Apoptosis in T-Lymphocyte Subsets in Human Immunodeficiency Virus-Infected Children Measured Immediately Ex Vivo and following In Vitro Activation

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Phosphatidylserine molecules are translocated to the outer plasma membrane of lymphocytes undergoing apoptosis and can be detected by the binding of fluorochrome-conjugated annexin V. Using the annexin V assay, we examined CD4 and CD8 T cells from human immunodeficiency virus (HIV)-infected children for apoptosis upon isolation or following in vitro culture. Immediate ex vivo analysis or overnight culture showed significantly higher levels of apoptosis in CD8 cells than in CD4 cells. Following culture with the activating stimulus phytohemagglutinin or anti-CD3 monoclonal antibody, we observed an increase in the percentage of apoptotic CD4 cells, whereas there was no change in the rate of CD8 cell death. These results demonstrate that in HIV-infected children, CD8 apoptosis may occur at a greater rate than CD4 apoptosis in vivo; greater CD4 depletion may be observed due to more efficient mechanisms for peripheral lymphocyte replacement in the CD8 compartment. Furthermore, our data suggest that CD8 lymphocytes may be maximally activated in vivo, a condition which may lead to the exhaustion of CD8-mediated immunity. These findings clarify the differences between the CD4 and CD8 apoptotic responses to HIV.

While an increased rate of lymphocyte apoptosis has been documented for patients with human immunodeficiency virus (HIV) infection (20), the precise mechanism(s) is still unclear. Different pathways leading to apoptosis have been proposed; there is evidence for direct cytopathic effects by viral components as well as indirect effects on bystander cells (9, 11, 18, 19). While both CD4 and CD8 T cells undergo apoptosis, the induction and kinetics of cell death may be different for each subset. For example, telomeres have been observed to be significantly shorter in CD8 cells than in CD4 cells of HIV-infected adults, suggesting faster turnover in the CD8 population (7, 21, 24). In addition, in vitro addition of interleukin-2 failed to rescue activated CD8 lymphocytes undergoing apoptosis (15), indicating that these cells may be committed to death in vivo. The deletion of activated responding CD8 T lymphocytes following an infection may be a homeostatic process serving to restore normal cell numbers, an event which may be amplified due to the chronic nature of HIV infection. Together, these data suggest that during HIV infection, CD8 cells primarily undergo activation-induced cell death due to an environment of persistent inflammation.

Cells undergoing apoptosis translocate phosphatidylserine (PS) to their outer cell membrane (23). Annexin V, which binds PS, was used to investigate lymphocyte subset apoptosis in a cohort of HIV-positive children and uninfected, healthy pediatric controls. Importantly, the method of annexin V labeling allowed us to quantitate apoptosis in peripheral blood mononuclear cells (PBMC) directly after isolation. To validate the specificity of annexin binding for apoptosis, we examined whether PBMC which bound annexin V simultaneously demonstrated DNA strand breaks by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method. We found that the percentage of CD8 T lymphocytes undergoing apoptosis was greater than that of CD4 cells when measured immediately ex vivo or following overnight culture. Furthermore, the addition of activating stimuli in vitro was able to increase the percentage of CD4 cells which were dying, whereas the CD8 subset was unchanged.

MATERIALS AND METHODS

Study subjects. Peripheral blood samples were obtained from 67 children with perinatal HIV infection. Children were grouped using Centers for Disease Control and Prevention classification by the level of immune suppression: category 1, none ($n = 23$, age $= 8.2 \pm 3.7$ years [mean $\pm$ standard deviation]); category 2, moderate ($n = 24$, age $= 7.5 \pm 4.0$ years); category 3, severe ($n = 20$, age $= 11.3 \pm 5.6$ years). Treatments consisted of no antiretroviral therapy ($n = 6$), reverse transcriptase inhibitor therapy ($n = 40$), or combination therapy with reverse transcriptase inhibitors and protease inhibitors ($n = 21$). In a subset of patients for whom viral load measurements were available ($n = 46$), the median number of RNA copies per ml was 2,050 (25th to 75th percentile, 400 to 11,000). Control blood samples were obtained from 10 HIV-negative healthy children (age $= 3.5 \pm 2.9$ years). These children were free of infection and were undergoing elective surgery for nonmalignant disorders (inguinal hernia or phimosis). In all cases, informed consent was obtained from the parents or guardians of the children per institutional review board-approved protocols.

Cell isolation and culture. Following collection into heparinized tubes, separation of mononuclear cells was performed by conventional Ficoll-Hypaque (Lymphoprep; Nymoed AS, Oslo, Norway) density gradient centrifugation. Identical conditions were used for samples from HIV-infected children and healthy uninfected controls. Each sample was processed within 1 h of collection. In some experiments, PBMC were cultured overnight at a concentration of $10^6$ ml at $37^\circ$C and $5\%$ CO$_2$ in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and 2 mmol of L-glutamine (Whittaker Bioproducts, Walkersville, Md.) per liter with...
Apoptosis measured immediately ex vivo in T-cell subsets. Since our objective was to account for all apoptotic lymphocytes within a sample, the large gate was used with monocytes excluded by gating on CD4\textsuperscript{bright} or CD8\textsuperscript{bright} cells, which limits analysis to T lymphocytes. In order to determine the in vivo levels of cell death, we measured apoptosis immediately after phlebotomy (Fig. 2) in CD4 and CD8 cells isolated from children of immune categories 1, 2, and 3 as well as from healthy uninfected control children. In HIV-positive children from all immune categories, apoptosis was significantly higher in CD8 cells than in CD4 cells (Fig. 3). The percentage of apoptotic CD4 cells was significantly higher than that of controls only in children with severe disease, while the percentage of apoptotic CD8 cells was significantly increased in all infected children. As the majority of the patients were receiving antiretroviral therapy at the time of this cross-sectional study, it was not possible to evaluate the effects of treatment on apoptosis. However, elevated levels of CD8 T-lymphocyte death existed despite effective control of viremia.

Apoptosis in T-cell subsets after in vitro activation. To further investigate the observed differences in cell death levels between CD4 and CD8 lymphocytes, apoptosis was examined in T-cell subsets of HIV-infected children after overnight culture in the absence or presence of activation stimuli (Fig. 4). Upon overnight incubation with PHA, CD4 cells from HIV-infected children showed an increase in the percentage of apoptotic cells, whereas the CD8 population did not change (Fig. 5). In cells from uninfected children, the percentage of apoptotic CD4 and CD8 cells did not change after overnight
stimulation with PHA. The addition of anti-CD3 monoclonal antibody also induced an increased percentage of apoptosis in CD4 T cells from HIV-infected children, whereas apoptosis in CD8 lymphocytes did not change (data not shown).

DISCUSSION

While the ability of HIV to induce lymphocyte cell death is well established, the causative mechanism(s) remains elusive. Putative pathways include induction via Fas ligand (2) or tumor necrosis factor-related apoptosis-inducing ligand (14) and loss of protective molecules such as Bcl-2 (3). Results of the analysis of T-cell apoptosis in freshly isolated blood samples from HIV-infected persons most likely reflect the rate of cell death in vivo, thus providing valuable insight into the pathogenesis of this disease. Previous reports of immediate ex vivo apoptosis in HIV-infected adults (12, 13, 16) and children (6), including a recent study which utilized the annexin V assay (16), indicated low levels of cell death. In contrast, a study utilizing the dye Apostain, which is reported to detect early apoptotic cells, found that over half of freshly isolated lymphocytes underwent apoptosis in HIV-infected adults (1). These differences may be explained by the patient cohorts studied but are more likely due to the apoptosis assays used and the analysis schemes employed. Our experiments were designed to account for all apoptotic lymphocytes while omitting potentially confounding monocytes from the analysis, and thus our results demonstrate higher levels of cell death than many previous reports. Indeed, levels of cell death observed in a conventional lymphocyte gate were of a magnitude similar to those previously reported, indicating that a basic variable such as gating can have tremendous influence on the level of apoptosis.

FIG. 2. Representative histograms demonstrating apoptosis measured immediately ex vivo in T-cell subsets. Samples from healthy children (CONT) or HIV-infected children (HIV+) were labeled with anti-CD4 (A) or anti-CD8 (B) PE and annexin V fluorescein isothiocyanate immediately upon isolation. Flow cytometric analysis consisted of a combination of a large light scatter gate and a gate on CD4bright or CD8bright cells (not shown). The percentages of apoptotic CD4 T cells (control, 8%; HIV positive, 18%) and apoptotic CD8 T cells (control, 11%; HIV positive, 53%) were then determined.

FIG. 3. Summary of immediate ex vivo apoptosis percentages in T-cell subsets. Mean percentages ± standard deviations of annexin binding CD4 and CD8 cells from HIV-infected children in immune categories 1 (n = 10), 2 (n = 10), and 3 (n = 11) and from healthy controls (n = 10) directly after isolation are shown. Asterisks indicate statistically significant differences between the percentages of apoptotic CD4 and CD8 cells within each immune category: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Differences between groups were determined in a paired manner using the paired Student t test or the Wilcoxon signed rank test. In addition, both CD4 and CD8 apoptosis levels in patients from category 3 were significantly higher than those from category 1 or 2 (for CD4, P was 0.004 for categories 1 and 3 and 0.01 for categories 2 and 3; for CD8, P was 0.004 for categories 1 and 3 and 0.008 for categories 2 and 3).
We observed that in the majority of samples from HIV-infected children, CD8 apoptosis was significantly higher than in controls at all disease stages and apoptosis was present at higher levels in the CD8 population than in the CD4 population. Because an earlier report of lymphocyte apoptosis in children (6) indicated that levels of immediate ex vivo CD4 and CD8 cell death were similar, we suggest that our effort to include all apoptotic lymphocytes in our analysis may explain this discrepancy.

The percentages of peripheral blood CD4 apoptosis were elevated only in children in immune category 3. Since most children were on antiretroviral therapies with moderate to complete virus suppression, relatively low CD4 apoptosis levels may reflect the adequacy of virus control. Alternatively, CD4 lymphocyte death may predominantly occur in locations other than the peripheral circulation. However, in agreement with our findings, in experiments conducted with tonsillar tissues from HIV-infected adults, Rosok and coworkers found higher levels of cell death in the CD8 population than in the CD4 population (22). CD8 lymphocyte death during HIV infection has been associated with cellular activation resulting in increased sensitivity to apoptosis (3, 5). The physiologic process of response to a pathogen, i.e., activation, may lead to a greater propensity for CD8 death, a pathway which may be augmented by the chronic nature of HIV. CD8 T lymphocytes from patients with acute Epstein-Barr and varicella-zoster virus infections have been demonstrated to be highly sensitive to apoptosis (4). Elevated levels of CD8 cell death may thus be a common feature of the immune system’s response to viral

FIG. 4. Representative histograms demonstrating apoptosis in T-cell subsets after activation. Samples from a healthy child (A) and an HIV-infected child (B) were cultured overnight in the presence or absence of PHA. Cells were then labeled with anti-CD4 or anti-CD8 PE and annexin V fluorescein isothiocyanate. Flow cytometric analysis consisted of gating on CD4bright or CD8bright events and quantifying the percentages of CD4 control apoptotic cells (resting, 8%; activated, 10%), CD8 control apoptotic cells (resting, 21%; activated, 21%), CD4 HIV-positive apoptotic cells (resting, 18%; activated, 38%), and CD8 HIV-positive apoptotic cells, (resting, 46%; activated, 59%).

FIG. 5. Summary of percentages of apoptosis in T-cell subsets following activation. Shown are mean percentages ± standard deviations of annexin binding CD4 and CD8 cells of HIV-infected children (n = 31) (A) and uninfected controls (n = 10) (B) after overnight culture with PHA or medium. For HIV-infected CD4 cells, the difference between the percentage of apoptosis with overnight culture in medium and that with overnight culture in PHA was statistically significant (p = 0.007). Differences between groups were determined in a paired manner using the paired Student t test or the Wilcoxon signed rank test. NS, not significant.
challenge. However, evidence which suggests that the CD8 compartment may inherently possess a greater regenerative capacity exists. In studies of lymphocyte reconstitution following cancer chemotherapy, Mackall and colleagues showed that the CD8 pool had returned to baseline by 3 months posttherapy, when the CD4 population was only at one-third of its starting value (17). Furthermore, in HIV-infected individuals, the fraction of proliferating CD8, but not CD4, T lymphocytes has been reported to be increased (10). Thus, massive amounts of CD8 turnover may occur during HIV infection, but the relative levels appear unperturbed, possibly due to their production and/or proliferation. In an important study differentiating the disease-causing abilities of immunodeficiency viruses, in which pathogenic and nonpathogenic strains of simian immunodeficiency virus were compared, CD8 apoptosis was detected in all cases, whereas CD4 cell death was limited to pathogenic infections (8). Limiting levels of virus via effective therapy may serve to decrease induction of apoptosis in the CD4 pool. These findings, together with our results, put forth the notion that one difference between HIV and other chronic pathogenic infections (8). Limiting levels of virus via effective therapy, when the CD4 population was only at one-third of its normal level, returned to baseline by 3 months postchemotherapy, Mackall and colleagues showed that the CD8 compartment may inherently possess a greater regenerative capacity.

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