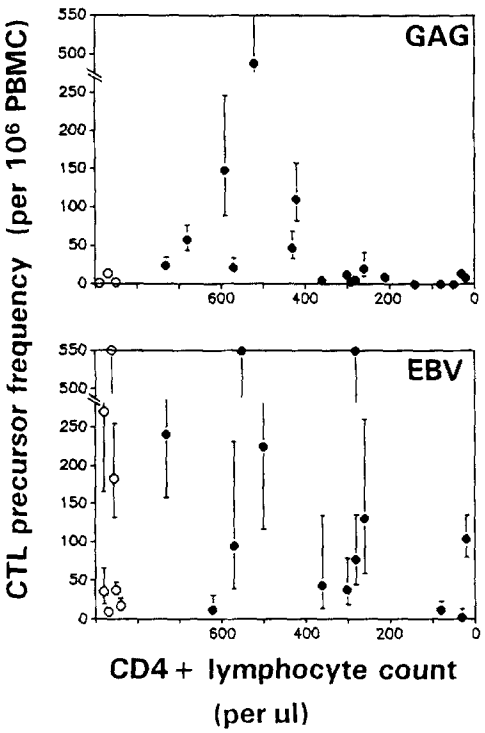

Figure 3. The frequency of EBV-specific CTL precursors may be maintained in advanced HIV-1 infection. Fresh PBMC from HIV-1 infected patients *(filled symbols)* and healthy controls *(open symbols)* were cocultured with autologous irradiated EBV-transformed lymphoblastoid B cells and IL-2 as described. On day 14, cultures were assayed for cytotoxicity against autologous and MHC-mismatched lymphoblastoid B cells, and the frequency of MHC-restricted CTL precursors was plotted against the $CD4^+$ lymphocyte count. In six of the HIV-1 infected patients whose $CD4^+$ lymphocyte count was <400 μl and whose HIV-1 specific response was very low or undetectable *(top)*, EBV-specific CTL precursors were detected at frequencies that are observed in healthy controls *(bottom)*.

Table 1. *Comparison of CTL Precursor Frequency with Unstimulated Cytotoxic Activity*

Patient	CD4+ count	CTL precursor frequency*				Cytotoxic activity in unstimulated PBMC‡							
						ENV		GAG		POL		T7	
		ENV	GAG	POL	T7	aut	mis	aut	mis	aut	mis	aut	mis
	per μl												
1	680	72	58	–§	–	9.3	11.9	1.7	8.8	2.2	14.8	1.5	11.9
2	570	47	22	7	25	4.0	0.0	4.2	0.0	0.0	0.1	0.0	1.6
3	510	7	5	6	5	1.5	0.7	5.2	0.1	1.9	0.0	0.0	1.3
4	450	17	489	93	–	9.8	13.7	10.8	9.4	5.8	6.4	6.0	8.6
5	420	73	110	23	6	10.6	6.2	8.7	4.7	7.0	6.7	3.4	8.9
6	280	4	13	1	1	0.0	0.0	1.9	0.0	0.6	0.0	ND	
7	210	15	9	12	3	7.1	14.1	2.6	5.4	10.3	0.0	3.8	7.3
8	140	9	21	54	8	13.0	4.9	13.4	2.9	11.0	5.8	7.1	3.2
9	50	0	0	1	0	7.8	11.3	0.0	4.7	2.9	5.3	4.3	5.2

* Expressed per 10^6 PBMC using threshold of control plus 10% specific release.
‡ Percent specific lysis at E/T ratio of 50:1 against autologous (aut) and MHC mismatched (mis) target cells. Results that show significant ($p < 0.05$) greater killing of autologous targets compared with mismatched targets are underlined.
§ Indicates that LDA plot was nonlinear.

CTL precursors against EBV remained equal to or higher than that observed in healthy control subjects (Fig. 3).

Direct Cytotoxicity Assays. MHC-restricted CTL in unstimulated PBMC were detected in three of the nine patients studied (Table 1). In patient 8, activated MHC-restricted CTL specific for env (Fig. 4 *a*) and for gag were detected; the corresponding env- and gag-specific CTL precursor frequencies in LDA were $9/10^6$ PBMC and $21/10^6$ PBMC. In patient 7, activated MHC-restricted CTL were detected against target cells expressing pol (Fig. 4 *b*); the corresponding pol-specific CTL precursor frequency in LDA was $12/10^6$ PBMC. In patient 5, activated MHC-restricted CTL were detected against target cells expressing gag (Fig. 4 *c*); the corresponding gag-specific CTL precursor frequency in LDA was $110/10^6$ PBMC. Non-MHC-restricted killing of both autologous and MHC-mismatched target cells was observed in several patients, and this may have obscured the presence of an underlying MHC-restricted component of the response. In the five patients whose unstimulated PBMC showed non-MHC-restricted killing of target cells expressing env, the corresponding frequencies of MHC-restricted env-specific CTL precursors in LDA were 0, 15, 17, 72, and $73/10^6$ PBMC. No correlation was observed between the presence of activated MHC-restricted CTL and the magnitude of the CTL precursor response in LDA (Table 1).

Discussion

The frequency of MHC-restricted CTL precursors in 23 HIV-1-infected patients ranged from 0 to $488/10^6$ PBMC, and the relative frequency against the different structural gene products varied from individual to individual. In cross-sectional

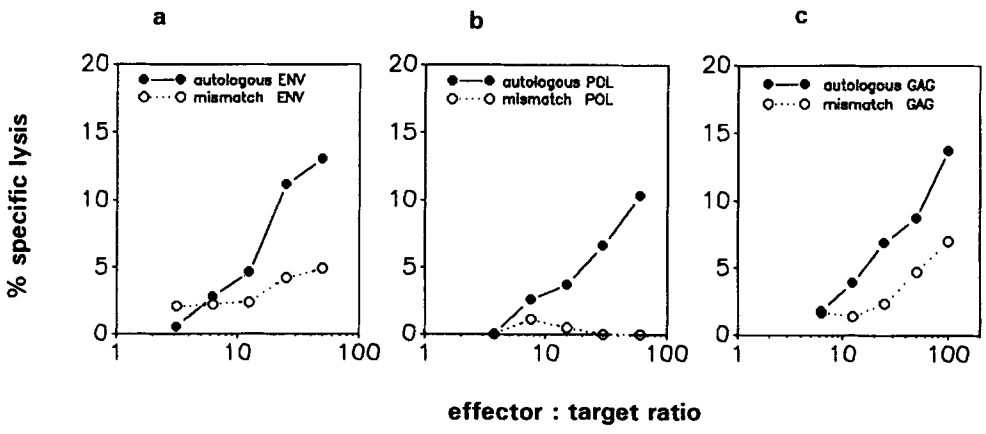

Figure 4. The direct cytotoxic response in unstimulated PBMC was MHC restricted in a proportion of patients. Unstimulated PBMC from three asymptomatic patients were assayed for cytotoxicity against autologous and MHC-mismatched radiolabeled target cells at different E/T ratios. *(a)* Patient 8; *(b)* patient 7; *(c)* patient 5.

analysis, the frequency of HIV-1-specific CTL precursors was reduced in patients with more severe CD4 lymphocyte depletion. In six of eight subjects who had very low HIV-1-specific CTL precursor frequencies, the CTL precursor frequency against EBV was maintained. Activated MHC-restricted CTL in unstimulated PBMC were detected in a proportion of the patients, but no correlation was observed with the magnitude of the CTL precursor response in LDA.

Virus-specific CTL precursors have been demonstrated during acute viral infections in humans, but for influenza-specific CTL after recovery from infection, the CTL activity progressively declines to undetectable levels (4). In contrast, the CTL response to persistent virus infections studied by LDA has shown that the frequency of virus-specific CTL precursors is maintained after initial infection, suggesting that during asymptomatic persistent infection the CTL response is subjected to a varying degree of restimulation in vivo and by inference may play a role in controlling viral replication. For instance, LDA has been used to examine the relative frequency of CTL precursors specific for viral antigens expressed at early and late stages of the virus replicative cycle during asymptomatic persistent infection with human cytomegalovirus (HCMV) (12, 13). LDA can be used to derive quantitative estimates of CTL precursor frequency, but the absolute frequency must be interpreted with caution. Small differences in culture conditions in the LDA, the number of aliquots of responder cells derived from each well, and differences in the threshold used to determine cytotoxicity or noncytotoxicity can all influence the absolute frequency estimate. The observed frequency estimate indicates the order of magnitude of the CTL response, and makes possible comparisons (between different antigen specificities or between different time points) within the same experimental system. Previous studies have shown that part of the cytotoxic cellular response against lentiviruses is non-MHC restricted, particularly against target cells expressing the env gene product (18–20). Therefore, in contrast to other recent studies in HIV and SIV (21, 22), we exploited a particular advantage of LDA in using split-well analysis to specifically exclude wells with non-MHC-restricted activity (NK activity) in every experiment, allowing the underlying MHC-restricted CTL precursor response to be quantified even in the presence of an NK response. Because LDA examines a large number of short-term CTL clones derived directly from and representative of the original lymphocyte population, LDA complements studies of the fine antigen specificity of long-term CTL clones where selection during prolonged in vitro culture may result in clones that are less representative of the original population.

CTL precursor frequencies against EBV, HCMV, and varicella-zoster virus (VZV) in healthy laboratory workers have been previously reported, using the threshold of control plus 3 SD in the analysis: EBV-specific CTL precursors (40–100/10^6 PBMC), HCMV-specific CTL precursors (20–80/10^6 PBMC), and VZV-specific CTL precursors (2–10/10^6 PBMC) (12–14). In comparison with these frequencies, the frequency of HIV-1-specific CTL precursors was high in asymptomatic HIV-1-infected patients, supporting the findings of previous studies (7, 10). Furthermore, in the previous analyses of herpes virus–specific CTL precursors, virus-infected cells were used as target cells, so that the observed responses reflect the aggregate CTL responses against cells expressing a constellation of different viral antigens, while the HIV-1-specific CTL responses in this study were directed against target cells expressing single HIV-1 gene products, either env, gag, or pol. Although in analyses of virus-specific CTL it is conventional to include whole virus-infected target cells, this presents difficulties in HIV-1 infection; the number of HIV-1-infected cells accessible from peripheral blood is low in asymptomatic HIV-1 infection, and target cells infected with laboratory strains of HIV-1 may not be representative of autologous viral strains. Work is in progress to develop an appropriate HIV-1-infected target cell system.

The relative frequency of CTL precursors against the different gene products varies from one individual to another. One explanation for this may be differences in antigen processing and presentation by the different MHC alleles in the patients studied. In addition, particular CTL epitopes present in each patient's own virus population may not have been represented in the target cells we used, which expressed the gene products of the HIV-IIIB strain. Nevertheless, in most asymptomatic patients a CTL response could be detected against more than one viral gene product (presumably recognizing epitopes conserved between the patient's own viruses and HIV-IIIB). As reported recently by others, a particular CTL epitope within gag may undergo sequence variation in vivo that is no longer recognized by the CTL response (23), and sequence variation in CTL epitopes within env may impair CTL recognition (24). However, the fact that the CTL response recognizes more than one protein in the same patient suggests that a change in single epitope would not necessarily abrogate the entire CTL response.

In the cross-sectional analysis of HIV-1-specific CTL precursors in this cohort of patients, the frequency of CTL precursors detected in LDA was reduced in those who had severe $CD4^+$ lymphocyte depletion. This might result from an inability to activate and detect in vitro the CTL precursors from patients with advanced HIV-1 infection. Possible mechanisms might include impaired antigen presentation in vitro, lack of lymphokines essential for activation and differentiation of CTL (including lymphokines secreted by $CD4^+$ T lymphocytes), the presence of suppressor cells, and failure of the target cell system to present particular antigenic variants that are recognized by the CTL responses of each individual. To determine whether there is a global impairment of the ability to generate a CTL response, or whether there might be a selective impairment of the HIV-1-specific CTL response, we carried out parallel experiments to determine the CTL precursor frequency against EBV in the same HIV-1-infected patients. The EBV-specific CTL response was maintained in six of eight patients whose HIV-1-specific CTL response was very low, suggesting that at least in some individuals there may be selective impairment of the HIV-1-specific CTL response. A possible explanation for this might be differential requirements for $CD4^+$ T cell help in the generation of the

CTL responses against the two different viruses, HIV-1 and EBV (25). Alternatively, there may be a genuine reduction in the numbers of HIV-1-specific CTL precursors as HIV-1 infection progresses. This raises the question of clonal exhaustion resulting from continuous stimulation of precursors, with inability to regenerate the memory cell pool because of impairment of dendritic cell function (26).

Little is known about the factors important in the terminal differentiation of human effector T cells. The direct cytotoxicity assays demonstrated the presence of circulating activated HIV-1-specific CTL (which did not require in vitro stimulation), but, as noted by others (10, 27), MHC-restricted HIV-1-specific CTL responses were detected in only a proportion of HIV-1-infected patients, while non-MHC-restricted cytotoxicity was observed in several patients, particularly against targets expressing env. No correlation was observed here between the magnitude of the CTL response detected in LDA and the direct unstimulated response against target cells expressing the same HIV-1 structural gene product.

The observed decline in CTL precursors specific for HIV-1 as disease progresses raises interesting questions concerning the possible protective role of the CTL response. This is now being addressed by a longitudinal study of the HIV-1-specific CTL response in the infected patients of this cohort. Detailed study of lentivirus-specific CTL presents greater complexities than those against other viruses, because the heterogeneous virus population in vivo gives rise to a changing mixture of viral epitopes that evolves with time (28) and has the capacity to evade CTL recognition (23). Studies are now in progress to analyze the CTL response in relation to the viral variants that are present in each patient's own HIV-1 quasispecies. In addition, the approaches described here are appropriate to study cellular responses to vaccination and immunotherapy.

We are grateful to Jean Rooney for excellent technical assistance, to Dr. Tim Wreghitt for the serological assays, and for the cooperation of our clinical colleagues Drs. Chris Carne, Chris Sonnex, and David Rowen.

This work was supported by the Medical Research Council's AIDS Directed Programme.

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Received for publication 7 August 1992 and in revised form 20 October 1992.

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