The relationships between primary human immunodeficiency virus type 1 (HIV-1) Gag–specific cytotoxic T lymphocyte (CTL) frequency, virus load, and CD4 T cell loss were evaluated in a group of 46 HIV-1–infected persons with hemophilia. Freshly isolated peripheral blood mononuclear cells in limiting dilution assays were used to measure HIV-1 Gag–specific CTL frequencies. Concurrent measurements of virus load and lymphocyte surface markers were obtained. No correlation between Gag-specific CTL frequency and concurrent CD4 cell count was observed. A significant inverse relationship was observed between HIV-1 Gag–specific CTL frequency and provirus load as measured by polymerase chain reaction. Subjects with higher CTL frequencies were found to have more stable CD4 cell counts over time. These results provide additional evidence to support the concept that the predominant role of this virus-specific cellular immune response is to limit viral replication and CD4 cell loss in HIV-1 infection.

A vigorous cytotoxic T lymphocyte (CTL) response is generated in most adults with human immunodeficiency virus type 1 (HIV-1) infection [1–6]. Compared with that occurring in other chronic viral infections, this immune response is unique with respect to its breadth, magnitude, and durability. The spectrum of CTL specificities observed in seropositive persons includes most structural and regulatory proteins of HIV-1 [3, 7–10]. Circulating major histocompatibility complex class I–restricted, CD8-positive, “primary” HIV-1–specific CTL have been demonstrated without in vitro stimulation in most adults throughout all phases of disease [1, 2, 6, 11–14]. The frequency of CTL precursors (“secondary” CTL), however, appears to decline as HIV-1 disease advances [13, 15, 16].

The presence of expanded populations of HIV-1–specific CTL in the face of disease progression has raised the issue of whether CTL responses contribute more to the protection of the host by limiting viral replication and spread or to the progression of disease by immunopathogenic mechanisms [17]. The effects of CTL extend beyond the cytolysis of infected cells [18]. Significant effects may also result from cytokines released on CTL activation [19]. These act locally, but less specifically, and bystander (uninfected, neighboring) cells may also be affected [20]. Cytokines elaborated by CTL include interferon-γ and tumor necrosis factors-α and -β. Each of these could contribute to positive or negative regulation of virus expression in infected cells, depending on the cell type. Uninfected cells may also respond to these cytokines with resultant immunopathology [21]. Finally, CD8-positive cells have been shown to secrete substances that inhibit HIV-1 replication in vitro [22–25]. Protective and pathogenic consequences of CTL activity undoubtedly coexist in vivo and counterbalance each other to a certain extent. The outcome of infection (i.e., rapidity of disease progression) may, in part, depend on the predominant role of this immune response.

We have previously demonstrated that a majority (86%) of HIV-1–infected persons with hemophilia have activated, Gag-specific cytotoxic CD8 T lymphocytes in the peripheral blood. Quantitation of these primary Gag-specific CTL demonstrated a frequency of 1/38,000 to 1/86,000 peripheral blood mononuclear cells (PBMC) [2, 4]. These observations have now been extended to better define the relative importance of protective and pathogenic consequences of CTL activity in the HIV-1 infection. We have studied the quantitative relationship of virus-specific CTL frequency, virus load, and correlated these measurements with the loss of CD4 T cells over time.

**Methods**

**Subjects.** The persons described here have been followed by the New England Area Comprehensive Hemophilia Center at the Medical Center of Central Massachusetts–Memorial Hospital, in Worcester, Massachusetts. All are males with hemophilia who...
were infected by contaminated factor infusions, and none included in this report seroconverted after 1985.

Overall, 46 subjects were studied between May 1990 and March 1994. Concomitant immunologic and virologic assays were done on blood drawn during a single clinic visit. Twenty-eight subjects were studied at a single time point, 12 at two different time points, 5 at three time points, and 1 at six time points. The duration of intervals between repeat samplings ranged from 8 to 169 weeks.

The age of these subjects ranged from 11 to 64 years (median, 30.5). The median CD4 T cell count was 324/μL, and 12 subjects (26%) had <200/μL at first measurement of CTL. Thirty-six subjects in this group had additional surface marker data from other visits that enabled calculations of CD4 cell slopes (see below). Thirty subjects (65%) were receiving antiretroviral therapy when first studied.

PBMC separations. Heparinized blood was processed for PBMC within 6 h of venipuncture by use of Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation.

Lymphocyte marker studies. Whole blood immunophenotypic analyses were done with fluorescein isothiocyanate– or phycoerythrin-conjugated monoclonal antibodies (Becton Dickinson, Mountain View, CA) and flow cytometry (FACScan; Becton Dickinson).

p24 antigen assays. Serum or plasma HIV-1 p24 antigen levels were measured by EIA following immune complex dissociation (Coulter Immunology, Hialeah, FL).

Quantitative HIV-1 cultures. End point dilution cultures of plasma or PBMC were done either as described by Ho et al. [26] or by the AIDS Clinical Trial Group consensus protocol (the latter was adopted after April 1991). Plasma cultures done before April 1991 were not included in the qualitative (positive vs. negative) analysis of results, as the sensitivity differed from that of later assays. Whole blood cultures were done as described by Alimenti et al. [27].

Polymerase chain reaction (PCR) studies. Quantitative proviral DNA PCR on PBMC was based on the methodology of Ou et al. [28] as described by Brettler et al. [29]. Dilutions of PBMC lysates (10¹−10⁸ cells) and 8E5 or ACH2 cell lines (2–100 copies of the HIV-1 genome at 1 copy/cell) were processed in parallel. Cell lysates were subjected to 25–30 cycles of amplification with SK38/SK39 gag primer pairs. A 5⁰ end-labeled SK19 probe was used to detect the PCR product after resolution on a 10% polyacrylamide gel. Appropriate negative controls (uninfected PBMC and CEM cell line) were included in each assay. The highest dilution at which an amplification product was detected was used for an end point readout in assays in which the controls demonstrated a sensitivity of <2 copies of HIV-1 genome. Later samples were analyzed by use of a modification of the Amplicor HIV-1 test system (Roche Diagnostic Systems, Branchburg, NJ). This assay uses the same primers to detect HIV-1 gag sequences in DNA prepared from lysates of whole blood. Before amplification, this DNA preparation is serially diluted. The cellular equivalents of DNA within each of these dilutions can be calculated from the complete blood count and differential white blood cell count. The HIV copy frequency in the population of lymphocytes is then determined from the end point of detection. Controls are included in each assay, which ensures a sensitivity of detection of 2–5 copies of proviral DNA. These assays give comparable results when tested in parallel by use of samples (lysates of PBMC and whole blood) from single blood collections (data not shown).

Quantitative viral RNA PCR was done by use of the Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems) with strict adherence to the manufacturer’s protocol. The serum or EDTA-anticoagulated plasma specimens used in these assays were processed within 6 h of phlebotomy and stored at −80°C.

Cytotoxic T lymphocyte assays. Previous studies by this laboratory and others have demonstrated activated or primary CTL in the circulation of infected persons [2, 4]. Freshly isolated PBMC were assayed in a limiting dilution format to quantitate most directly the level of CTL activity. Without in vitro stimulation, these measurements circumvent numerous variables that may affect the clonal expansion of CTL. Of the primary responses present, Gag-specific activity is the most frequently detected and was therefore selected as the antigenic target for limiting dilution analyses.

Target cells in all cases were autologous B-lymphoblastoid cell lines (B-LCL) generated by the transformation of peripheral blood B cells with Epstein-Barr virus as previously described [2]. The recombinant vaccinia virus, vABT141 (expressing HIV-1 Gag, p55; Thorien Biologics, Cambridge, MA) and control vaccinia virus, NYCBH, were used to infect target cells for CTL assays. Standard limiting dilution assays were done as previously described by use of freshly isolated, unstimulated PBMC with dilutions ranging from 100,000 to 1500 PBMC/well [4]. For each dilution of effector cells, the fraction of negative responses was calculated from the number of wells that failed to exhibit ⁵¹Cr release in excess of 3 SD above the mean of spontaneous release controls. CTL frequency was estimated by the maximum likelihood method (statistical program provided by S. Kalamis, Massachusetts General Hospital, Boston) [30]. The HIV-1 Gag–specific CTL frequency was taken to be the difference between frequencies calculated for activity against B-LCL targets infected with recombinant vaccinia virus expressing HIV-1 Gag and activity against B-LCL targets infected with control vaccinia virus. HIV-1 Gag–specific CTL frequency was expressed in terms of CTL/10⁶ CD8±positive T lymphocytes.

Depletion studies. For selected CTL assays, parallel limiting dilution studies were done in which CD8-positive lymphocytes were depleted from freshly isolated PBMC by use of immunomagnetic beads coated with anti-CD8 antibodies (Dynal, Great Neck, NY). For the assays reported, depletion of the CD8 cell population exceeded 90%.

CD4 cell slope calculations and statistical analyses. CD4 cell slopes were calculated by use of least squares regression analyses. The absolute CD4 T cell count at the time of the CTL assay was designated the baseline count. All subjects with three or more absolute CD4 T cell values available for >1 year after measurement of CTL frequency were included. The mean number of CD4 T cell determinations from which the CD4 cell slopes were calculated was 7.44 (range, 3–15). The mean time interval was 2.11 years (range, 1.0–4.3).

The relationships between Gag-specific CTL frequencies, virus titers, and absolute CD4 T cell counts were examined by use of Pearson’s correlation coefficient (r). Significance of the correlation coefficient was assessed with Fisher’s transformation. Mean virus load values for groups defined by quartiles of CTL frequency were compared by the Student’s t test for independent samples. Multiple regression analysis was applied to determine the relative strengths
of relationships between continuous variables. The mean CD4 T cell loss, age, proviral DNA titers, and viral RNA titers for groups defined by median CTL frequency were compared by the Student’s t test. The use of antiretroviral therapy in these groups was compared with Fisher’s exact test. All available data were included in statistical analyses; however, not all assays could be done on all study visits because of limitations in PBMC yield or lack of properly stored plasma samples. The software package StatView version 4.0 (Abacus Concepts, Berkeley, CA) was used for all calculations.

**Results**

**Summary of immunologic and virologic data.** Primary Gag-specific CTL frequencies ranged from 0 (no difference from background) to 516/10⁶ CD₈ lymphocytes, with a mean of 7.9 and a median of 1. Depletion studies on selected subjects in this study and those previously reported have all demonstrated Gag-specific lysis to be CD8 cell–mediated (data not shown) [4]. Virus burden was measured with a variety of assays with differing sensitivities. Plasma culture was positive in 19 (28%) of 68 cases, and serum p24 antigen measured after immune complex dissociation was positive in 15 (21%) of 70 assays. Cell-associated virus titers were measurable in 58 (80.6%) of 72 samples and ranged between 1 and 3125 TCID/10⁶ PBMC (mean, 88). Whole blood cultures were of intermediate sensitivity; 46 (73%) of 63 assays were positive. Proviral DNA was detectable by PCR in 47 (95.9%) of 49 samples and 36 (97.3%) of 37 subjects tested, and frequencies ranged from 3 to 42,000 copies/10⁶ PBMC in positive samples (mean, 2345). Viral RNA was detectable by PCR in 46 (98%) of 47 measurements and 33 of 33 subjects tested, and titers ranged from 732 to 318,412 copies/mL of plasma in positive samples (mean, 29,567). These immunologic and virologic studies are summarized in table 1.

**Primary HIV-1 Gag–specific CTL and virus burden.** All analyses of the relationship between CTL frequency and virus load showed a negative correlation coefficient. The strength of this relationship varied with the assay used for defining the virus burden.

As described above (and in table 1), the quantitative PCR studies provided the most direct and sensitive measurements of virus load in the circulation. There was a significant inverse correlation between the Gag-specific CTL frequency and the number of proviral copies measured by quantitative DNA PCR (figure 1A; n = 46; r = –.401; Fisher’s test, P = .005). The Gag-specific CTL frequency measurements appeared to show an inverse relationship with quantitative plasma viral RNA levels, but this did not reach significance in this group of subjects (figure 1B; n = 43; r = –.241; P = .120).

Corresponding Gag-specific CTL frequency measurements for all available provirus and virus PCR measurements were divided into quartiles for further analysis. The quartile of lowest Gag-specific CTL frequency measurements (quartile 1) was associated with a mean copy frequency of proviral DNA in PBMC that was higher than for each of the remaining quartiles (figure 2A). These differences were significant when quartile 1 was compared with quartiles 2 and 3 (t test, P < .05) but not when quartile 1 was compared with 4 (t test, P = .2). There were no significant differences in mean absolute CD4 T cell counts between these groups (data not shown). The lowest quartile included CTL frequencies of up to 15 Gag-specific CTL/10⁶ CD₈ lymphocytes. A similar quartile analysis of CTL frequencies and viral RNA in plasma indicated a similar trend (figure 2B), but differences in mean levels of viral RNA in plasma were not significant between these groups.

Interestingly, no correlation was found between CTL activity and CD4 T cell concentration (figure 3). This suggested that the strong relationship observed between CTL frequency and provirus load was independent of the CD4 T cell count. As would be expected, proviral DNA titers were inversely related to CD4 T cell counts in a univariate analysis (n = 48; r = –.422; P = .003). In a multiple regression analysis, quantitative proviral DNA PCR results were significantly correlated with both Gag-specific CTL frequencies (r = –.360; P = .008) and CD4 T cell counts (r = –.346; P = .011).

**HIV-1 Gag–specific CTL frequency and disease progression.** All subjects with at least three measurements of absolute CD4 T cell concentrations available for >1 year after the initial measurement of CTL were included in an analysis of CD4 T cell loss over time in relation to CTL activity (n = 36). Comparisons were made between those with ≥70 and <70 Gag-specific CTL/10⁶ CD₈ lymphocytes, the median CTL frequency. Of the 36 subjects, those with CTL frequencies greater than the median had a significantly slower loss of CD4 T cells (t test, P = .017). We reasoned that CD4 cell slopes for subjects with low baseline CD4 T cell counts (baseline defined as the

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<thead>
<tr>
<th>Table 1. Summary of immunologic and virologic measurements of 46 persons with HIV-1 infection.</th>
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</thead>
<tbody>
<tr>
<td>Measurement (n)</td>
</tr>
<tr>
<td>CD4 cell Percentage (72)</td>
</tr>
<tr>
<td>Absolute count/µL (72)</td>
</tr>
<tr>
<td>Gag CTL frequency* (68)</td>
</tr>
<tr>
<td>PBMC culture† (72)</td>
</tr>
<tr>
<td>Whole blood culture‡ (63)</td>
</tr>
<tr>
<td>DNA PCR§ (49)</td>
</tr>
<tr>
<td>RNA PCR∥ (47)</td>
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NOTE. BQL = below quantitation limit.

* No./10⁶ CD₈ T cells.
† TCID/10⁶ peripheral blood mononuclear cells (PBMC).
‡ TCID/mL of whole blood.
§ Proviral DNA copies/10⁶ lymphocytes.
∥ Viral RNA copies/mL of plasma.
groups (table 2). Furthermore, no significant difference in other parameters known to affect disease progression (baseline CD4 T cell count, age, virus load, or treatment) could be demonstrated between these groups.

Similar results were obtained by use of CD4 cell slopes calculated from an interval that included all time points from 0.5–2 years before the CTL measurement to 1–2 years after the measurement (data not shown). This was an effort to increase the number of CD4 cell values from which slopes were derived while focusing on a time interval proximate to the CTL measurement.

Figure 1. Correlation of Gag-specific CTL frequency with proviral DNA in lymphocytes and viral RNA in plasma. A: Gag-specific CTL frequencies (no./10^6 CD8 T cells) with concurrent proviral DNA copy titers (copies/10^6 lymphocytes); n = 46; r = −.401; P = .005. B: Gag-specific CTL frequencies with concurrent viral RNA copy titers (copies/mL of plasma); n = 43; r = −.241; P = .120.

Figure 2. Correlation of Gag-specific CTL frequency with proviral DNA in lymphocytes and viral RNA in plasma: quartile analysis. A: Proviral DNA copy titers (copies/10^6 lymphocytes) associated with quartiles of Gag-specific CTL frequency measurements. Means ± SEs are shown. * t test, P < .05, vs. first quartile. B: Viral RNA copy titers (no./mL of plasma) associated with quartiles of Gag-specific CTL frequency measurements. Means ± SEs are shown.
measurement that would minimize “plateau” effects on CD4 cell slopes as counts decline to low levels.

**HIV-1 Gag–specific CTL frequency over time.** Sequential measurements of CTL frequencies showed no consistent trends; 8 subjects demonstrated a reduction in CTL frequency over time, while 8 subjects demonstrated no change or an increase. Of the 3 subjects who showed a progression to AIDS (CD4 T cells <200/μL) between measurements, only 1 had a decline in CTL frequency. This subject still had a Gag-specific CTL frequency of 180/10^6 CD8 T cells at a CD4 T cell count of 131/μL (a decrease from 290/10^6 CD8 T cells at a CD4 T cell count of 289/μL).

**Discussion**

This study adds to the accumulating information suggesting that the major effects of HIV-specific CTL responses are protective. Perhaps the most compelling of prior observations is the temporal link between emerging CTL responses and dramatic declines in circulating virus loads during acute infection [31, 32]. The significance of this relationship, however, has recently been called into question by mathematical modeling of virus loads in acute infection, which suggests that the decline in virus load is largely a function of the availability of activated CD4 T cells for free virus to infect [33].

Beyond the acute phase of infection is a period of relative clinical quiescence. During this phase, it is now well established that active viral replication is ongoing [34, 35]. Virus-specific CTL activity is also detectable by use of freshly isolated effector cells in most persons during this phase. It has been argued that this persistent, vigorous CTL response is partly responsible for limiting viral replication and CD4 T cell loss during this phase [2, 6]. The loss of HIV-1–specific CTL precursors as disease progresses has lent further supporting evidence to this hypothesis [13, 15, 16]. The relative lack of virus-specific CTL in vertically infected children is a potential explanation for the rapid pace of progression in many of them [36]. Other evidence that cellular immune responses are contributing to the lack of disease progression in some persons was described by Cao et al. [37], who found strong inhibition of viral replication by CD8 T cells from nonprogressors. Finally, animal studies have correlated high levels of CTL activity against simian immunodeficiency virus with lower virus loads and an improved prognosis [38–40].

The beneficial effects of virus-specific CTL may be counterbalanced by immunopathologic effects under certain circumstances. These effects could result from direct cytolytic activity or the release of soluble factors that adversely affect uninfected “bystander” cells [19–21, 41–43]. Surface marker studies measuring the number of activated CD8 T lymphocytes with cytolytic capacity indicate that higher levels of such cell populations are correlated with a greater risk of disease progression [44]. Furthermore, high levels of CTL activity have been associated with specific HIV-1–related conditions: lymphocytic alveolitis [41] and AIDS dementia complex [42]. Analyses of lymph node histopathology over the course of disease have demonstrated high levels of immune activation (including CD8 T cells with HIV-specific cytolytic capacity) in response to
persistent viral replication and trapping in the lymphoid tissues [45]. These pathologic changes occur early and progress to destruction of the lymphoid architecture and the ultimate loss of responsiveness to pathogens. It is not clear to what extent disease progression is attributable to viral replication itself [34, 35] or to the virus-specific cellular immune response.

Overall, our results support the concept that HIV-1–specific CTL responses are primarily protective. Our observations complement and expand on those of previous reports. Rinaldo et al. [13] demonstrated a lack of association of primary Gag-specific CTL activity (in freshly isolated PBMC) with disease stage while showing a lower level of secondary (CTL precursor frequency) activity in persons with advanced disease compared with those with long-term nonprogressive infection. Virus load was tested but not correlated directly with CTL activity. From these results, it was concluded that the level of primary CTL activity is not significantly correlated with disease progression. We have, in this report, similarly shown a lack of correlation between primary CTL activity and disease stage (CD4 cell count) in a cross-sectional analysis. The longitudinal, prospective analysis of CD4 T cell loss over time, however, has allowed a clearer demonstration of the role of CTL and a different interpretation: that primary CTL activity is inversely correlated with disease progression. Furthermore, our analysis of the relationship between virus load and Gag-specific CTL frequencies would argue that this immune response is limiting disease progression regardless of the CD4 T cell concentration.

Our results are also consistent with the findings of Kundu and Merigan [46], who demonstrated a significant inverse association between the Env-specific CTL activity and proviral copy numbers in asymptomatic persons. In that study, however, proviral copy frequencies were correlated with qualitative (positive or negative) Env-specific responses, since such activity was demonstrated in only 11 of 25 persons studied. Our results demonstrate an inverse relationship between primary Gag-specific CTL levels and virus load. While it seems more likely that CTL activity is limiting viral replication, it is acknowledged that the converse may be true: Active viral replication might be limiting activation of CTL. Arguing against the latter possibility are in vitro studies in which CTL clones or CD8 T cells from infected persons are combined with actively infected CD4 T cells [37]. In such assays, it is readily demonstrated that there is a reduction in viral replication in a dose-dependent manner. In vivo, the most compelling data that CTL limit viral replication is derived from infusion studies of a Nef-specific CTL clone, which led to the appearance of virus lacking the epitope for which the clone was specific [47]. Such direct evidence of virus escape from CTL activity argues strongly for the concept that CTL activity inhibits viral replication.

Finally, the lack of Gag-specific CTL activity in freshly isolated PBMC has been correlated with disease progression to AIDS or AIDS-related complex in a study by Riviere et al. [48]. Our analysis provides quantitative measures of both Gag-specific CTL and surrogate markers of disease progression that support this finding.

No strong correlation between the absolute CD4 T cell counts and frequency of circulating CTL was found in our cross-sectional analysis of these persons with hemophilia, as others have shown previously in other cohorts [13, 49]. Furthermore, in this series, no clear trends in CTL frequency over time were observed in 16 persons for whom there was more than one determination. These data demonstrate that a decline in primary CTL activity is not seen over the course of disease. High CTL frequencies are observed in some persons with advanced disease. Studies that have demonstrated a decline of CTL activity with loss of CD4 T cells have used in vitro
stimulation to measure CTL precursors [13, 16]. Pantaleo et al. [50] found a “defective clonogenic potential” of CD8-positive T cells in persons with advanced disease. Differences in CTL frequency would be apparent if, indeed, there were disparate responses to in vitro stimulation protocols dependent on disease state. The assay used here is the most direct means of quantification of primary, activated CTL in PBMC but may not reflect the size of the reservoir of CTL precursors. The reserves of competent, virus-specific CTL precursors may also contribute to the outcome of infection.

We have included in these analyses 5 persons with long-term nonprogressive HIV-1 infection (asymptomatic, with CD4 T cell counts >600/µL without treatment >10 years after seroconversion). It could be argued that these persons may not be representative of the group as a whole. Indeed, 1 of these long-term nonprogressors has been shown to harbor virus with uniformly defective nef genes [51]. It is possible that the protective influence of CTL in such a setting becomes less important. It is noteworthy, however, that this person has had high Gag- and Env-specific CTL frequencies measured on three separate occasions in conjunction with low virus loads. This suggests that ongoing antigenic stimulation (i.e., viral replication) is maintaining activated CTL in the circulation. Furthermore, animal studies have shown that defective simian immunodeficiency virus in certain hosts can be pathogenic, presumably because of the relative inability of the immune response to control viral replication [52]. No other nonrevertible viral defect has been found to be uniformly present in the other long-term nonprogressors as yet. Exclusion of the long-term nonprogressor with nef-defective virus from the analyses presented here does not alter the findings (data not shown).

In conclusion, the results presented here provide further evidence that CTL activity limits disease progression. This study has demonstrated a significant inverse correlation between cell-associated viremia and Gag-specific CTL frequency in HIV-1–infected persons with hemophilia. It is noteworthy that this association was independent of the absolute CD4 T cell concentrations. Thus, results suggest that CTL influence virus burden and would thus be expected to limit the pathogenic effects associated with viral replication. To support these conclusions, a longitudinal examination of CD4 T cell loss in this group showed that those with higher CTL frequencies had a less rapid decline of this integral cell population. The implications of these observations with respect to vaccine development are readily apparent. Applications of vaccines that would augment CTL hinge on whether or not this immune response is beneficial long-term nonprogressors. J Exp Med

References

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