Accelerated Apoptosis in Peripheral Blood Mononuclear Cells (PBMCs) From Human Immunodeficiency Virus Type-1 Infected Patients and in CD4 Cross-Linked PBMCs From Normal Individuals

By Naoki Oyaizu, Thomas W. McCloskey, Maria Coronesi, Narendra Chirmule, Vaniambadi S. Kalyanaraman, and Savita Pahwa

This study investigates apoptosis as a mechanism for CD4+ T-cell depletion in human immunodeficiency virus type-1 (HIV-1) infection. Although several recent studies have suggested that T cells of HIV-infected individuals show enhanced susceptibility to cell death by apoptosis, the mechanisms responsible for apoptosis are largely unknown. By using a flow cytometric technique and by morphology, we have quantitated the percentage of cells undergoing apoptosis in peripheral blood mononuclear cells (PBMCs) from HIV-seronegative donors and from HIV-infected asymptomatic patients. The PBMCs were cultured without any stimulus or with staphylococcus enterotoxin B, anti-T-cell receptor (TCR) αβ monoclonal antibody WT-31, or phytohemagglutinin for periods up to 6 days. In addition, we sought to determine whether cross-linking of CD4 followed by various modes of TCR stimulation in vitro could induce apoptosis in normal PBMCs. Here we show that (1) patient PBMCs undergo marked spontaneous apoptosis; (2) stimulation of T cells of patients as well as normal donors results in increased apoptosis; and (3) cross-linking of CD4 molecules is sufficient to induce apoptosis in CD4+ T cells if cross-linking is performed in unfractioned PBMCs, but not if CD4 molecules are cross-linked in purified T-cell preparations. These observations strongly suggest that accelerated cell death through apoptosis plays an important role in the pathogenesis of HIV-1 infection. At the same time, our observations implicate cross-linking of CD4 in vivo as a major contributor to this mechanism of accelerated cell death in HIV infection.

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Materials and Methods

Cells and culture conditions. PBMCs were obtained from 17 HIV-seropositive adult patients at North Shore University Hospital. All patients were asymptomatic and their CD4 counts ranged from 257 to 670 (mean 432)/mm3 at the time of examination. Controls were HIV-seronegative healthy donors. PBMCs were isolated from heparinized venous blood by Ficoll-Hypaque density gradient (Lymphoprep; Nycomed AS, Oslo, Norway) centrifugation. In the cell selection experiments, purified T cells were prepared by rosette formation with sheep red blood cells followed by Petri dish adherent cell depletion as described.15 These cells were greater than 97% CD2+ and contained less than 1% CD14+ monocytes as determined by flow cytometry. RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), 2 mmol/L L-glutamine (Whittaker Bioproducts...
APOPTOSIS IN CD4 CROSS-LINKED PBMCs

Inc, Walkersville, MD), 100 U/mL penicillin G, and 100 μg/mL streptomycin was used for all cultures.

Antibodies and reagents. Reagents and sources were as follows: monoclonal antibody (MoAb) to CD4 (Leu-3a; IgG1) and to TCRαβ (WT-31, IgG1) (Becton Dickinson, Mountain View, CA); MoAb to CD1a (IgG1; AMAC Inc, Westbrook, ME); goat-antimouse IgGs, (GAM; Tago, Inc, Burlingame, CA); staphylococcal enterotoxin B (SEB; Toxin Technology, Inc, Madison, WI); phytohemagglutinin (PHA; Difco, Detroit, MI); sodium lauryl sarkosinate (Sigma Chemicals, St Louis, MO); proteinase K (Sigma); RNase (Type I-A, Sigma); propidium iodide (PI; Sigma). Fab', fragments of Leu-3a antibody were prepared as described.15

Induction of CD4 cross-linking. PBMCs were treated with various anti-CD4 MoAbs at concentrations of 3 μg/2 × 10⁶ cells/mL for 1 hour at 4°C. Thereafter, cells were cultured in 24-well Nunc plates (Nunc, Roskilde, Denmark) coated with GAM IgGs (100 μg/mL).

TCR stimulation. Freshly isolated PBMCs or the cells that had been subjected to CD4 cross-linking were cultured with one of the following: medium alone, SEB (0.1 μg/mL), WT-31 (1 μg/mL), or PHA (10.5 μg/mL) for indicated periods. In some cultures, ZnS04 (Sigma) was added at a concentration of 2 mmol/L.

Measurement of apoptosis. For the present study, the percentage of cells undergoing apoptosis was quantitated by a modification of the flow cytometric method for determining fragmented nuclei with PI staining as described.16 Briefly, 2 × 10⁶ cells were washed in Hanks’ balanced salt solution (HBSS), and fixed in 70% ethanol for 3 hours at 4°C. The cells were then washed and resuspended in 0.5 mL HBSS. To this suspension, 0.5 mL RNase solution (1 mg/mL HBSS) was added followed by 1 mL PI (100 μg/mL HBSS). After gentle mixing, cells were incubated overnight at 4°C in the dark. The PI fluorescence of individual cells was measured using a flow cytometer (Epics Elite; Coulter Electronics, Hialeah, FL). Cell debris and cell clumps were excluded by gating for single cells under forward- and side-light scatter. The specificity of this technique was confirmed using human thymocytes. Treatment with dexamethasone, a known inducer of apoptosis in thymocytes, specifically induced a characteristic hypodiploid apoptotic (Ao) DNA peak, whereas sodium azide treatment, which induces cell death through a nonapoptotic mechanism, failed to induce this hypodiploid Ao DNA peak.17 Moreover, to verify this method for the evaluation of apoptosis, a cytopspin smear was made of the same samples used for flow cytometry and examined morphologically for the presence of apoptotic cells under the fluorescent microscope (Leitz, Wetzlar, Germany). Percentage of cells undergoing apoptosis was also determined morphologically, based on the criteria described.18

For analysis of DNA fragmentation, conventional electrophoresis for total DNA19 was performed with slight modifications. In brief, pellets from 4 × 10⁶ cultured cells were resuspended in 20 μL of lysis buffer (10 mmol/L EDTA/50 mmol/L Tris-HCl [pH 8.0]/0.5% [wt/vol] sodium lauryl sarkosinate/0.5 mg/mL proteinase K) and incubated at 50°C for 1 hour. Thereafter, RNase was added at a final concentration of 0.5 mg/mL and the sample incubated at 50°C for 1 hour. Samples were then loaded into the dry wells of a 2% agarose gel containing 0.1 μg/mL ethidium bromide. Electrophoresis was performed at 30 V for 16 hours.

Phenotype analysis and subset separation of PBMC. Surface phenotypes of cells were identified by using MoAb by single- or dual-color immunofluorescence. The fluorescein and phycoerythrin (PE)-conjugated MoAbs used were CD3-fluorescein isothiocyanate (FITC)/CD4-PE, CD3-FITC/CD8-PE, CD20-FITC/CD19-PE, CD14-PE (Coulter, Hialeah, FL), CD3-FITC/CD16 plus 56-PE (Becton Dickinson). All incubations were for 10 minutes at room temperature followed by washing with HBSS. These stained cells were analyzed on a flow cytometer (Epics Elite). In some experiments, CD8+ cells or CD19+ cells were purified by se-
RESULTS

Culture-induced spontaneous apoptosis and activation-induced apoptosis. The apoptotic process is characterized by extensive morphologic changes involving chromatin condensation and by the regular fragmentation of genomic DNA into oligonucleosome fragments of 180 to 200 bp units. The conventional DNA electrophoresis technique has technical restrictions; in addition to its low sensitivity, it is not suitable for quantitative estimation. To circumvent these shortcomings, we have used a flow cytometric approach to quantitate the cells undergoing apoptosis after staining of nuclei with PI. It has been shown that the induction of apoptosis in murine thymocytes is accompanied by the appearance of sub-G0/G1 peak in the DNA histogram. When this method was applied to cultured PBMC obtained from HIV- healthy donors and from HIV+ asymptomatic patients, patient PBMCs showed marked spontaneous apoptosis (Fig 1C) as manifested by a sub-G0/G1 Ao peak in the DNA histogram. Morphologic examination (Fig 1D) of identical samples showed the presence of cells with characteristics typical of apoptotic cell death that correlated quantitatively with the flow cytometry assay. As shown in Fig 2, activation-induced apoptosis in PBMCs from HIV-seronegative normal individuals, elicited by superantigen SEB or PHA, was readily demonstrable by the flow cytometry analysis, and Zn2+-treatment, a known inhibitor of endonuclease activity, completely abrogated the Ao peak. The reliability of this method is further supported by several recent publications; the percent Ao peak has been shown to correlate very well with percent DNA fragmenta-
A decrease in apoptosis was noted upon TCR stimulation using treated cells (Table I). PBMCs of normal individuals showed minimal apoptosis without any stimulus, and the levels of apoptotic cells were already enhanced. However, when compared with viability, there was no statistically significant difference between controls and patients except in WT-31-treated samples. Next, we tried to determine whether the cells in the Ao fraction represented the same cells that constantly stain throughout the culture period, or if there was a continuous turnover in apoptosis, resulting in actual cell loss. To address this question, we performed another kinetic study using PBMCs from HIV+ healthy donors and from HIV+ patients with examined cell counts, viability, and percentage of apoptotic cells by flow cytometry. As shown in Table 2, cell counts were reduced to 69% in controls and 46% in patients; apoptotic cells increased to 25% in controls and 52% in patients; and the cellular viability was 90% in controls and 74% in patients, respectively, after 6 days of culture in medium. The percentage of apoptotic cells was always greater than the percentage of trypan-blue positive cells and apparent of significant numbers of apoptotic cells preceded the decline of cellular viability (Table 2 and Fig 3). These data suggest that (1) the observed apoptosis did actually result in cell loss; (2) the process of apoptosis is continuous turnover, which is initiated by nuclear fragmentation and membrane integrity; and (3) the vital dye test only identifies cells in later stages of apoptosis whereas cells in the Ao fraction determined by flow cytometry represent cells in both early (containing fragmented nuclei but with intact membrane integrity) and later stages of apoptosis.

**Table 2. Kinetics of Cell Number, Cellular Viability, and Apoptosis, in Cultured PBMCs From Normal Donors and HIV-1+ Asymptomatic Patients**

<table>
<thead>
<tr>
<th>Time</th>
<th>Controls (x10^6)</th>
<th>Controls (Viability)</th>
<th>Patients (x10^6)</th>
<th>Patients (Viability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>1.00 ± 0</td>
<td>0.2 ± 0.1</td>
<td>1.00 ± 0</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>2 d</td>
<td>0.99 ± 0.19</td>
<td>12.1 ± 9.9</td>
<td>0.81 ± 0.34</td>
<td>33.3 ± 10.0*</td>
</tr>
<tr>
<td>4 d</td>
<td>0.81 ± 0.18</td>
<td>20.3 ± 11.0</td>
<td>0.54 ± 0.18</td>
<td>45.0 ± 12.5*</td>
</tr>
<tr>
<td>6 d</td>
<td>0.69 ± 0.11</td>
<td>24.8 ± 11.8</td>
<td>0.46 ± 0.10*</td>
<td>51.6 ± 19.0*</td>
</tr>
</tbody>
</table>

PBMCs were obtained from healthy donors (n = 3) and from HIV+ asymptomatic patients (n = 5). Cells were resuspended in complete medium and cultured for indicated periods at 37°C. Cell number was counted on a Coulter automatic cell counter; cellular viability was assessed by trypan-blue exclusion, and the percentage of cells found in the Ao fraction by flow cytometry as described. Values significantly different from HIV+ controls at *P < .01 and **P < .05, respectively, by the Student’s t-test are indicated.
Pretreatment primed patient PBMC for apoptosis. Therefore, we sought to determine whether cross-linking of CD4 followed by various modes of TCR stimulation in vitro could induce apoptosis in normal PBMC. As shown in Fig 4 and Table 3, anti-CD4 MoAb (Leu-3a)/GAM Ig treatment-induced cross-linking of CD4 molecules did indeed induce apoptosis in normal PBMCs. The induction of apoptosis was specific for CD4 cross-linking because control-antibody CD1 or GAM treatment alone failed to induce a significant increase in apoptosis. Leu-3a MoAb treatment alone also induced significant apoptosis that was probably due to Fc receptor-mediated CD4 cross-linking. The degree of apoptosis correlated with the degree of CD4 cross-linking because the F(ab')2 form of Leu-3a was much less effective in the induction of apoptosis. Although TCR stimulation significantly augmented the percentage of cells undergoing apoptosis in untreated cells, these stimuli had a minor effect on the augmentation of apoptosis when the CD4 molecules had already been cross-linked. Importantly, cross-linking of CD4 by itself was sufficient to induce apoptosis in PBMCs without any additional T-cell stimulation. Further, we examined the effects of CD4 cross-linking in unfractioned PBMC and in purified T cells. As shown in Fig 5 and Table 4, we confirmed that CD4 cross-linking was sufficient to induce apoptosis in PBMCs when it was introduced in unfractioned PBMCs whereas in purified T cells, CD4 cross-linking alone did not significantly induce apoptosis. These data strongly suggest that the non-T cell fraction plays an important role in CD4 cross-linking-induced apoptosis in PBMC.

Phenotype analysis of cells undergoing apoptosis. In a final set of experiments, we investigated the phenotype of the cells undergoing apoptosis induced by CD4 cross-linking. First, we analyzed surface phenotype in PBMC that had been treated with medium or with Leu-3a + GAM (Table 5). The effect of CD4 cross-linking can be summarized as follows: (1) Although the total percentage of CD3+ T cells

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Step 1</th>
<th>Step 2</th>
<th>Medium</th>
<th>SEB</th>
<th>WT-31</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>16.2 ± 7.8</td>
<td>30.3 ± 14.4</td>
<td>28.8 ± 11.21</td>
<td>49.4 ± 14.71</td>
</tr>
<tr>
<td>CD1</td>
<td>GAM</td>
<td>23.3 ± 4.6</td>
<td>35.6 ± 7.8</td>
<td>40.8 ± 13.8</td>
<td>53.7 ± 18.75</td>
<td></td>
</tr>
<tr>
<td>Leu3a</td>
<td>GAM</td>
<td>51.2 ± 11.0</td>
<td>45.9 ± 13.3</td>
<td>39.9 ± 11.8</td>
<td>54.4 ± 13.6</td>
<td></td>
</tr>
<tr>
<td>—</td>
<td>GAM</td>
<td>21.9 ± 0.7</td>
<td>43.8 ± 14.08</td>
<td>40.5 ± 16.3</td>
<td>46.3 ± 17.55</td>
<td></td>
</tr>
<tr>
<td>Leu-3a</td>
<td>—</td>
<td>47.0 ± 10.21</td>
<td>44.4 ± 7.7</td>
<td>41.9 ± 10.6</td>
<td>51.7 ± 17.0</td>
<td></td>
</tr>
<tr>
<td>F(ab')2</td>
<td>—</td>
<td>29.9 ± 4.2</td>
<td>45.4 ± 0.85</td>
<td>32.1 ± 4.7</td>
<td>40.6 ± 9.6</td>
<td></td>
</tr>
</tbody>
</table>

Cross-linking of CD4 was induced by the various MoAbs indicated and described in the Fig 3 legend. Cells were then cultured with medium alone or with SEB, WT-31, or PHA, respectively for 3 days. Values denote the percentage of cells found in Ao peak (mean ± SD) determined by flow cytometry. Experiments were independently repeated at least four times. Values significantly different from unstimulated (medium) controls at *P < .001, †P < .01, ‡P < .02, §P < .05, respectively, and values significantly different from CD1/GAM-treated controls at ‡P < .001, †P < .01, respectively, by the Student's t-test are indicated.
Table 4. Comparison of the Effect of CD4 Cross-linking in Unfractioned PBMCs and in Purified T Cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>Treatment</th>
<th>% Viability</th>
<th>% Ao Cells</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>medium</td>
<td>92.3 ± 2.5</td>
<td>17.8 ± 1.7</td>
<td>8.0 ± 1.0</td>
</tr>
<tr>
<td>PBMC</td>
<td>xlink</td>
<td>78.0 ± 5.0*</td>
<td>45.5 ± 6.7*</td>
<td>33.0 ± 2.0*</td>
</tr>
<tr>
<td>T-cell</td>
<td>medium</td>
<td>79.0 ± 1.5</td>
<td>19.0 ± 4.7</td>
<td>12.3 ± 3.8</td>
</tr>
<tr>
<td>T-cell</td>
<td>xlink</td>
<td>80.6 ± 10.8</td>
<td>23.8 ± 1.7</td>
<td>17.3 ± 3.7</td>
</tr>
</tbody>
</table>

PBMCs and purified T cells from a normal donor were treated with medium or with Leu-3a/GAM as described in Fig 4. Cells were then cultured for 4 days. After PI staining, percent Ao cells was assessed by flow cytometry and percent apoptosis was determined by morphology. Cellular viability was assessed by trypan-blue exclusion. Values denote mean ± SD of three independent experiments. Values significantly different from medium controls at * P < .01.

and the percentage of CD8+/CD3+ T cells was unchanged, we were unable to detect CD4+ cells by staining with FITC-conjugated anti-CD4 MoAb in the CD4 cross-linked samples; (2) the percentage of B cells (CD19+20+) and natural killer (NK) cells (CD16+/56+/CD3-) did not significantly change with CD4 cross-linking, and the cells harvested from CD4 cross-linked culture contained less than 2% monocytes (virtually no monocytes were detected in 4 of 6 experiments). This is probably because apoptotic cells are tightly adherent to the GAM-coated culture plate. These results indicate that the major population in CD4 cross-linked cells subjected to DNA analysis consisted of T cells. Secondly, after induction of CD4 cross-linking, we positively selected CD19+ B cells and CD8+ cells and examined them for apoptosis by flow cytometry and by morphology (Table 6). These cells did not manifest significant apoptosis. Again, we were unable to positively select CD4+ cells in a CD4 cross-linked population even when we used magnetic beads conjugated with anti-CD4 MoAb that recognize the OKT4 epitope. Thirdly, we examined the percentage of apoptosis in the cells that had been depleted of CD8+ and CD19+ cells (Table 6). This CD3+ (CD8-, CD19-, monocyte free) cell population was found to be the major population undergoing apoptosis. However, the percentage of cells undergoing apoptosis was not enriched in this population. This is probably because apoptotic cells are labile and some are lost during the cell-separation procedures. The cell recovery was 75% in CD19+, 63% in CD8+ and 67% in CD19-CD8- cell fraction, respectively. Although we cannot rule out the possibility that some apoptotic cells lose their surface molecules...
Table 5. Phenotypic Analysis of PBMCs With or Without CD4 Cross-linking

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Medium (day 0)</th>
<th>Medium (day 3)</th>
<th>xlink (day 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+4-8-</td>
<td>74 ± 4</td>
<td>80 ± 8</td>
<td>86 ± 5</td>
</tr>
<tr>
<td>CD3+4+</td>
<td>51 ± 3</td>
<td>50 ± 3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CD3+8+</td>
<td>23 ± 3</td>
<td>31 ± 6</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>CD3+4+8-</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>B cells</td>
<td>CD19<em>20</em></td>
<td>5 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>NK cells</td>
<td>CD3<em>16</em>56*</td>
<td>8 ± 6</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD14*</td>
<td>16 ± 7</td>
<td>6 ± 5</td>
</tr>
</tbody>
</table>

Freshly isolated PBMCs or PBMCs with or without CD4 cross-linking cultured for 3 days were analyzed for surface phenotype by flow cytometry. Values denote mean ± SD of percent positive cells from six healthy individuals. Percent recoveries of the cells after 3 days of culture in three experiments were 93% ± 7% in medium and 86% ± 3% in CD4 cross-linked samples, respectively.

and hence are unable to be positively selected, and also that some NK cells present in fraction (Fr) 3 (6%) were undergoing apoptosis, these findings suggest that CD4+ T cells constitute the major cell population to undergo apoptosis as a result of CD4 cross-linking.

**DISCUSSION**

In this report, we have used a quantitative flow cytometric technique and morphologic analysis for showing apoptosis and have shown that (1) PBMC from HIV-seropositive asymptomatic individuals undergo marked spontaneous apoptosis that results in cell loss; (2) in vitro stimulation of T cells of HIV-seronegative as well as HIV-seropositive individuals by SEB, WT-31 or PHA, results in activation-induced cell death by apoptosis; and (3) induction of CD4 cross-linking in PBMC by anti-CD4 MoAb Leu-3a is sufficient to induce apoptosis in normal CD4+ T cells. A large body of evidence has been accumulating that cell death through apoptosis plays an important role in the pathogenesis of HIV-1 infection. At the same time, our observations imply that cross-linking of CD4 in vivo may contribute greatly to this accelerated cell-death mechanism.

The observation by Newell et al28 that cross-linking of CD4 followed by TCR stimulation results in apoptosis of murine CD4+ lymphocytes prompted us to hypothesize that circulating gp120/anti-gp120 immune complexes in HIV-infected individuals could trigger human CD4+ T cells for PCD after cell activation. Our study shows that in vitro CD4 cross-linking of normal PBMC can induce apoptosis that, quantitatively, closely approximates the spontaneous apoptosis observed with cultured patient samples. In this context, our results differ from those recently reported by two other groups.29 Groux et al9 observed activation-induced cell death of CD4+ cells from HIV-infected individuals, but they failed to observe activation-induced cell death of normal CD4+ T cells even after cross-linking of CD4 followed by TCR stimulation. The discrepancy may be related to the difference in the culture period and the method used for analyzing apoptosis; the investigators evaluated apoptosis by conventional DNA electrophoresis in overnight-cultured PBMCs, and by means of trypan-blue exclusion in PBMCs and in purified T-cell subsets that had been cultured for 48 hours. In a time-course study (Fig 3), we also did not observe activation-induced effects by vital-dye exclusion test in samples cultured for 2 days; however by flow cytometry, we clearly showed that significant activation-induced cell death occurs in PBMCs of normal controls and HIV+ patients that have been cultured for 3 days. The hallmarks of apoptosis are a rapid onset of oligonucleosomal DNA fragmentation followed by a delayed loss of membrane integrity.23 Our time kinetics study also supports this notion. Thus, vital-dye exclusion alone is not an appropriate means for assessing apoptosis. Banda et al29 recently reported that cross-linking of CD4, induced either by anti-CD4 MoAb or by gp120, induced apoptosis in normal CD4+ T cells subsequent to TCR stimulation, but that cross-linking CD4 alone failed to induce apoptosis. They used purified CD4+ T cells that had been cultured for 72 hours and analyzed for DNA fragmentation by means of agarose electrophoresis followed by colorimetric quantitation with the diphenylamine method. A major difference between their study and ours is the cell population used; we used whole PBMC, and not purified T cells. In fact, we ourselves confirmed that CD4 cross-linking by itself is not sufficient to induce apoptosis in purified T cells but did induce CD4+ T-cell apoptosis if CD4 cross-linking was introduced in unfractioned PBMC. These data strongly suggest that for purified CD4+ T cells, signaling through the CD4 molecule alone may not be sufficient for triggering endonuclease activation and that TCR-me-

Table 6. Phenotypic Analysis of the Cells Undergoing Apoptosis

<table>
<thead>
<tr>
<th>Subset</th>
<th>% Recovery*</th>
<th>% Viability</th>
<th>Ao Cell</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>med unfractioned (Fr 0)</td>
<td>96 ± 1</td>
<td>14 ± 3</td>
<td>9 ± 7</td>
<td></td>
</tr>
<tr>
<td>xlink unfractioned (Fr 0)</td>
<td>100</td>
<td>81 ± 6</td>
<td>37 ± 8</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>CD19 enriched (Fr 1)</td>
<td>75 ± 7</td>
<td>92 ± 6</td>
<td>7 ± 9</td>
<td>9 ± 9</td>
</tr>
<tr>
<td>CD8 enriched (Fr 2)</td>
<td>63 ± 6</td>
<td>93 ± 4</td>
<td>9 ± 8</td>
<td>7 ± 8</td>
</tr>
<tr>
<td>CD8/19 depleted (Fr 3)</td>
<td>67 ± 7</td>
<td>87 ± 5</td>
<td>29 ± 7</td>
<td>27 ± 9</td>
</tr>
</tbody>
</table>

PBMCs from three donors were treated with medium (med) or with Leu-3a/GAM as described. After culture for 3 days, cells were harvested (Fr 0) and subsequently selected with anti-CD19 MoAb (Fr 1) and CD8 MoAb-coated magnetic beads (Fr 2), respectively. Fr 3 represents the cell fraction depleted of CD19+ and CD8+ cells. After PI staining, percent Ao cells was assessed by flow cytometry and percent apoptosis was determined by morphology. Cellular viability was assessed by trypan-blue exclusion. Values denote mean ± SD of three experiments. Purity of each cell fraction as determined by flow cytometry is as follows: Fr 1: >95% CD19*20*; Fr 2: >90% CD3*8*; Fr 3 <1% CD19*, <1% CD3*8*, 86%-90% CD3*8*, 6% CD3-16*56* and 0% CD14+ cells.

* Percent recovery of cells in Fr 1-3 was calculated by the following formula: (net cell recovery in each fraction/absolute number of cells in xlink-Fr 0 [based on described phenotype]) × 100.
diated signaling might be required in addition. Alternatively, CD4 cross-linking not only elicits signals into CD4+ T cells but also stimulates other CD4-expressing cells such as monocytes to secrete apoptosis-inducing factor or cytokine.45

Our results and those of Banda et al imply that HIV-mediated CD4 cross-linking plays an important role in inducing depletion of uninfected CD4+ T cells. The recent evidence that gp120/160, predominantly in the form of immune complexes, can be identified in circulation39 as well as in cell-associated form on CD4+ lymphocytes of HIV-infected patients43 supports this contention. Autoantibodies against CD4 T cells, which are frequently detected in HIV infection,32 could also lead to CD4 cross-linking. Circulating lymphocytes comprise only 2% of total body lymphocytes.33 Recent studies show that a heavy load of HIV is trapped as immune complexes by the follicular dendritic cell network in the lymph node.34-37 There is also evidence implying that T cells constantly move through lymphoid follicles.38 Therefore, the lymph node is most likely the site where T cells are primed for apoptosis. The observed accelerated apoptosis of patient cells was manifested in cultured but not in freshly isolated cells, suggesting constant clearance of apoptotic cells in vivo. Cell death by apoptosis includes several steps leading to progressive alteration in the plasma membrane that result in swift recognition and ingestion by macrophages.39

In contrast to irradiation or dexamethasone treatment, which rapidly induces apoptosis in thymocytes within several hours,21,22 cross-linking of CD4 and/or TCR-stimulation–triggered apoptosis in mature peripheral blood T cells required a relatively long induction period.29,40-42 Therefore, it is unlikely that intracellular signals elicited by these molecules directly trigger endonuclease activation, but rather some indirect mechanism appears to be responsible. Thus, CD4 cross-linking per se is not the only mechanism of apoptosis induction in HIV infection as suggested,4 and it is possible that some factor or receptor could also be involved in inducing apoptosis. Indeed, we and others have previously reported that gp120 is able to induce secretion of IL-6 as well as TNF-α (N.C., unpublished observation, January 1991) from CD45RO+–cloned CD4+ T cells and from macrophages.5 In this context, several recent studies have shown that certain novel surface molecules such as Fas antigen,43 which is identical to the APO-1 antigen,44 and belongs to the nerve growth factor/TNF receptor family, mediates apoptosis-triggering signals. Anti-Fas MoAb treatment has been shown to induce selective killing of chronically HIV-infected cells.45 Defective Fas-antigen expression results in a lymphoproliferative disorder in mouse46 and human CD45RO+ T cells preferentially expressing Fas antigen.47

We observed that cross-linking of CD4 could lead to increased Fas-antigen expression on T cells (N.O. in preparation). Further study is required to elucidate the mechanism of CD4 cross-linking–mediated apoptosis, so that novel therapeutic approaches can be designed to prevent CD4+ T-cell depletion in HIV-infected individuals and for designing safe vaccines using gp120/gp160.

ACKNOWLEDGMENT

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Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals

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