Human Immunodeficiency Virus Type 1 (HIV-1)– and Epstein-Barr Virus–Specific Cytotoxic T Lymphocyte Precursors Exhibit Different Kinetics in HIV-1–Infected Persons

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The frequencies of human immunodeficiency virus type 1 (HIV-1) Gag– and Epstein-Barr virus (EBV)–specific cytotoxic T lymphocyte precursors (CTLp) were studied longitudinally in peripheral blood mononuclear cells from 9 HIV-1–infected persons. By antigen-specific stimulation, HIV-1 Gag–specific CTLp were detected in vitro throughout the course of HIV-1 infection, even after the onset of overt disease. In 4 patients, however, HIV-1 Gag–specific CTLp frequencies declined over time in the presence of maintained EBV-specific CTLp. This decline was correlated with decreasing CD4 ($r = .38; P < .05$) and CD8 ($r = .75; P < .001$) cell numbers. The maintenance of EBV-specific CTLp in patients with low CD4 cell numbers indicated that EBV-specific CTL-mediated immunity may remain longer unaffected by HIV-1–induced immune dysfunction. Consistent with this observation, the growth of EBV-specific CTL could be supported in vitro by EBV-infected lymphoblastoid B cell lines, independent of both CD4 cells and exogenous cytokines.

Human immunodeficiency virus type 1 (HIV-1)–specific cytotoxic T lymphocytes (CTL) are generated in most HIV-1–infected persons and are generally believed to play a role in the containment of virus spread [1–4]. The relatively conserved HIV-1 Gag protein is a well-described target of the CTL response against HIV-1: Gag-specific CTL of the CD8 and major histocompatibility complex (MHC) class 1–restricted phenotype have been detected in most asymptomatic HIV-1–infected persons, even without in vitro restimulation [5]. In addition, limiting dilution analyses have shown that the frequencies of Gag-specific CTL precursors (CTLp), although varying among HIV-1–infected persons, can be remarkably high in peripheral blood mononuclear cells (PBMC) from some asymptomatic persons [6–9]. Even though this strong response appears eventually incapable of controlling HIV-1 infection and preventing progression to AIDS, its decline as disease advances may suggest that significant, albeit temporary, antiviral effects are provided by HIV-1–specific CTL [9–12]. The mechanisms of CTL decline, however, have not been fully elucidated and it cannot be ruled out that this decline is a consequence rather than the cause of the progression of HIV-1 infection.

Like HIV-1, Epstein-Barr virus (EBV) causes a life-long infection and induces strong and long-lasting CTL immunity [13]. Unlike HIV-1 [14], EBV establishes a state of true latency in its host, interrupted by intermittent cycles of replication [15]. Substantial evidence indicates that virus-specific CTL play a part in controlling EBV infection of B lymphocytes and in preventing the outgrowth of EBV-induced lymphoproliferative disorders [16–18]. Lymphoproliferative disorders occur with increased incidence in patients with primary or acquired immunodeficiency, including transplant recipients undergoing immunosuppressive therapy and patients with AIDS. Interestingly, EBV sequences have been detected in virtually all transplant-related but in only 25%–50% of AIDS-related lymphoproliferative disorders [19, 20]. It is also noteworthy that in HIV-1–infected persons, the majority of EBV-induced lymphoproliferative disorders occur in the central nervous system, where they may escape immunosurveillance. These observations suggest that HIV-1–infected persons may retain sufficient immunocompetence to control EBV infection in advanced stages of disease. Consistent with this hypothesis, a cross-sectional study has suggested that the frequencies of EBV-specific CTLp detectable in HIV-1–infected persons can be similar to those measurable in HIV-1–seronegative healthy persons [9]. In contrast, an earlier cross-sectional study showed that the levels of EBV-specific CTL immunity are low in HIV-1–infected patients and decline further during disease progression [21]. The occurrence of a selective dysfunction of HIV-1–specific CTL in the presence of maintained CTL activity against EBV and the possible underlying mechanisms remain a matter of debate.
Here we present the results of a longitudinal study aimed at gaining further insight into the kinetics of HIV-1 Gag- and EBV-specific CTLp, and their relationship with circulating CD4 and CD8 cells, during progression of HIV-1 infection.

Materials and Methods

Study Group

The study included 9 HIV-1-infected adults recruited from a cohort monitored at Utrecht University Hospital. HIV-1 infection was related to intravenous drug use in 1 case, to high-risk heterosexual contacts in 6 cases, and to high-risk homosexual or bisexual contacts in 6 cases. Six patients (Ew03, Fw19, Fw07, Gw21, Gw38, Gw39) remained asymptomatic throughout the study, whereas 3 patients (Gw43, Hw39, and Lw40) had clinical manifestations of AIDS (CDC stages IV-A and IV-D) at the baseline visit. Total leukocyte counts were determined according to established National Institutes of Health procedures.

PBMC Preparation

Blood samples were obtained from each patient at the baseline visit and at two or three consecutive time points. PBMC were separated from heparinized blood by density gradient centrifugation on ficoll-hypaque (Lymphoprep; Nycomed, Oslo), then washed three times in RPMI 1640 containing penicillin (100 U/mL), streptomycin (100 μg/mL), 10^{-5} M β-mercaptoethanol, and 2 mM L-glutamine (complete medium), and cryopreserved in liquid nitrogen until use. Cell viability (as assessed by trypan blue exclusion) exceeded 95% after thawing.

Immunomagnetic Cell Fractionation

Magnetic beads coated with anti-CD8 or anti-CD4 monoclonal antibodies (MAbs) (Dynabeads M-450; Dynal, Oslo) were used for cell separation as described [22]. Briefly, cells were resuspended in complete medium supplemented with 2% fetal calf serum (FCS) and mixed with magnetic beads at a 10:1 target cell-to-bead ratio. After incubation for 60 min at 4°C on a Rock-n-Roller (Snijders TachaBead; Dynal) as described [22].

Generation of B Lymphoblastoid Cell Lines

EBV-transformed B lymphoblastoid cell lines (B-LCL) were established by incubation of PBMC with cell-free supernatant from the EBV-producing marmoset cell line B95-8 (American Type Culture Collection, Rockville, MD), in the presence of 3 μg/mL cyclosporin A (Sandoz Pharma, Basel, Switzerland). B-LCL were maintained in complete medium supplemented with 10% FCS (R-10).

Preparation of HIV-1 Gag Antigen—Presenting Cells (APC)

APC expressing the p55 protein of HIV-1(A1) were prepared as described [23]. Briefly, autologous B-LCL were infected overnight with the recombinant vaccinia virus (rVV) TG1144 (Transgene, Strasbourg, France) [24] at an MOI of 10 and then fixed in 1.5% paraformaldehyde. Antigen expression was confirmed by immunofluorescent analysis using a polyclonal bovine anti-vaccinia serum (RIVM, Bilthoven, Netherlands) and the murine anti-p24 MAb CLB14 (CLB, Amsterdam) as described [23].

HIV-1 Gag—Specific CTL

Limiting dilution cultures. At least four PBMC dilutions in R-10 were seeded in 96-well round-bottomed plates, and each dilution was done in at least 24 replicate wells. Appropriate numbers and ranges of dilutions and numbers of replicate wells were predetermined for each patient with baseline PBMC samples. Cells were cultured with HIV-1 Gag APC (10^5/well), autologous irradiated (2500 rad) feeder PBMC (10^4/well), and recombinant interleukin-2 (rIL-2) at 10 U/mL (final concentration) from day 3. To study CTLp activation requirements, PBMC and positively isolated CD8 cells were cultured in limiting dilution with HIV-1 Gag APC (10^5/well), with or without rIL-2 (10 U/mL), as indicated. After 14 days, two 50-μL aliquots from each well were screened for cytotoxicity against autologous B-LCL infected overnight with either HIV-1 Gag rVV TG1144 or vaccinia control 186 poly (10 MOI). To analyze MHC restriction, HIV-1 Gag—infected B-LCL from MHC class I–mismatched donors were used as targets in additional experiments.

Bulk cultures. Positively selected CD8 or CD4 cells (2 × 10^6 well) in R-10 were seeded in 96-well round-bottomed plates, in the presence of HIV-1 Gag APC (10^5/well), with or without autologous irradiated (2000 rad) CD4 cells (2 × 10^6 well), and with or without rIL-2 (20 U/mL) from day 3, as indicated. After 14 days, cultures were screened for cytotoxicity against autologous B-LCL infected with either HIV-1 Gag rVV or vaccinia control, at indicated effector-to-target cell (E:T) ratios.

EBV-Specific CTL

Limiting dilution cultures. Parallel cultures were set up as described above using PBMC from the same cell sample. Autologous irradiated (5000 rad) B-LCL infected overnight with EBV were used as stimulator cells at 10^2–10^5/well (stimulator-to-effector cell ratio, <1:1), in the presence of autologous irradiated feeder PBMC (10^5/well) and rIL-2 (10 U/mL) from day 5. To study CTLp activation requirements, positively isolated CD8 cells were cultured with irradiated stimulator B-LCL alone. After 14 days, two 50-μL aliquots from each well were screened for cytotoxicity against autologous and MHC class I–mismatched EBV-infected B-LCL.
Bulk cultures. PBMC (10⁶/mL) were cultured in 24-well plates with autologous irradiated stimulator B-LCL (10⁶/mL), autologous irradiated feeder PBMC (10⁵/mL), and rIL-2 (10 U/mL) from day 5. Alternatively, positively selected CD8 or CD4 cells (10⁵/mL) were cultured with or without irradiated (2000 rad) CD4 cells (10⁵/mL), and with or without rIL-2 (10 U/mL), in the presence of autologous irradiated stimulator B-LCL at indicated stimulator-to-effector cell ratios. In additional experiments, autologous B-LCL were treated with paraformaldehyde as described [23] before use as stimulator cells at 10⁶/mL. After 10 days, cultures were screened for cytotoxicity against autologous and MHC class I–mismatched or partially matched B-LCL, at indicated E:T ratios. EBV-specific CTL lines were established by periodic restimulation (every 14–20 days) with irradiated stimulator B-LCL, in the presence of autologous irradiated feeder PBMC and rIL-2 (20 U/mL).

Immunofluorescence Analysis

Circulating CD3, CD4, and CD8 cells, cell fractions after immunomagnetic separation, and expanded HIV-1 Gag– and EBV-specific CTL were analyzed in double-color immunofluorescence analysis using fluorescein isothiocyanate– or phycoerythrin-conjugated anti-CD3, anti-CD4, and anti-CD8 MABS (Becton Dickinson, Etten-Leur, Netherlands).

Cytotoxicity Assay

Cytotoxicity was measured in standard ⁵¹Cr release assays. Targets were labeled for 1 h with 100 μCi of ⁵¹Cr at 37°C in 7% CO₂, washed three times, resuspended in R-10 at 10⁵/mL, then added to effector cells at 50 μL (5000 cells)/well in 96-well round-bottomed plates (Costar, Cambridge, UK). After a 5-h incubation at 37°C in 7% CO₂, supernatants were harvested (Skatron harvester; Skatron, Oslo), and the release of ⁵¹Cr was measured in a gamma counter. Maximum ⁵¹Cr release was determined by detergent (5% Triton X-100) lysis of targets. Spontaneous release was determined by incubation of target cells in R-10 alone. Spontaneous release was <25% of maximum release in all reported assays.

Calculation of Results

Percentages of lysis were calculated as follows: % lysis = [(experimental release - spontaneous release)/(maximum release - spontaneous release)] × 100. Individual wells of limiting dilution cultures were considered positive when lysis of specific targets exceeded by 10% that of control targets if the latter was <10% or by 20% if the latter was >10%. The reliability of these thresholds was tested by pilot experiments in which, after split-well CTL assays, residual cells of limiting dilution cultures were restimulated to generate short-term CTL lines (data not shown). CTLp frequencies were estimated by the maximum likelihood method using the statistical software package described by Strijbosch et al. [25]. All frequencies were normalized to the number of CTLp/10⁶ PBMC. Rates of change (slopes) and correlation coefficients (r) were calculated by linear regression analysis.

Results

Kinetics of HIV-1 Gag– and EBV-Specific CTLp

The frequencies of HIV-1 Gag– and EBV-specific CTLp were measured longitudinally in 9 HIV-1–infected subjects. Of these, 3 (Ew03, Fw19, Gw21) remained asymptomatic and maintained CD4 cell numbers above 200/μL throughout the study, 3 (Fw07, Gw38, and Gw39) remained asymptomatic but their CD4 cell numbers declined to below 200/μL, and 3 others (Hw39, Lw40, and Gw43) had clinical manifestations of AIDS (CDC stages IV-A and IV-D; figure 1). Essentially two response patterns could be distinguished: HIV-1 Gag–specific CTLp were maintained or increased in 5 patients (figure 1A), whereas they declined in the remaining 4 patients (figure 1B). In contrast, the frequencies of EBV-specific CTLp were maintained or even increased over time in all 9 patients. Figure 2 summarizes the rates of change (slopes) of CTLp frequencies, as calculated by linear regression analysis of the longitudinal data.

Reproducibility of CTLp Frequency Estimates

Precautions were taken to reduce the influence of variations in experimental conditions on the outcome of CTLp measurements. First, to allow comparison, HIV-1 Gag– and EBV-specific CTLp were measured in parallel in the same PBMC sample. Second, to improve the goodness of fit of limiting dilution analysis lines, pilot limiting dilution assays were carried out with baseline PBMC samples, providing optimal numbers and ranges of dilutions and numbers of replicate wells for each subject [25]. Subsequently, PBMC from three or four time points were tested simultaneously using identical culture and assay conditions. The reproducibility of CTLp measurements was verified in 22 replicate experiments, which showed a mean coefficient of variation (as 100× SDs of the residuals/mean CTLp frequency estimate) of 7.4% ± 3.8% (data not shown).

Correlation of HIV-1 Gag–specific CTLp Frequencies with CD4 and CD8 Cell Numbers

The patterns of response in the 9 subjects appeared to be independent of the clinical status at baseline, as shown by the presence of symptomatic patients (i.e., Gw43, Lw40, and Hw39) in each group. A clear relationship was detected between the kinetics of HIV-1 Gag–specific CTLp and those of circulating CD8 and, to a lesser extent, CD4 cell numbers (figure 1). A cumulative analysis of this relationship is shown in figure 3. The frequencies of HIV-1 Gag–specific CTLp showed a significant correlation (r = .38; P < .05) with the absolute numbers of CD4 cells. A stronger and more significant correlation (r = .75; P < .001) was found between HIV-1 Gag–specific CTLp frequencies and the absolute numbers of CD8 cells. A significant correlation was also observed between
Figure 1. Kinetics of HIV-1 Gag– and EBV-specific cytotoxic T lymphocyte precursors (CTLp): their relationship to absolute nos. and % of CD4 or CD8 cells in 9 HIV-1 infected subjects with maintained or increasing (A) or decreasing HIV-1 Gag–specific CTLp (B). CTLp frequencies were determined by limiting dilution analyses of peripheral blood mononuclear cells (PBMC). Frequencies were normalized to no. of CTLp·10^6 PBMC. Error bars indicate 95% confidence intervals.
**Figure 2.** Rates of change (slopes) of HIV-1 Gag- and EBV-specific cytotoxic T lymphocyte precursor (CTLp) frequencies, determined from linear regression analysis of longitudinal CTLp data in figure 1. Arrows indicate symptomatic (CDC stage IV) patients. Positive slopes indicate increase, negative slopes correspond to decrease. CTLp frequencies were considered maintained if slope with SE overlaps range of $-1$ to $+1/10^6$ PBMC/month (dotted lines).

**Figure 3.** Frequencies of HIV-1 Gag (right)– and EBV (left)-specific cytotoxic T lymphocyte precursors (CTLp) in peripheral blood mononuclear cells (PBMC) from 9 HIV-1-infected subjects at 3 or 4 time points, plotted against patients' absolute CD4 (top) and CD8 (bottom) cell nos. Frequencies were normalized to no. of CTLp/10⁶ PBMC.
Figure 4. Single-well analysis of cytotoxic responses against HIV-1 Gag. Baseline peripheral blood mononuclear cells (PBMC) from patient Fw07 were seeded in 6 dilutions (range: 20,000–1000 cells/well), each including 24 replicate wells. After 14 days, 3 aliquots from each well were screened for cytotoxicity against autologous (A) and major histocompatibility complex (MHC) class I–mismatched (B) B lymphoblastoid cell lines (B-LCL) infected with HIV-1 Gag recombinant vaccinia virus (rVV) and against autologous B-LCL infected with vaccinia control (C). D, % of negative wells plotted against initial cell no. Linearity of relationship is consistent with single-hit Poisson model. Frequency of HIV-1 Gag–specific CTLp (1/10,100, or 99/10⁶ PBMC) was estimated by maximum likelihood method (solid line interpolated at 37% negative wells). Dotted lines indicate 95% confidence intervals (74–124/10⁶ PBMC).

Phenotype of HIV-1 Gag–specific CTLp

We have previously shown that in vitro stimulation of PBMC from HIV-1–infected subjects with HIV-1 Gag APC promotes the expansion of HIV-1 Gag–specific CTL of the CD8 and MHC class I–restricted phenotype [23]. In agreement with our previous observations, immunofluorescence analyses done on day 14 or 15 of limiting dilution cultures showed that cell populations in wells containing HIV-1 Gag–specific CTLp were predominantly (>85%) of the CD3CD8 phenotype and <1% were of the CD3CD4 phenotype (data not shown). Furthermore, as shown in figure 4 for patient Fw07, additional limiting dilution analyses confirmed that cytotoxic responses to HIV-1 Gag were directed predominantly against autologous rather than MHC class I–mismatched targets.

Activation Requirements of HIV-1 Gag–specific CTL

Limiting dilution assays. We studied the effects of CD4 cell depletion on the expansion of HIV-1 Gag–specific CTLp in patients Gw43 (baseline) and Hw39 (at 2 months). Limiting dilution analyses were done with PBMC and highly pure (>99%) positively isolated CD8 cells in the presence of HIV-1 Gag APC and rIL-2. As shown in table 1, the frequencies of HIV-1 Gag–specific CTLp estimated in the positively isolated CD8 cell fractions were only slightly lower than those measured in PBMC. This indicated that the absence of CD4 cells did not have adverse effects on CTLp activation and expansion.

To study the requirement for exogenous rIL-2, PBMC and the positively selected CD8 cell fraction from patient Gw43 were

<table>
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<th>Effector cells</th>
<th>rIL-2*</th>
<th>Gw43</th>
<th>Hw39</th>
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<tbody>
<tr>
<td>PBMC</td>
<td>+</td>
<td>259</td>
<td>33</td>
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<tr>
<td>CD8' fraction</td>
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<td>NT</td>
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<tr>
<td>CD8’ fraction</td>
<td>-</td>
<td>31</td>
<td>NT</td>
</tr>
</tbody>
</table>

NOTE. NT, not tested because of limited supply of PBMC samples. To facilitate comparison, all frequencies were normalized to no. of CTLp/10⁶ PBMC (95% confidence intervals).

* Recombinant interleukin-2 (rIL-2) was added to cultures on days 3, 7, and 9.
cultured in limiting dilution with HIV-1 Gag APC alone. The expansion of HIV-1 Gag–specific CTLp was reduced in the PBMC and, to a greater extent, in the positively isolated CD8 cell fraction (table 1). This indicated that, particularly in the absence of CD4 cells, exogenous rIL-2 was required to promote CTLp growth.

**Bulk assays.** The results of limiting dilution assays were confirmed in bulk assays of highly pure (>99%) positively isolated CD8 and CD4 cell fractions from patient Gw43. As shown in figure 5 (line A), HIV-1 Gag–specific CTL were expanded from CD8 cells cultured with HIV-1 Gag APC, autologous irradiated CD4 cells, and rIL-2. The positively selected CD4 cell fraction cultured in the same fashion showed a cytotoxic response mostly directed against control targets (figure 5, line B). Consistent with the results obtained in limiting dilution, HIV-1 Gag–specific CTL were expanded from CD8 cells cultured with HIV-1 Gag APC and rIL-2 in the absence of CD4 cells (figure 5, line C). Finally, no CTL were detected in CD8 cells cultured with the same APC in the absence of both CD4 cells and rIL-2.

### Effects of CD4 Cell Enrichment on the Expansion of HIV-1 Gag–specific CTLp

Patient Hw39 showed a marked decrease of both HIV-1 Gag–specific CTLp and CD4 cell numbers, while maintaining stable CD8 cell numbers, between the second (2 months) and third (10 months) assessment (figure 1B). To study whether CTLp detection at 10 months could be enhanced by CD4 cell enrichment, the positively isolated CD8 cell fraction from time point 10 months (>99% CD8 cells) was reconstituted with the CD8 cell–depleted fraction from time point 2 months (31% CD4 cells, <2% CD8 cells) before stimulation in limiting dilutions. The reconstituted population contained 57% CD8 cells from time point 10 months and 13% CD4 cells from time point 2 months. In parallel, limiting dilution cultures were set up with PBMC from time points 2 months (14% CD4 cells, 57% CD8 cells) and 10 months (5% CD4 cells, 59% CD8 cells). Despite CD4 cell reconstitution, the frequency of HIV-1 Gag–specific CTLp in the reconstituted population (figure 6, bottom) was similar to that measured in PBMC from time point 10 months (figure 6, center) and still considerably lower than that measured in PBMC from time point 2 months (figure 6, top).

**Phenotype of EBV-specific CTLp**

As also observed with HIV-1 Gag–specific CTLp, immunofluorescence analyses done on day 14 or 15 of limiting dilution cultures showed that cell populations in wells containing EBV-specific CTLp were predominantly of the CD3CD8 phenotype (data not shown). This observation suggested that EBV-specific CTL were indeed CD8 cells and was consistent with the detection of cytotoxic responses restricted predominantly to autologous targets. However, in both limiting dilution (data not shown) and bulk cultures of positively isolated CD8 cell fractions (figure 7), the phenotype of cytotoxic cells against EBV was influenced by the ratio of stimulator to effector cells: Ratios below 1:1 were required to favor the growth of MHC class I–restricted CTL over that of unrestricted cytotoxic cells.

**Characterization of EBV-specific CTL in Patient Gw43**

To analyze further the phenotype of EBV-specific CTL, bulk PBMC cultures were done with stimulator B-LCL at 1:10 stimulator-to-effector cell ratios and separated into positively iso-
Figure 6. Frequency analysis of HIV-1 Gag-specific cytotoxic T lymphocyte precursors (CTLp) in patient Hw39. Effector cells were peripheral blood mononuclear cells (PBMC) from time points 2 and 10 months (PBMC<sub>2</sub>, PBMC<sub>10</sub>, respectively) and positively selected CD8 cells from time point 10 months (CD8<sup>+</sup><sub>10</sub>) reconstituted with CD8-depleted fraction from time point 2 months (CD8<sup>-</sup><sub>2</sub>). Parallel cultures were set up in presence of HIV-1 Gag antigen-presenting cells and recombinant interleukin-2 from day 3. After 14 days, cytotoxicity was measured against autologous B lymphoblastoid cell lines infected with either HIV-1 Gag recombinant vaccinia virus or vaccinia control. CTLp frequencies were 33 (23-44), 7 (3-11), and 6 (2-9)/10<sup>6</sup> PBMC, respectively.

Figure 7. Effects of stimulator-to-effector cell ratios on cytotoxicity against EBV. Positively isolated CD8 cells from patient Gw43 were cultured with irradiated stimulator B lymphoblastoid cell lines (B-LCL), autologous irradiated CD4 cells, and recombinant interleukin-2 from day 5. After 10 days, cytotoxicity was measured against autologous and major histocompatibility complex (MHC) class I-mismatched B-LCL. Results are expressed as mean specific lysis with SE from triplicate well estimations at effector-to-target cell ratio of 30:1.

Figure 8. Frequency analysis of autologous CD8 and CD8-depleted fractions of effector cells. PBMC 100 (top) and CD8<sup>+</sup><sub>10</sub> (bottom) from patient Gw43 were cultured with HIV-1 Gag antigen-presenting cells and recombinant interleukin-2 from day 3. Cytotoxicity was measured against autologous B-LCL infected with either HIV-1 Gag recombinant vaccinia virus or vaccinia control. CTLp frequencies were 141/10<sup>6</sup> PBMC for PBMC<sub>10</sub> (top) and 242/10<sup>6</sup> cultured CD8<sup>-</sup> cells, equal to 141/10<sup>6</sup> PBMC (bottom).

Figure 9. Frequency analysis of EBV-specific CTLp. Limiting dilution assays were done with the positively isolated CD8 cell fraction from patient Gw43 (baseline) in the presence of irradiated stimulator B-LCL alone. As illustrated in figure 9, the frequency of EBV-specific CTLp estimated in the positively isolated CD8 cell fraction (224/10<sup>6</sup> cultured CD8 cells, equal to 141/10<sup>6</sup> PBMC) was similar to that measured in parallel in the PBMC (154/10<sup>6</sup> PBMC).

Activation Requirements of EBV-Specific CTL

Limiting dilution assays. To study the dependence of EBV-specific CTLp on CD4 cells and exogenous rIL-2, limiting dilution analyses were done with the positively isolated CD8 cell fraction from patient Gw43 (baseline), in the presence of irradiated stimulator B-LCL alone. As illustrated in figure 9, the frequency of EBV-specific CTLp estimated in the positively isolated CD8 cell fraction (224/10<sup>6</sup> cultured CD8 cells, equal to 141/10<sup>6</sup> PBMC) was similar to that measured in parallel in the PBMC (154/10<sup>6</sup> PBMC). These results indicated that the activation and expansion of
EBV-specific CTLp was independent of both CD4 cells and exogenous rIL-2.

**Bulk assays.** Bulk cultures of highly pure (99%) positively isolated CD8 and CD4 cell fractions from patient Gw43 confirmed and extended the observations made in limiting dilution. As shown in figure 10, the presence of CD4 cells and rIL-2 (figure 10, line A) was not required to promote the growth of EBV-specific CTL, since this could be entirely supported by irradiated stimulator B-LCL (figure 10, line B). No CTL were detected in the CD4 cell fraction cultured in the same fashion (figure 10, line C). Similarly, no CTL were detected in the CD8 cell fraction cultured with rIL-2 alone, indicating that the presence of stimulator B-LCL was required for CTL activation (figure 10, line D).

**Effects of B-LCL Fixation in Paraformaldehyde**

As also shown previously by Fishwild et al. [26], the ability of stimulator B-LCL to support the growth of EBV-specific CTL was abolished by fixation with paraformaldehyde (figure 10, line E). This inhibition was not corrected by the presence of autologous irradiated CD4 cells but could be partially overcome by the addition of exogenous rIL-2 at the beginning of culture (figure 10, line F). The latter finding indicated that paraformaldehyde-treated B-LCL had retained their ability to activate EBV-specific CTL but lost their ability to promote CTL expansion, probably because of reduced release of soluble factors.

**Discussion**

We report a longitudinal study showing that HIV-1–infected persons may retain EBV-specific CTL responses while losing both their CD4 cells and their CTL responses against the Gag protein of HIV-1.

For CTLp detection we used an antigen-specific stimulation protocol that proved highly efficient in inducing selective expansion of MHC class I–restricted CD8 CTL against HIV-1 Gag [23] (figure 4). As also observed by Lubaki et al. [27], antigen-specific stimulation allowed the detection of HIV-1 Gag–specific CTL responses even in patients with advanced disease (figure 1). The frequency of HIV-1 Gag–specific CTLp differed considerably among patients, ranging from 5 to 306/10^6 PBMC. This is consistent with results obtained in cross-sectional studies of HIV-1–infected persons [6–9]: Frequencies ranging from 58 to 588/10^6 PBMC were reported by Koup et al. [7], whereas Carmichael et al. [9] measured HIV-1 Gag–specific CTLp at frequencies ranging from 0 to ~488/10^6 PBMC. Similarly, the frequency of EBV-specific CTLp differed considerably from patient to patient, ranging from 18 to 355/10^6 PBMC (figure 1), which is also in agreement with observations made by others [9, 13]. We studied extensively the reliability and reproducibility of the limiting dilution analyses in assessing changes in CTL immunity over time. Optimal assay parameters were first determined for each patient with baseline samples, and subsequently PBMC from all time points

![Figure 8](https://example.com/figure8.png)

**Figure 8.** CD8 cell–mediated cytotoxicity against EBV. Peripheral blood mononuclear cells (PBMC) from patient Gw43 were cultured with irradiated stimulator B lymphoblastoid cell lines (B-LCL), autologous irradiated feeders, and recombinant interleukin-2 from day 5. After 10 days, cytotoxicity of total cell population and CD8-enriched (CD8+) and CD8-depleted (CD8−) fractions was measured against autologous (solid symbols) and major histocompatibility complex (MHC) class I–mismatched (open symbols) B-LCL. Et, effector-to-target cell ratio.

**Table 2.** Major histocompatibility complex (MHC) class I–restricted cytotoxicity against EBV.

<table>
<thead>
<tr>
<th>Targets*</th>
<th>MHC class t</th>
<th>% lysis</th>
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<tr>
<td>Gw43</td>
<td>A10, A24 (9), B35, B62 (15)</td>
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<tr>
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<td>A2, A11, B35, B62 (15)</td>
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<td>RV</td>
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<td>Gw38</td>
<td>A3, A31 (19), B44 (12), B13</td>
<td>8</td>
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* Autologous and partially matched EBV-infected B lymphoblastoid cell lines at 15:1 effector-to-target cell ratio.

t Shared MHC class I alleles are underlined.
were tested simultaneously using identical culture and assay conditions. This resulted in a high reproducibility of CTLp measurements, with mean coefficient of variation among replicate experiments of 7.4%.

In two earlier cross-sectional studies, the EBV-specific CTL responses of AIDS patients were compared with those of asymptomatic HIV-1-seropositive and healthy seronegative persons: The relatively high numbers of EBV-specific CTLp detected in some HIV-1-infected persons by Carmichael et al. [9] are in agreement with our observations but seem to contradict the low levels of EBV-specific CTL immunity observed in HIV-1-infected persons by Blumberg et al. [21]. It should be pointed out, however, that the study by Blumberg et al. compared the results of bulk CTL assays rather than those of limiting dilution analyses. The latter approach may indeed increase CTL detection in HIV-1-infected persons [8]. Our longitudinal study extends these initial cross-sectional observations by providing data on individual patients followed for up to 25 months (figure 2), together with evidence of a significant relationship between the kinetics of HIV-1 Gag CTLp frequencies and those of circulating CD4 and CD8 cell numbers (figures 1, 3). Furthermore, we attempted to investigate possible mechanisms underlying EBV-specific CTLp maintenance in otherwise immunocompromised patients.

Several mechanisms have been proposed to explain HIV-1-specific CTL decline during disease progression, including impairment of APC function [28], infection of CD8 cells [29], emergence of CTL suppression [30, 31], and antigenic variation in HIV-1 Gag leading to escape from CTL surveillance [32]. The hypothesis that persistently high levels of antigenic stimulation may first lead to clonal expansion and subsequently to clonal exhaustion of virus-specific CD8 CTL has also been postulated [33, 34]. CD4 T helper cell dysfunction is likely to play an important part in the decline of HIV-1 specific CTL immunity [35–37]. In support of this view, we detected a significant correlation between the numbers of HIV-1 Gag–specific CTLp and those of circulating CD4 cells (figure 3, top left). Previous cross-sectional studies have either denied [38] or confirmed [39] this association, a discordance probably due to different methods of CTL measurement as well as different compositions of the groups of patients studied.

CTL differentiation in vivo is a complex multistep process, which obviously can only be partially explored through in vitro studies. In particular, the degree of dependence on CD4 cell-mediated help is likely to vary in relation to the stage of CTL differentiation and may become less stringent during in vitro recall. In an attempt to study this dependence, we found that the expansion of HIV-1 Gag–specific CD8 CTLp, although requiring the addition of exogenous rIL-2, was not significantly influenced by removal (table 1, figure 5) or enrichment (figure 6) of CD4 cells.

These findings imply that HIV-1–induced CD4 cell dysfunction may affect HIV-1–specific CTL responses at an early stage of CTL differentiation. As a consequence, HIV-1–specific CTL may lose their responsiveness in vitro [12] and eventually disappear [40–42]. Indeed, a progressive disappearance of HIV-1–specific CTL was suggested by the finding that the decline of HIV-1 Gag–specific CTLp was strongly correlated with the decline of circulating CD8 cells (figure 3, bottom left). This strong correlation seems to confirm the view that in the course of HIV-1 infection, the kinetics of circulating CD8 cells are mostly a reflection of the host response against HIV-1 [38, 39].
In contrast with the frequency of HIV-1 Gag-specific CTLp, the frequency of EBV-specific CTLp was not correlated with either CD4 or CD8 cell numbers. Indeed, the most evident differences between the kinetics of HIV-1 Gag-specific CTLp and those of EBV-specific CTLp were seen in the presence of marked CD4 cell decline (figure 1B, figure 3 [top]). This observation indicates that EBV-specific CTL immunity may remain longer unaffected by HIV-1-induced CD4 cell and cytokine dysfunction. Results of in vitro studies seem to support this view. We found that EBV-transformed B-LCL were able to finely regulate (figure 7) and support the growth of EBV-specific CD8 CTL (figure 8) in the absence of both CD4 cells and exogenous cytokines (figures 9, 10), as also reported in HIV-seronegative persons [26]. This ability of EBV-transformed B-LCL efficiently to activate EBV-specific CTL may reside in their high expression of MHC antigens and accessory molecules. Support for CTL expansion may derive from their rich production of soluble factors (figure 10) [26, 43], including IL-12, a cytokine that by itself can induce differentiation of CTLp into mature CTL [44]. Therefore, EBV infection of B lymphocytes in vivo may result in the induction of high-affinity CTL [45], displaying a low degree of dependence from CD4 cell-mediated help from the early stages of their differentiation.

In summary, our data indicate that HIV-1 Gag-specific CTLp can be detected in vitro even after the onset of symptomatic disease, although, in some persons, their numbers decline in correlation with declining CD4 and CD8 cells. The prolonged maintenance of EBV-specific CTLp in these persons may reflect the relative independence of EBV-specific CTL function from HIV-1−induced CD4 cell and cytokine dysfunction, possibly providing one explanation for the relatively low incidence of EBV-related lymphoproliferative disorders in patients with AIDS.

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References


