Diminished Spontaneous Apoptosis in Lymphocytes from Human Immunodeficiency Virus–Infected Long-Term Nonprogressors

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The relationship between peripheral lymphocyte apoptosis and human immunodeficiency virus disease progression was studied in infected subgroups with distinct profiles of progression. Long-term nonprogressors (LTNP) and seronegative controls had levels of spontaneous apoptosis significantly lower than those for recent seroconverters who had CD4 cell counts similar to those of nonprogressors but with a high likelihood of disease progression. Lymphocytes from nonprogressors and seronegative controls also showed negligible spontaneous caspase-3 activity, a biochemical indicator for apoptosis, whereas early progressors exhibited substantial activity. In contrast, when activated with mitogens, the lymphocytes from both LTNP and progressors displayed indistinguishable levels of heightened apoptosis. Spontaneous apoptosis and plasma viremia levels correlated positively in progressors, but not in LTNP. These findings demonstrate that increased lymphocyte apoptosis is evident prior to CD4 T cell decline and that LTNP are relatively resistant to the factors that induce accentuated levels of spontaneous but not mitogen-induced cell death.

Despite considerable study, the pathogenic mechanisms underlying the progression of human immunodeficiency virus (HIV) disease remain enigmatic. In developed countries, the average time from seroconversion to AIDS is ~10 years [1, 2], but there is significant variation between individuals. Some patients progress to AIDS within a few months [3–5], whereas others fail to manifest signs or symptoms of progression for many years. The San Francisco City Clinic Cohort (SFCCC) was the first to identify HIV-infected long-term nonprogressors (LTNP) [7]. Representing ~5% of the study participants, LTNP are gay and bisexual men with well-defined seroconversion dates as early as 1978 [6, 7]. Strikingly, not only are these men healthy, but their CD4 T cell counts have remained high (>500/mm³) and stable despite HIV infection for up to 20 years. These LTNP provide a unique resource in the search for factors that contribute to HIV disease progression.

Accelerated and aberrant lymphocyte apoptosis has been proposed as a central mechanism of HIV pathogenesis [8, 9]. Spontaneous and activation-induced apoptosis is higher in peripheral blood T cells from HIV-infected subjects than in those from seronegative controls [10–15]. CD4 T cell apoptosis correlates with the development of disease in simian models of pathogenic lentivirus infection [14, 16, 17] but not in nonpathogenic models, such as simian immunodeficiency virus (SIV) infection of African green monkeys. In addition, HIV-1 and HIV-2 infections of peripheral blood mononuclear cells (PBMC) or CD4 T cell lines in vitro produce significant apoptosis [18–20]. Lymph node biopsies from HIV-1–infected humans and SIV-infected rhesus macaques at various stages of disease progression reveal a breakdown in lymph node architecture and widespread evidence of apoptosis [21, 22]. Finally, in SCID-hu mice, HIV infection of human fetal thymus/liver grafts results in massive apoptosis of thymocytes, including CD4⁺, CD8⁺, and CD4⁺/CD8⁺ cells [23, 24]. These latter findings raise the intriguing possibility that HIV infection may also deplete lymphocyte progenitor populations.

Despite these various results, it remains unclear whether accentuated lymphocyte apoptosis contributes to HIV disease progression or is a noncontributing secondary effect of chronic viral infection [25, 26]. Of note, peripheral lymphocytes from persons with AIDS generally display higher levels of in vitro apoptosis than those from persons with asymptomatic HIV infection; however, no clear correlation between lymphocyte apoptosis and disease progression is observed when asymptomatic HIV patients are ranked for disease stage by CD4 T cell count [12, 13] or by longitudinal sampling from the time of seroconversion to the development of AIDS [27]. Clearly, alternative approaches are needed to delineate what role, if any, apoptosis plays in initiating or maintaining HIV-induced disease progression.

In the present study, we analyzed morphologic and biochemical markers of spontaneous and activation-induced apoptosis...
together with plasma virus load in 4 distinct clinical subgroups differentiated by HIV serostatus and profile of disease progression, including HIV-infected LTNP with CD4 T cell counts >500/mm$^3$; recently seroconverted asymptomatic HIV-infected persons (RSC) with equally high CD4 T cell counts and a high statistical likelihood of normal disease progression; former long-term nonprogressors with a recent and consistent drop in CD4 T cell numbers (LTNP-D), indicating disease progression; and healthy HIV-seronegative controls. The goal of this study was to determine if LTNP have lower levels of spontaneous or activation-induced apoptosis than other HIV-infected persons at the early stages of progression and how levels of apoptosis correlate with virus load.

**Patients and Methods**

**Study Participants**

The HIV-positive study subjects were participants of the SFCCC, which was originally composed of homosexual and bisexual men recruited for studies of the prevalence and incidence of hepatitis B infection in 1978–1980 [6]. Stored blood samples from the SFCCC study made it possible to establish long-term HIV infection for many cohort participants. Study subjects were selected from ~200 cohort members with well-characterized seroconversion dates, who were undergoing scheduled follow-up examinations during the enrollment period of this study. All subjects participating in the current study were free of AIDS-defining illnesses and had no history of antiretroviral drug therapy. The inclusion criteria for the HIV-positive subgroup were as follows: LTNP were HIV positive for >10 years and had a consistent CD4 T cell count >500/mm$^3$. RSC were HIV positive for <10 years and had a consistent CD4 T cell count >500/mm$^3$. LTNP-D were former LTNP who experienced a drop in CD4 T cells to <500/mm$^3$ on at least two consecutive samplings. HIV-negative subjects consisted of healthy, age-matched adults from the SFCCC and the San Francisco community. HIV antibody seronegativity was confirmed by ELISA. Specimens were coded by number so that the clinical status of donors was unknown until after the apoptosis assays were completed.

**Specimen Processing and Cell Culture**

Human peripheral blood samples were collected in vacuum tubes containing sodium heparin and stored at room temperature for a maximum of 8 h before processing. PBMC were enriched by density gradient centrifugation (histopaque $\rho = 1.077$; Sigma, St. Louis). Cells were plated at $2 \times 10^6$ cells/mL in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 $\mu$g/mL streptomycin (Mediatech, Herndon, VA ) in 6- or 12-well plates (Corning Glass Works, Corning, NY). For mitogen stimulation, cultures were incubated with 5 $\mu$g/mL pokeweed mitogen (PWM) or 1 $\mu$g/mL staphylococcal enterotoxin B (SEB) (Sigma) at 37°C in 5% CO$_2$ and 95% air for 72 h before quantification of apoptosis or for 16 h before lysis preparation. For caspase inhibition studies, cells were pretreated with the irreversible inhibitor Z-VAD-fmk (50 $\mu$M) prepared from a 100 mM stock solution in DMSO (Enzyme Systems Products, Livermore, CA) for 60 min at 37°C before addition of mitogens. Control cultures were treated with an equivalent amount of DMSO for a final concentration of 0.05%. This concentration of DMSO was not toxic to PBMC (unpublished data).

**Flow Cytometric Analysis of Apoptosis**

*Acridine orange–ethidium bromide (AO-EB) staining.* PBMC (10$^6$, including live and dead cells as assessed by trypan blue dye exclusion) were pelleted at 250 g in a 12 × 75–mm tube. Cells were resuspended in 400 $\mu$L of PBS, and a combined AO-EB stock solution was added to a final concentration of 0.1 $\mu$M for AO (Molecular Probes, Eugene, OR) and 0.25 $\mu$M for EB (Sigma). Cells were incubated at room temperature for 5 min in the presence of both dyes and then analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) bench-top cytometer with standard argon laser settings (488 nm) and filter sets (530-, 585-, and >650-nm bypass filters). Data from the cytometer were acquired and analyzed with Consort 30 and LYSIS II software (Becton Dickinson Immunocytometry Systems), respectively. Single live and dead lymphocytes were gated by light scatter parameters, using linear scale amplification; red blood cells, debris, and nonadherent macrophages were excluded. Fluorescence signals were logarithmically amplified with fluorescence compensation. AO (green) and EB (red) fluorescence was collected (FL-1 and FL-3, respectively) and plotted as dual-parameter dot plots. For each sample, rectangular gates used for quantification were set around events representing live and early-stage apoptotic cells, excluding necrotic and late-stage apoptotic cells showing the highest levels of EB staining [28].

*TdT-mediated dUTP-biotin nick end labeling (TUNEL) analysis.* TUNEL protocol as described by Gorczyca et al. [29] was used with minor modifications. PBMC (10$^6$, both live and dead as assessed by trypan blue dye exclusion) were fixed in 2% fresh paraformaldehyde-PBS for 15 min on ice, pelleted at 250 g, and incubated in 80% ethanol for a minimum of 30 min at −20°C. Cells were washed in PBS and resuspended in 50 $\mu$L of TdT assay solution (Boehringer Mannheim, Indianapolis) consisting of 200 mM potassium cacodylate, 25 mM Tris-HCl, 250 $\mu$g/mL bovine serum albumin (BSA; pH 6.6), 2.5 $\mu$M cobalt chloride, 0.1 mM dithiothreitol, and 0.5 nmol of biotin-16–dUTP, with or without 5 U of TdT (Boehringer Mannheim) per sample. Reactions lacking TdT were used to determine background fluorescence. Samples were incubated for 30 min at 37°C, washed in rinsing buffer (0.1% Triton X-100, 5% wt/vol BSA in PBS) at room temperature, pelleted at 250 g, and resuspended in 100 $\mu$L of avidin–fluorescein isothiocyanate (avidin-FITC) solution (4 × standard saline citrate, 0.1% Triton X-100, 5% wt/vol nonfat dry milk, and 0.5 mM avidin–FITC [Boehringer Mannheim]). Samples were incubated in the dark at room temperature for 60 min, washed twice with 1 mL of rinsing buffer, and resuspended in 0.5 mL of 2% paraformaldehyde–PBS. Cell-associated green fluorescence was assessed by use of the cytometer. Contaminating platelets, erythrocytes, and cellular debris, characterized by low forward and side light-scattering properties, were gated out to permit analysis of intact live or apoptotic cells only.
Immunoblot Analysis of Caspase-3–Dependent D4-GDI Cleavage

Cellular lysates were prepared from PBMC by resuspending cells in 2× Laemmli sample buffer and incubating at 95°C for 15 min. Protein from ~2 × 10^6 cells was separated on 14% SDS-PAGE gels and electrophoretically transferred to Immobilon PVDF membranes (Millipore, Bedford, MA). Nonspecific protein binding sites on these membranes were blocked with 0.1% Tween–PBS–5% nonfat dry milk. The membranes were then incubated first with rabbit anti-human D4-GDI anti-serum (1:1000 in 0.1% Tween–PBS, a gift from Dennis Danley, Pfizer, Groton, CT) and then with protein A–horseradish peroxidase (1:5000 in 1% Tween–PBS–1% nonfat dry milk: Amersham Life Sciences, Arlington, IL). Bands representing D4-GDI were visualized on radiographic film by enhanced chemiluminescence (Amersham).

Quantification of Plasma HIV-1 RNA Concentrations

Plasma was separated from blood cells collected in vacuum tubes containing acid citric and dextrose within 24 h of collection and stored at ~70°C. HIV-1 RNA in plasma was quantified (RNA molecules/mL) by the branched DNA (bDNA) signal-amplification assay (version I; Chiron, Emeryville, CA) with a linear dynamic range of 10,000–1,600,000 RNA molecules/mL plasma [30]. For samples that registered below the limits of detection of this bDNA assay, HIV RNA was quantified by quantitative competitive polymerase chain reaction (QC-PCR) [31], which has a lower detection limit of 167 RNA molecules/mL plasma.

Quantitative and Qualitative HIV-1 Coculture

HIV from PBMC was propagated and isolated by use of established procedures [32]. In brief, PBMC were isolated from acid citric and dextrose-treated whole blood by Ficoll-Paque (Pharmacia, Piscataway, NJ). For the qualitative culture, 1 × 10^6 cells were cocultured with an equal number of phytohemagglutinin A blasts from an uninfected donor. The culture supernatant was assayed every 3–4 days for HIV-1 gag p24 antigen by ELISA. For the quantitative culture, five 5-fold dilutions of 1 × 10^6 PBMC were cocultured in duplicate with an equal number of phytohemagglutinin blasts from an uninfected donor. At day 14, culture supernatants were assayed for HIV-1 p24 antigen by ELISA, and the infectious units per million cells were calculated with Dataworks RLMP (Seattle, WA) software.

Statistical Analyses

Pairwise comparisons of data sets were done by the Wilcoxon nonparametric test. Significance was defined as P < .05 by use of a two-tailed test. Linear and repeated measures regression analysis was done using SAS software (version 6.11; SAS Institute, Cary, NC).

Results

Characteristics of study subjects. Sixteen LTNP were enrolled in this study. The mean duration of infection in the subjects at the time of testing was 14.9 years (range, 10.9–16), and their mean CD4 T cell count was 815/mm^3 (range, 534–1383) at the time of evaluation. Despite their lengthy infection, LTNP showed a slow rate of CD4 T cell depletion (mean, 11 cells/mm^3/year). RSC (n = 16) were seropositive for an average of 3.3 years (range, 4–6.6) and had a mean CD4 T cell count of 680/mm^3 (range, 503–1318) at the time of sampling. Their mean rate of T cell loss (139 cells/mm^3/year) was substantially higher than that for the LTNP. Statistical norms for this cohort indicate that ~80% of the RSC will progress to AIDS within 15 years after seroconversion and only 5% may be nonprogressors. LTNP-D (n = 14) were similar to the LTNP with respect to length of infection (mean, 13.9 years; range, 12–16.2), but their CD4 T cell counts were significantly lower (mean 343/mm^3; range, 231–496), indicating a more rapid course of CD4 T cell decline and disease progression.

Quantification of apoptosis. Apoptotic lymphocytes from HIV-positive and HIV-negative donors were identified and quantified by two independent flow cytometric assays that measure distinct features of apoptosis. AO-EB staining differentiates live, apoptotic, and necrotic lymphocytes on the basis of chromatin condensation, DNA loss, and plasma membrane permeability. Apoptotic cells exhibit diminished AO staining as evidenced by EB exclusion [28]. TUNEL staining differentiates live and apoptotic cells by end-labeling of fragmented DNA with FITC-conjugated nucleotides [29].

Figure 1 shows the dual-parameter (AO-EB) and single-parameter (TUNEL) fluorescence profiles of representative control and RSC lymphocytes undergoing spontaneous cell death and activation-induced cell death (AICD) after in vitro culture for 72 h. Control lymphocytes showed low levels of spontaneous apoptosis by both assays. Treatment with PWM significantly increased the levels of apoptosis. A similar effect was seen with RSC lymphocytes; however, the level of spontaneous apoptosis was significantly higher than in control lymphocytes (28.8% vs 7.2%). In analyses of cells from the same culture, AO-EB staining consistently yielded relatively more apoptotic cells than TUNEL staining. This difference may reflect comparatively limited DNA fragmentation in apoptotic peripheral lymphocytes measured by TUNEL staining, as opposed to other nuclear and membrane changes measured by AO-EB staining [28]. Nevertheless, both assays were equally reproducible. Triplicate measurements of cultured lymphocytes from HIV-positive or HIV-negative donors show a standard error within 5% of the mean using either assay.

Peripheral lymphocyte apoptosis. To assess whether spontaneous or activation-induced apoptosis increases with the onset of disease progression, we analyzed apoptosis in lymphocytes from LTNP, RSC, LTNP-D, and HIV-seronegative controls by both AO-EB and TUNEL measurements after 72 h in culture. The results are shown in figure 2 and in table 1.

AO-EB staining demonstrated significantly lower median levels of spontaneous apoptosis in resting PBMC from LTNP...
compared with levels in PBMC from RSC or LTNP-D (13% vs. 29.5% and 25.5%; \( P = .0001 \) and .0006, respectively). Of interest, the levels of apoptosis in lymphocytes from LTNP and seronegative controls were statistically indistinguishable (median, 6.5%; \( P = .09 \)). TUNEL staining also showed lower median levels of spontaneous apoptosis in cells from LTNP than in those from RSC or LTNP-D (14% vs. 22% and 20%, respectively). The median spontaneous apoptosis values from LTNP and seronegative controls were statistically indistinguishable (9%; \( P = .08 \)). Similar results were obtained by using
repeated-measures regression models to analyze all available samples for each donor (data not shown).

An alternative analysis showed that the relationships between the 3 groups also hold among individuals. To test whether the between-group differences in apoptosis reflected special characteristics of the LTNP, or simply the independent influences of CD4 cell count and duration of infection, we considered linear regression models for the dependence of apoptosis on these factors among individuals in the combined LTNP, LTNP-D, and RSC groups. We found that the best predictor of apoptosis was the product of CD4 cell count and years since seroconversion (regression slope $-0.14$, $P = 0.0001$), a constructed variable that is largest among LTNP, who are characterized by high CD4 cell counts and long duration of infection; substantially smaller among LTNP-D, who have low CD4 cell counts; and smallest among RSC, who have a short duration of infection. In a model including this variable, CD4 cell count and years since seroconversion considered separately had no independent influence. Thus, this alternative analysis points to the special character of the LTNP and suggests that apoptosis is correlated with the rate of progression.

When treated with mitogens, lymphocytes from HIV-positive patients undergo higher levels of AICD than do lymphocytes from seronegative subjects [11, 12, 15]. To determine if activated lymphocytes from LTNP show lower AICD levels, resembling those in HIV-negative persons, or higher levels, similar to those in seropositive progressors, PBMC were treated with the polyclonal activator PWM or the CD4 T cell superantigen SEB for 72 h, and apoptosis was quantified by AO-EB and TUNEL staining (figure 2). In agreement with previous studies, the median level of apoptosis was higher in the activated cultures than in the corresponding unstimulated cultures for any particular subgroup. However, in contrast to the pattern observed measuring spontaneous apoptosis, the levels of AICD in PBMC from LTNP were not significantly different from those for RSC or LTNP-D, and they were higher than levels in seronegative controls. Of note, the TUNEL assay did not show a statistically significant difference between SEB-treated LTNP and normal controls ($P > .10$), possibly because of the rather blunted response to SEB in this assay (figure 2F).

**Caspase-3 activation and D4-GDI cleavage.** Apoptosis induced by extracellular signals is mediated by a family of cysteine proteases or caspases that cleave and disable substrates critical in homeostatic processes and cellular structure [33]. Caspase-3 is a mammalian caspase that is instrumental in the execution phase of apoptosis [34]. One intracellular substrate for caspase-3 is D4-GDI, a GDP-dissociation inhibitor of the Rho family of GTP-ases [35]. Following Fas-induced apoptosis in Jurkat T cells, the 28-kDa D4-GDI protein is rapidly cleaved into a 23-kDa product by caspase-3 or a closely related protease. Using substrate cleavage as a biochemical marker for caspase-3 activation, we analyzed lysates of resting lymphocytes from LTNP, RSC, and HIV-negative controls by Western blot to determine if the higher levels of spontaneous apoptosis occurring in the RSC group were associated with an increased incidence of D4-GDI cleavage (figure 3A). The immunoblots are shown in figure 3A. D4-GDI cleavage was considered significant if $\geq 10\%$ of total D4-GDI measured by densitometry was in the 23-kDa form. Consistent with the results of the fluorescence-based assays, the immunoblots showed significant D4-GDI cleavage in PBMC from RSC (lanes 1, 3, 5, 6, and 8). In contrast, D4-GDI cleavage was infrequent in PBMC lysates from LTNP and HIV-negative controls, reflecting the significantly lower basal levels of spontaneous apoptosis in these groups.

The combination of PWM and SEB is a potent and rapid inducer of AICD in lymphocytes from HIV-positive subjects [15]. Cells from RSC, LTNP, and LTNP-D were treated with PWM and SEB to induce AICD and D4-GDI cleavage and then cultured for 16 h. Immunoblots from 4 representative HIV-positive subjects probed with anti–D4-GDI antiserum are shown in figure 3B. In nearly all the lysates analyzed (11/13 donors), D4-GDI cleavage was enhanced in activated cells (figure 3B, compare lanes 1 and 3). Treatment with the potent caspase inhibitor Z-VAD-fmk during culture completely or substantially blocked activation-induced cleavage (compare lanes 3 and 4) and apoptosis, as measured by the TUNEL assay (data not shown). These results indicate that in lymphocytes from HIV-positive subjects, apoptosis is mediated through an active pathway involving caspase-3 or a caspase-3–like protease.

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**Figure 1.** Differentiation and quantification of live and apoptotic peripheral blood lymphocytes by acridine orange–ethidium bromide (AO/EB) and TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining. Peripheral blood mononuclear cells (PBMC) from HIV-negative control and recently seroconverted asymptomatic HIV-infected person were incubated in complete medium with or without 5 μg/mL pokeweed mitogen (PWM) for 72 h and assayed for activation-induced or spontaneous apoptosis, respectively. A, B, Dual-parameter fluorescence dot plots representing AO/EB stained lymphocytes, including cells showing low (dead cells), high (live cells), and very high (activated blasts) forward light scatter. Apoptotic lymphocytes are differentiated from live cells by diminished FL-1 fluorescence reflecting decreased AO staining of apoptotic nuclei. Late-stage apoptotic and necrotic cells exhibiting increased membrane permeability show very high FL-1 fluorescence and are not included in apoptotic gate. Events displaying equal green and red fluorescence (on diagonal) represent cellular particulate and debris that were not removed on basis of light scatter properties. Events in both high– and low–AO staining populations were electronically quantified with LYSIS II software (Becton Dickinson Immunocytometry Systems, San Jose, CA) and represent relative proportions of live and apoptotic cells, respectively, among all cells. % of apoptotic cells is indicated. C, D, PBMC from cultures described above were fixed and labeled with biotinylated dUTP and avidin–fluorescein isothiocyanate (avidin-FITC) and analyzed by TUNEL assay. Log-scale single-parameter fluorescence histograms are shown from cells undergoing spontaneous or activation-induced apoptosis. % of apoptotic cells showing increased FL-1 fluorescence is indicated for each sample.
Relationship between lymphocyte apoptosis and plasma virus load. A persistently high level of plasma viremia after seroconversion correlates with more rapid progression of HIV disease [36–38]. We examined the relationship between virus load and spontaneous apoptosis in LTNP and likely progressors (RSC). Plasma HIV-1 RNA concentration was determined by the bDNA signal-amplification assay. When values were undetectable by this method, duplicate samples were quantified by a more sensitive method, QC-PCR [31]. Specimens for virus load were collected at the same time as those used for immunophenotyping and apoptosis measurements. In both the RSC and LTNP, there was a 4-log range in HIV-1 plasma virus concentration. However, the HIV-1 RNA concentrations were overall lower in the LTNP (n = 16; range, <167–114,000 RNA molecules/mL; mean, 1995) than in the RSC (n = 16; range, 600–203,000 RNA molecules/mL; mean, 28,840). Despite readings at the limits of detection by QC-PCR in 4 of the 16 LTNP, PBMC from all LTNP yielded measurable virus in coculture assays. In addition, HIV-specific cytotoxic T lymphocyte responses have been reported in these LTNP with low virus load, indicating chronic HIV infection [39].
Table 1. Levels of spontaneous and activation-induced apoptosis determined by acridine orange–ethidium bromide and TdT-mediated dUTP-biotin nick end labeling (AO-EB) TUNEL staining of cultures of peripheral lymphocytes from HIV-positive and HIV-negative subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>AO-EB staining</th>
<th>TUNEL staining</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SD (%)</td>
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<tr>
<td>Spontaneous apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>20</td>
<td>10.3 ± 8.7</td>
</tr>
<tr>
<td>LTNP</td>
<td>16</td>
<td>12.4 ± 5.3</td>
</tr>
<tr>
<td>RSC</td>
<td>16</td>
<td>28.3 ± 8.9</td>
</tr>
<tr>
<td>LTNP-D</td>
<td>14</td>
<td>25.1 ± 10.3</td>
</tr>
<tr>
<td>PWM-induced apoptosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neg</td>
<td>18</td>
<td>27.0 ± 9.5</td>
</tr>
<tr>
<td>LTNP</td>
<td>16</td>
<td>39.3 ± 17.4</td>
</tr>
<tr>
<td>RSC</td>
<td>16</td>
<td>44.0 ± 12.6</td>
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<tr>
<td>LTNP-D</td>
<td>14</td>
<td>45.6 ± 15.1</td>
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<tr>
<td>SEB-induced apoptosis</td>
<td></td>
<td></td>
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<tr>
<td>Neg</td>
<td>20</td>
<td>27.8 ± 13.3</td>
</tr>
<tr>
<td>LTNP</td>
<td>16</td>
<td>49.3 ± 16.5</td>
</tr>
<tr>
<td>RSC</td>
<td>16</td>
<td>45.8 ± 11.8</td>
</tr>
<tr>
<td>LTNP-D</td>
<td>14</td>
<td>50.6 ± 12.6</td>
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</table>

NOTE. Neg, HIV-seronegative; LTNP, long-term nonprogressors; RSC, recently seroconverted asymptomatic; LTNP-D, former LTNP with recent and consistent drop in CD4 cell no., indicating disease progression; PWM, pokeweed mitogen; SEB, staphylococcal enterotoxin B.

The relationship between plasma viremia and spontaneous lymphocyte apoptosis by AO-EB staining is shown in figure 4. In a linear-regression model, the log concentration of HIV RNA was a significant predictor of spontaneous lymphocyte apoptosis in the RSC (P = .03) but not in LTNP (P = .68). The average increase in spontaneous apoptosis per log virus load was 7.5% in likely progressors, but it was only 1.8% in LTNP. LTNP with either relatively low (<10,000) or high (>10,000) plasma viremia showed similar levels of apoptosis (low [n = 8]: mean, 11.9%; range, 6%–22%; high [n = 8]: mean, 12.9%; range, 2%–19%). Thus, although some LTNP had substantial plasma viremia, their levels of spontaneous apoptosis were independent of virus load, possibly reflecting the contribution of undefined protective factors provided by the virus or host.

Discussion

In this study, we compared the levels of spontaneous and activation-induced lymphocyte apoptosis in HIV-infected LTNP with those in other seropositive subgroups showing distinct patterns of disease progression and in seronegative controls. Despite their long-term chronic infection, LTNP had basal levels of spontaneous apoptosis that were indistinguishable from those of seronegative controls, although our sample sizes limit the power to detect minor differences. Conversely, spontaneous apoptosis in LTNP was significantly lower than that found in other seropositive groups (RSC and LTNP-D) likely to be in the early stages of disease progression. Consistent with these observations, unstimulated lymphocytes from LTNP and seronegative controls showed decreased evidence of caspase-3 activation compared with the RSC. These results demonstrate that the levels of spontaneous lymphocyte apoptosis are increased in HIV-positive subjects in the early stages of disease progression, before or during the onset of CD4 T cell decline or clinical signs of immunosuppression.

Aberrant lymphocyte apoptosis has been reported in other virus infections [23, 24], raising the possibility that apoptosis is a nonspecific consequence of virus infection per se. As such, if chronic infection drove apoptosis, higher apoptosis levels would be evident in persons with measurable virus loads. We observed a positive association between plasma virus load and the degree of apoptosis in the RSC; however, there was no such positive association between apoptosis and plasma virus concentration in the LTNP, despite significant virus load in many of these subjects. These findings indicate that chronic HIV-1 infection per se does not necessarily result in increased spontaneous apoptosis and that nonprogressors appear to be qualitatively different in controlling apoptosis.

Our linear regression models for the dependence of apoptosis on CD4 cell count and years since seroconversion among persons in the LTNP, LTNP-D, and RSC groups also suggest that the LTNP constitute a qualitatively different group. In that analysis, the product of CD4 cell count and years since seroconversion most clearly predicted apoptosis. This constructed variable can be interpreted as a measure of the rate of progression since it takes on large values only among the LTNP, who maintain high CD4 cell counts despite a long duration of
infection, and is smaller in both of the other groups. While the relationships between virus load, apoptosis, and rate of disease progression remains uncertain, stable and significant virus loads in this and other cohorts of LTNP [22, 40] suggest that factors beyond the quantity of plasma viremia contribute to apoptosis and disease progression.

These systematic differences between individuals are not necessarily inconsistent with the previously reported lack of longitudinal effects on CD4 T cell decline and apoptosis [12, 13]. Our results are consistent with the notion that the determinants underlying the initiation of disease progression also influence lymphocyte apoptosis. Once persistent T cell decline has begun, spontaneous apoptosis may remain at a higher steady-state level and may not be a particularly useful marker of subsequent progression.

Recently, interest has grown in defining virologic, genetic, and immunologic factors associated with long-term non-progression of HIV disease [6, 7, 22, 41–43]. It is important to discern whether LTNP are truly more resistant to HIV disease progression than other HIV-positive subjects or if they represent random statistical outliers. As the length of infection of LTNP in the SFCCC increases up to 20 years, with individuals maintaining high and extremely stable CD4 cell counts (compare annual CD4 T cell changes in HIV-negative controls at +1 cell/mm$^3$ vs. LTNP at −11 cells/mm$^3$ and RSC at −139 cells/mm$^3$), it becomes increasingly likely that the LTNP do, in fact, have undefined elements that protect against or delay progression of disease. Indeed, recent studies of this and other cohorts have defined unique host genetic [44, 45] and virologic [46, 47] factors that may contribute to the observed protection. Because of the high individual variability in the rate of HIV disease progression and the lack of sensitive surrogate markers, the LTNP in this study are probably a heterogeneous group, including both true nonprogressors and misclassified slower progressors. Our study included subjects previously classified as LTNP who had a consistent drop in CD4 T cell counts to steady-state level and may not be a particularly useful marker of subsequent progression.

Of interest, LTNP-D showed higher levels of spontaneous apoptosis (similar to those in RSC) rather than lower levels like those in LTNP and HIV-negative controls. These findings suggest that in LTNP-D, processes leading to enhanced lymphocyte apoptosis have been initiated that will lead to continued CD4 T cell loss and immunosuppression. Follow-up of these
subjects will be necessary to determine if increased spontaneous lymphocyte apoptosis predicts progression of HIV disease.

Although LTNP have high and stable CD4 T cell counts, they also exhibit hematologic abnormalities not seen in healthy HIV-negative persons, such as increased serum β₂-microglobulin, a modestly diminished CD4 T cell count, and an increased CD8 T cell count [6, 7]. All of the LTNP had significantly increased levels of activation-induced apoptosis that were indistinguishable from the levels in asymptomatic progressors. HIV infection may drive this accelerated program of cell death, especially in CD4 cells, by altering cellular components involved in the control of cell stasis, proliferation, or cell suicide. Aberrant expression or modification of cellular gene products, such as bcl-2 [48, 49], p53 or p53-associated products [50–53], myc [54, 55], abl [56], and raf [57], or proteins participating directly in cell-cycle regulation [58] may influence pathways of cell growth and differentiation by effectively altering the cellular threshold for the induction of apoptosis. Recent experimental data support this possibility during HIV infection.

Cohen et al. [59] have shown aberrant phosphorylation of the cell cycle kinase p34cdc2 in HIV-infected T cells, indicating a G2/M block. G2 arrest has also been observed in cells transfected with the HIV-1 vpr gene product [60]. Reduced levels of bcl-2 in lymph nodes and PBMC have also been described in subjects with acute and chronic HIV infection [25]. Similarly, in vitro infection of Epstein-Barr virus–transformed B cells with HIV resulted in decreased bcl-2 expression and increased apoptosis [61]. Taken together, these results raise the possibility that HIV infection alters multiple pathways that contribute to the regulation of cell growth and differentiation, resulting in aberrant patterns of apoptosis in response to normally nontoxic extracellular signals. Nonprogressors may be intrinsically more resistant to these HIV-induced alterations underlying spontaneous apoptosis but equally sensitive to apoptotic signals of intracellular stress, including activation by mitogens.

In summary, our findings suggest that HIV-induced apoptosis is detectable at the early stages of HIV infection in persons likely to progress. In contrast, spontaneous programmed cell death is significantly attenuated in LTNP. These results suggest that apoptosis likely represents a cellular process that contributes to the progression of HIV disease.

**Acknowledgments**

We thank members of the San Francisco City Clinic Cohort and the San Francisco community for their continued participation in research studies. We acknowledge David Colbert for specimen handling, Tom Bersot for access to his cohort members, and Dennis Danley for the anti–D4-GDI antisera. We also thank Silvija Staprans, Stephen Ordway, and Gary Howard for critical reading of this manuscript.

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