Peripheral Blood from Human Immunodeficiency Virus Type 1–Infected Patients Displays Diminished T Cell Generation Capacity

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An organ culture chimera system was used to assess the effect of human immunodeficiency virus type 1 (HIV-1) infection on the T cell–generation capacity of precursors derived from human peripheral blood. Peripheral blood mononuclear cells from HIV-1–infected patients and uninfected controls were placed on fetal thymus lobes of NOD/LtSz-scid/scid mice. Blood from the HIV-1–infected patients consistently produced fewer CD4 and CD8 cells compared with blood from controls (P < .01). Addition of zidovudine to the cultures did not alter this profile. Limit dilution experiments suggested that there were fewer functional precursors in the infected patients. These results were not dependent on the patient’s level of peripheral CD4 cells; even samples from patients with normal CD4 cell counts were unable to generate T cells in organ cultures. The results are consistent with a loss in the capacity of HIV-1–infected patients to produce functional T cell progenitors in their peripheral blood.

Infection with human immunodeficiency virus type 1 (HIV-1) is characterized by progressive depletion in the number of CD4+ T lymphocytes circulating in the peripheral blood. As the primary site for production and selection of T cells, the thymus is the first site at which CD4+ T cells are produced. It has been previously shown that the thymus of children infected with HIV-1 is atrophied [1], with severe depletion of lymphocytes, Hassal’s corpuscles, and thymic epithelial cells [2]. In a 3-day-old neonatal subject with HIV-1 infection, levels of CD4+CD8+ and CD4+CD8− cells were low, suggesting that HIV-1 infection depletes T cells at a very early stage of their differentiation [3].

In addition, HIV-1 infection diminishes the capacity of adult bone marrow to generate new cells of the erythrocyte, granulocyte, and megakaryocyte lineages [4, 5]. Hematopoietic cells in fetal and adult life are derived from CD34+ stem cells in the bone marrow. A small subset of these cells have been shown to be infected with HIV-1 in some adult patients [6]. These data suggest that bone marrow of HIV-1–infected patients also would have a diminished capacity to develop T cells.

Model systems have been developed recently to more directly address the mechanisms involved in CD4+ T cell depletion during HIV-1 infection. One of these models is the SCID-hu mouse model, in which human fetal thymus is colonized with stem cells from human fetal liver under the kidney capsule of an immunoincompetent scid/scid mouse (e.g., C.B-17 scid/scid). Human T cells developing in these systems can be infected successfully with HIV-1, resulting in a thymic pathology that is very similar to that observed in HIV-1–infected pediatric patients [7]. An in vitro organ culture system for human thymus has also been reported that can be infected by HIV-1 with similar results [8]. These observations indicate that T cell development is abnormal in persons infected with HIV-1.

With the models of T cell development and HIV-1 infection utilizing human thymic tissue to support T cell differentiation, it has been difficult to distinguish the effects of HIV-1 infection on T cell precursors from the effects on thymic stroma. To obviate this problem, we have used a xenogeneic in vitro organ culture system [9], which can be infected with HIV-1 [10], to examine the effects of HIV-1 infection on T cell development. This system uses murine fetal thymus lobes as recipients for T progenitor cells from the peripheral blood of HIV-1–infected patients. Since HIV-1 does not infect murine stromal cells, the use of murine fetal thymus in organ culture reduces any effect of HIV-1 infection of thymic stromal cells on T cell development. The organ culture system should, therefore, isolate the effects of HIV-1 infection on T cell progenitors from its effects on thymic stromal tissue.

Materials and Methods

Mice. NOD/LtSz-scid/scid mice (Jackson Laboratory, Bar Harbor, ME) were maintained and bred in microisolator cages in the animal care facility at the University of Arizona. Fetal thymus...
lobes were removed from 14- to 16-day gestation mouse fetuses and placed in organ culture as described previously [10].

Human subjects. HIV-1–infected male subjects (13) and uninfected controls (3 male; 3 female) were recruited from the Tucson VA Medical Center. As shown in table 1, patients who had various levels of CD4 cell depletion (1072–20 CD4+ cells/μL) and who were or were not receiving antivirals were selected.

Peripheral blood mononuclear cell (PBMC) isolation. Peripheral blood (30 mL) was obtained by venous puncture and collected into tubes containing 5% sodium heparin. PBMC were isolated by centrifugation on separation medium (Ficoll-Paque; Pharmacia, Piscataway, NJ), according to the manufacturer's instructions, and washed in magnesium- and calcium-free Hanks' balanced salt solution.

Organ culture. Fetal murine thymus lobes (8–12) were placed in an organ culture system on Millipore (Bedford, MA) filters (25-μm thick, 0.45-μm pore size) supported on surgical Gelfoam (Upjohn, Kalamazoo, MI). Organ cultures were grown in Dulbecco's MEM (Sigma, St. Louis) with 20% fetal bovine serum (Hyclone, Salt Lake City), 1 mg/mL penicillin, 1 U/mL streptomycin, and 3.4 g/L sodium bicarbonate and maintained at 37°C with 5% CO2. Then 105 total PBMC were placed on each of the lobes, unless otherwise noted, as described [9]. In brief, cells were placed on each thymus lobe by direct application of broken pellets of cells in 0.2-μL aliquots until the designated total number of donor cells per lobe was reached. In some experiments, 1 μg/mL (4 μM) 3'-azido-3'-deoxythymidine (zidovudine; Sigma) was added to the organ culture medium. This amount is 4 times the dose previously reported to inhibit the effects of HIV-1 in organ culture [8]. After the designated number of days in culture, lobes were enzymatically digested in collagenase [11] and stained for three-color flow cytometry. Cell yield and viability were assessed by trypan blue staining and counting. Viability, as assessed by trypan blue, was 83.7% ± 8.7% SD in 16 determinations for cultures derived from uninfected donors and 81.4% ± 11.5% SD in 40 determinations for cultures derived from HIV-1–infected donors.

Table 1. Characteristics of HIV-1–positive subjects whose peripheral blood was used to study the effect of HIV-1 infection on the T cell–generation capacity of progenitor cells.

<table>
<thead>
<tr>
<th>Subject</th>
<th>No. of CD4+ cells/μL</th>
<th>Antiretroviral therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1072</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>776</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>650</td>
<td>None</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>590</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>563</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>520</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>510</td>
<td>Zidovudine</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>349</td>
<td>Didanosine</td>
</tr>
<tr>
<td>9</td>
<td>230</td>
<td>Stavudine</td>
</tr>
<tr>
<td>10</td>
<td>207</td>
<td>Zidovudine + zalcitine</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>84</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>Zidovudine + lamivudine</td>
</tr>
<tr>
<td>13</td>
<td>20</td>
<td>Zidovudine + didanosine</td>
</tr>
</tbody>
</table>

Flow cytometry. Cells from collagenase digestion were stained for three-color flow cytometry with monoclonal antibodies to human surface molecules CD3 (fluorescein), CD8 (phycoerythrin), and CD4 (tricolor) as described [9]. All monoclonal antibodies and isotype controls were from Caltag (South San Francisco). Cells were run on a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, Braintree, MA) and analyzed with Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were gated on the basis of forward scatter characteristics. Gated cells were analyzed for the expression of CD4 and CD8 cell subsets: CD4+CD8−, CD4−CD8+, CD4+CD8+, and CD4−CD8−. Each subset was further analyzed for the expression of CD3. Cells that expressed little or no CD3 were considered immature, while cells that expressed moderate to high levels of CD3 were considered mature.

Statistical analysis. Flow cytometry data were calculated on a per lobe basis by multiplying the frequency of a given T cell subset by the total number of viable cells produced per lobe as assessed by trypan blue exclusion of cells counted in a hemocytometer. These data were entered into a statistical program (Statview; Abacus, Berkeley, CA), which provided the mean ± SE for each data point. Differences between nonpaired continuous data in 2 groups were analyzed by use of Student’s t test. Differences between nonpaired continuous data in >2 groups or data with repeated measures were analyzed by analysis of variance (ANOVA). A P value with 95% confidence was considered statistically significant.

Reverse transcriptase (RT) assay. HIV-1 replication in culture was determined by RT assay, using supernatant from 12-day cultures as described [12, 13]. The amount of RT activity was expressed as counts per minute (cpm) per milliliter. HIV-1NL4-3 (from the National Institutes of Health AIDS Research and Reagent Program) was used as a control. This strain had a TCID50 of 2,511,886 or 105/mL, as determined on the A3.01 cell line.

Results

Peripheral blood was obtained from 6 uninfected control subjects and 13 HIV-1–infected patients (table 1) for determination of CD4 cell counts. Patients were stratified into 4 groups based on CD4 cell counts to determine whether levels of CD4 depletion were related to the ability to develop T cells in organ culture: Group I consisted of patients with >600 CD4+ cells/μL; group II had 400–599; group III had 200–399; and group IV had <200. PBMC from subjects and controls were placed onto murine fetal thymus lobes (105 total cells/lobe). We had shown previously [9] that peak development of human T cells in the xenogeneic fetal thymus organ culture system occurs from day 10 to 14 of culture, so the cultures were grown for 12 days.

As shown in figure 1, after 12 days in organ culture, the numbers of CD4+ and CD8+ cells were significantly decreased in cultures derived from HIV-1–infected blood compared with cultures from HIV-1–negative control blood (P = .001 and .005 for CD4+ and CD8+ cells, respectively, for all cultures derived from HIV-1–infected PBMC; Student’s t test). The
number of CD4⁺CD8⁻ cells that developed in organ culture was also lower with PBMC from HIV-1–infected patients (P = .04, Student’s t test; data not shown). In addition, as shown in figure 1, there were no statistical differences in the numbers of CD4⁺ (P = .94, ANOVA) or CD8⁺ (P = .61, ANOVA) cells that developed in culture for the 4 groups of HIV-1–infected patients. These data show that PBMC from HIV-1–infected patients are less capable of developing T cells of both the CD4 and the CD8 subsets in organ culture. The inability to develop T cells occurred in all cases; even patients who had not experienced any peripheral CD4 depletion displayed a lower capacity to develop T cells in this system.

The degree of T cell development was further examined by the level of CD3 cell expression. The number of mature (those with high to moderate levels of CD3) and immature (those with no or little CD3) cells was compared for cultures derived from HIV-1–infected and uninfected PBMC. As shown in table 2, the number of immature cells of both the CD4 and CD8 subsets that developed in cultures derived from HIV-1–infected PBMC was 2- to 3-fold lower than the number that developed in cultures with control PBMC (P = .03 and .05, respectively; Student’s t test). The number of mature cells (for both subsets) in cultures derived from HIV-1–infected PBMC was more than 10-fold lower than the number for control cultures. At days 3, 6, and 9 of culture, development of immature cells in cultures derived from HIV-1–infected PBMC showed the same 2- to 3-fold decrease compared with that for controls; few mature cells were seen at these time points (data not shown). Since the development of both immature and mature cells, as well as CD4⁺CD8⁻ cells, was diminished, the developmental capability of HIV-1–infected PBMC was affected very early in the T cell differentiation pathway.

Since T cell development was diminished in cultures derived from HIV-1–infected PBMC, limit dilution analysis was done to determine if there was a change in the number of functional progenitor cells. Figure 2 shows that 10⁴, 10⁵, and 10⁶ peripheral blood cells from uninfected 12-day cultures display, with increasing size of inoculum, a linear increase in development of both CD4⁺ and CD8⁺ cells. These data suggest a frequency of progenitor T cells to be of the order of 1 in 10⁴ total cells. This value is similar to what we have found previously [9] and suggests that the T cells produced in this system arise from immature progenitors rather than from contaminating mature T cells. Among the HIV-1–infected patients, there was a failure to develop substantial numbers of cells of either subset at any inoculum (P = .001 for CD4⁺ cells, P = .02 for CD8⁺ cells; ANOVA for HIV status and inoculum). These data show that there were fewer functional precursors in the peripheral blood of HIV-1–infected donors.

Since previous studies had shown that the number of productively infected cells in the peripheral blood of asymptomatic patients was about 1 in 10⁵ [14], it was possible that a few infected cells had been transferred to the culture from the original inoculum. Therefore, we checked the culture fluid of 12-day cultures for HIV-1 replication, using the RT assay. A very low level of virus was found in cultures derived from HIV-1–positive donors. Supernatant of cultures derived from PBMC of 8 HIV-1–positive donors, 116–1072 CD4⁺ cells/μL, gave a range of RT activity of 2275–39,465 cpm/mL. The RT activity of the HIV-1NL4-3 control (TCID₅₀ = 125,594/mL) was 1,602,097 cpm/mL. There was no correlation between the CD4 cell count in the blood of the patients and the amount of RT activity in culture, and the development of T cells in organ culture for PBMC from all of these patients was comparable (figure 1). These data show that the amount of viral replication in the organ culture was extremely low.

Since a low level of viral replication could be detected in cultures derived from infected donors, zidovudine was added to the organ cultures to block infection of newly developing

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**Figure 1.** CD4 (A) and CD8 (B) expression in 12-day chimeras constructed from 10⁶ peripheral blood mononuclear cells of 6 controls (black bars) and HIV-1–infected (clear bars) donors. Infected donors were stratified into 4 groups (minimum of 3/group) based on peripheral CD4⁺ T cell counts: I = >600, II = 400–599, III = 200–399, IV = <200 cells/μL (see table 1). Data are mean no. of cells/thymus lobe ± SE, P = .001 and .005 for CD4 and CD8 cells, respectively, (Student’s t test) for comparison of control and all HIV-1–infected samples. P = .94 and .61 (ANOVA) for CD4 and CD8 cells, respectively, when comparing across 4 HIV-1–positive groups.
cells. Others have shown that 0.25 \( \mu g/mL \) zidovudine could block the infection of organ culture by 10\(^2\) TCID\(_{50}\) of exogenously added HIV-1 [8]. When 1 \( \mu g/mL \) zidovudine was added to cultures derived from uninfected subjects, there was no difference in the number of CD4\(^+\) or CD8\(^+\) cells that developed, indicating that zidovudine at this dose had no effect on T cell development. There was also no difference in either CD4\(^+\) or CD8\(^+\) cells that developed in cultures derived from HIV-1–infected PBMC. Of interest, the number of CD8\(^+\) cells that developed in organ cultures was also diminished even though CD8\(^+\) cell depletion does not occur in patients until very late stages of the disease. The relative contribution of this mechanism to the eventual depletion of peripheral CD4\(^+\) cells in AIDS is the result of a failure to generate new T cells [15]. Taken together, these observations support the contention that part of the eventual depletion of peripheral CD4\(^+\) T cells remains to be determined and may differ between adults and children infected with HIV-1.

Of interest, the number of CD8\(^+\) cells that developed in organ cultures was also diminished even though CD8\(^+\) cell depletion does not occur in patients until very late stages of the disease.

### Discussion

The present study shows that progenitors in peripheral blood of HIV-1–infected patients display a diminished capacity to generate T cells in an in vitro organ culture system. Cultures derived from control, uninfected PBMC developed immature and mature cells of both the CD4\(^+\) and CD8\(^+\) subsets. Compared with control cultures, cultures derived from HIV-1–infected PBMC developed significantly fewer cells of either the CD4\(^+\) or CD8\(^+\) subsets. This inability to develop T cells was observed in both the immature and mature CD4\(^+\) and CD8\(^+\) subsets. The data show that immature T cell development was reduced 2- to 3-fold in cultures derived from HIV-1–infected donors, which suggests that T cell precursor activity is reduced but not completely abrogated in HIV-1–infected persons. The total number of cells recovered from 12-day organ cultures derived from HIV-1–infected PBMC was lower than that for control cultures, regardless of the level of CD4\(^+\) cell depletion of the patient. There was no difference in the ability of progenitors in peripheral blood to develop T cells from patients with widely varied levels of peripheral CD4\(^+\) cells; even patients with peripheral blood CD4\(^+\) cell counts that are considered normal were less capable of generating new T cells. These data suggest that HIV-1 infection has an effect on T cell generation that occurs early in infection, even before there is depletion of peripheral CD4\(^+\) cells.

Of interest, the number of CD8\(^+\) cells that developed in organ cultures was also diminished even though CD8\(^+\) cell depletion does not occur in patients until very late stages of the disease.

### Table 2. Comparison of the development of immature and mature cells in CD4 and CD8 cell subsets for 12-day organ cultures derived from 10\(^5\) peripheral blood mononuclear cells of HIV-1–positive and –negative donors.

<table>
<thead>
<tr>
<th>Subset, HIV status of donor</th>
<th>Immature cells</th>
<th>Mature cells</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0.69 ± 0.19</td>
<td>1.81 ± 0.62</td>
<td>.03</td>
<td>.02</td>
</tr>
<tr>
<td>Positive</td>
<td>0.32 ± 0.06</td>
<td>0.16 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0.74 ± 0.34</td>
<td>0.84 ± 0.38</td>
<td>.05</td>
<td>.01</td>
</tr>
<tr>
<td>Positive</td>
<td>0.20 ± 0.06</td>
<td>0.061 ± 0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Cells were considered immature if they expressed little or no CD3 and mature if they expressed moderate to high levels of CD3. Patients were pooled without regard to peripheral CD4 cell counts. Data are mean no. of cells/lobe × 10\(^3\) ± SE. P values were determined by use of Student’s t test.

### Figure 2.

CD4 (A) and CD8 (B) cell expression of limited dilution experiments. PBMC from control (○) and HIV-1–infected patients with >500 CD4\(^+\) cells/\(\mu L\) (□) were added to organ cultures (at 10\(^4\), 10\(^5\), and 10\(^6\) cells/lobe) and cultured for 12 days. Data are mean no. of cells/thymus lobe ± SE for 3 experiments. P = .001 and .02 (ANOVA) for CD4 and CD8 cells, respectively, when comparing HIV status and inoculum size.
Disease [16]. This dichotomy can be explained by the fact that while CD4+ cells are a target for HIV-1 and are being lost in the periphery, CD8+ cells are not. In addition, the CD8+ cells undergo peripheral expansion due to immune activation in response to antigens from HIV-1 and opportunistic pathogens [17]. The combination of proliferation and lack of direct killing of CD8 cells would serve to keep the numbers of CD8+ cells in the periphery relatively high despite a lack of differentiation of new cells.

We found that organ cultures derived from HIV-1–infected donors produced very low levels of replicating virus. If the infection of newly developing cells was completely responsible for the observed differences in T cell production among cultures derived from HIV-1–infected and uninfected PBMC, then a substantial increase in the number of CD4+ T cells would be expected when cultures were treated with zidovudine. In fact, cultures derived from HIV-1–infected PBMC that were treated with zidovudine produced the same low numbers of T cells as untreated cultures. Since zidovudine is known to interfere with new exogenous HIV-1 infection in organ culture [8], these data suggest that the infection of newly developing T cells in culture was not responsible for the observed lack of T cell development with HIV-1–infected PBMC. However, we cannot rule out that virus or viral products made by a small number of T cells that had been infected prior to organ culture, and therefore unaffected by zidovudine treatment in the culture, could have affected T cell development. To determine the contribution of virus or viral products in organ culture to the decrease in T cell development observed with HIV-1–infected PBMC, the production of virus and viral products would have to be completely abrogated.

The results of limit dilution experiments in the organ culture system suggest that there is a reduction in the frequency of functional T cell precursors in the peripheral blood of HIV-1–infected persons even if peripheral depletion of CD4+ cells has not yet begun. This loss of functional activity could be due to several mechanisms. First, the number of precursors in peripheral blood could be lower. This effect could occur by infection causing the stem cells to migrate out of the peripheral blood and home to some other site in the body. Second, the production of T cell precursors can be decreased in the bone marrow by alteration of bone marrow stromal cells or the hematopoietic stem cells themselves. Alternatively, the same number of precursors may be present in the peripheral blood, but they or a portion of them are less functional due to inactivation by the virus or its products. Finally, the precursors could be infected and, therefore, unable to develop or they could be killed outright. Some HIV-1–infected CD34+ cells can be detected in the bone marrow of some infected patients [6]. One mechanism whereby HIV-1 can affect the ability of cells in bone marrow to develop has been described. CD34+ cells of the bone marrow have been shown to make transforming growth factor (TGF-β) in response to the binding of envelope gp120 to CD4 on their surfaces even in the absence of productive infection [18]. TGF-β is a potent negative regulator of hematopoiesis, which acts by inhibiting entry into the cell cycle [19] and by inducing apoptosis [20].

The TGF-β production activity of a few affected CD34+ cells could affect neighboring cells of the bone marrow, and thus reduce the number of functional progenitors. TGF-β made by CD34+ cells has been shown to reduce the number of functional progenitors of the myeloid and erythroid lineages [5]. This mechanism may also explain the greater decrease in the production of mature T cells compared with that for immature cells in cultures derived from HIV-1–infected donors (table 2). The production of TGF-β by even a small number of infected T cells during the culture period could also further decrease the development of mature T cells. At present, we cannot determine the mechanism by which T cell generation capacity is diminished in these patients.

The data presented here show that the ability of HIV-1–infected persons to develop T cells is severely impaired. The data also demonstrate that the reduction in T cell development capacity occurs in an early T cell progenitor cell population, suggesting that our results may also apply to the generation of T cells in pediatric patients infected with HIV. The inability to develop substantial numbers of T cells occurs very early in infection; even patients who are considered immunologically normal displayed this defect, and patients treated after initiation of infection showed no improvement in their ability to produce T cell progenitors. Our data on T cell lineage development, combined with the data on other hematopoietic lineages, suggest that future therapeutic regimens for HIV-1 infection must include therapies designed to be used before T cell progenitors are affected.

Acknowledgments

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References