Elevated Adenosine Deaminase and Purine Nucleoside Phosphorylase Activity in Peripheral Blood Null Lymphocytes From Patients With Acquired Immune Deficiency Syndrome

By James L. Murray, Karin C. Loftin, C. Gwyneth Munn, James M. Reuben, Peter W.A. Mansell, and Evan M. Hersh

The purine metabolic enzymes adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) are important in lymphocyte differentiation, and genetic deficiencies of either enzyme have been associated with hereditary immunodeficiency states. Both ADA and PNP activity were measured in null cell-enriched and T cell-enriched peripheral blood lymphocytes from 16 patients with the acquired immune deficiency syndrome (AIDS), seven patients with the AIDS-related symptom complex (ARC), and seven asymptomatic homosexuals. ADA activity in nmo/10^9 lymphocytes/h was significantly elevated in null lymphocytes from AIDS (161 ± 12) as compared with 23 healthy heterosexual controls (127 ± 8; P < .025). PNP activity was also significantly increased in null lymphocytes from AIDS patients (96 ± 10; P < .005) as well as those from ARC patients (84 ± 11; P < .025) relative to controls (61 ± 6).

No significant differences in enzyme activity were noted in T cell-enriched cells in any group. Along with elevated enzyme activity, AIDS patients had small yet significant increases in the percentages of HLA-DR (P < .025), terminal deoxynucleotidyl transferase (TdT) (P < .0001), and peanut agglutinin receptor (P < .0001) positive lymphocytes in the null fraction compared with controls. TdT-positive cells appeared morphologically as large lymphoblasts with irregular nuclei. The data imply that the cellular immune deficiency in AIDS is not a result of deficiencies in lymphocyte ADA or PNP activity, but is more likely associated with an increase in an immature and/or activated lymphocyte subset.

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MATERIALS AND METHODS

Subjects

Purine enzymes were studied in peripheral blood lymphocytes from 30 homosexual males. Seven were asymptomatic and were self-referred because of their concern about AIDS. Seven had two or more of the AIDS-related symptoms, and 16 had documented Kaposi's sarcoma, opportunistic infections, or both (AIDS). No patients had received immunotherapy or chemotherapy prior to the study. Twelve healthy heterosexual males and 11 healthy females...
served as controls. In addition, peripheral blood lymphocytes from 12 of the original 16 patients with AIDS and 15 controls were examined for immature or activated lymphocyte markers as described below.

**Lymphocyte Separation Techniques**

Peripheral blood mononuclear cells (PBMCs) were purified from defibrinated blood by Ficoll-Hypaque solution density centrifugation. PBMCs were depleted of monocytes and B cells and enriched for null and T cells by two methods. In the first method, nylon wool-purified lymphocytes were incubated for ten minutes with neuraminidase-treated sheep RBCs (SRBCs), pelleted by centrifugation, and placed on ice for 30 minutes. The E-rosette-containing pellet was gently resuspended, layered on Ficoll-Hypaque, and centrifuged for 15 minutes at 800 g. Pelleted SRBCs were lysed with 0.14 Tris-NH₄Cl and resuspended in phosphate-buffered saline (PBS). In method 2, PBMCs were enriched for T cells by panning. An amount of 3 x 10⁶ PBMCs in 3 mL of PBS was placed on F(ab')₂ goat anti-human Ig (150 μg/mL) (Cappel Laboratories, Cochranville, Pa)-coated 20 x 100-mm polystyrene Petri dishes (Falcon Products, Oxnard, Calif). After one hour of incubation at 20 °C, nonadherent cells were eluted, washed twice, resuspended in 1 mL of PBS, and incubated with 1 μg of anti-Leu 1 (Becton Dickinson, Mountain View, Calif) for 30 minutes on ice. Five micrograms of affinity-purified goat anti-mouse Ig F(ab')₂ fragment (Tago, Burlingame, Calif) was coupled to 0.2 mL of packed ox erythrocytes (OEs) using 0.2 mL of chromic chloride solution. OEs were washed three times in PBS and resuspended to a concentration of 0.5% in RPMI media containing 5% fetal calf serum (FCS). Equal volumes of anti-Leu 1-treated cells at a concentration of 4 x 10⁶ cells per milliliter and pretreated OEs were centrifuged at 300 g for five minutes and incubated on ice for 60 minutes. Following resuspension, rosette-positive lymphocytes were separated from rosette-negative lymphocytes by centrifugation at 500 g for 30 minutes in Ficoll-Hypaque. Red cells were lysed by Tris-NH₄Cl. Lymphocytes were washed twice in RPMI and resuspended in PBS. Null cell-enriched and T cell-enriched cells obtained by either technique were examined for monocyte contamination using fluorescein isothiocyanate (FITC)-conjugated polyvalent rabbit anti-human Ig (Cappel). Fluorescent-positive lymphocytes were enumerated by using a Leitz Ortholux (Wetzlar, Germany) phase contrast microscope.

**Lymphocyte Surface Markers in Null Cell- and T Cell-Enriched Subsets**

From 0.5 to 1 x 10⁶ null cell-enriched and T cell-enriched lymphocytes from 15 controls and 12 homosexuals with AIDS were aliquoted into 12 x 75-mm glass tubes (Fisher Scientific, Houston). Ten microliters (1 μg) of the fluorescein-conjugated monoclonal antibodies anti-HLA-DR and anti-Leu 1 (Becton Dickinson), OKT3 (Ortho Diagnostics, Raritan, NJ), and the unconjugated antibody OKT10 (Ortho) were added. Lymphocytes were incubated at 4 °C for 20 minutes. Following incubation, cells were washed twice in PBS and the percentages of HLA-DR+, Leu 1+, and OKT3-positive cells were determined by flow cytometry using an Ortho Spectrum III cytofluorograph. Cells labeled with OKT10 had indirect staining performed with 50 μL of a 1:100 dilution of fluoresceinated F(ab')₂ goat anti-mouse Ig (Kallestad Laboratories, Dallas) for an additional 20 minutes before analysis. OKT3 was used to check the purity of T cells enriched for anti-Leu 1 by the ox-rosetting technique as a result of blocking and/or modulation of anti-Leu 1 from the cell surface.

**TdT Determination**

Cytoplasmic TdT was assessed according to the method of Bollum using an assay kit supplied by Bethesda Research Laboratories (Gaithersburg, Md).

**Peanut Agglutinin (PNA) Receptor Assay**

From 0.5 to 1 x 10⁶ null cell- and T cell-enriched lymphocytes were pelleted in 15 x 75-mm glass tubes (Fisher) and 50 μg of FITC-labeled PNA (Vector Laboratories, Burlingame, Calif) was added. Following a 20-minute incubation at 4 ℃, cells were washed twice and pelleted, and a drop of cell suspension was placed on a microscope slide. The percentage of fluorescence-positive lymphocytes was enumerated by phase contrast (100 x oil immersion objective).

**Measurement of ADA and PNP**

ADA and PNP activity were measured in null cell- and T cell-enriched lymphocytes using a modification of the technique of Van Laarhoven et al. One-microliter aliquots containing from 1,000 to 3,000 lymphocytes in normal saline and 0.5% bovine serum albumin (BSA) (Sigma Chemical, St Louis) were added to individual wells of Terasaki microtiter plates (Falcon Plastics), frozen at −30 ℃, and lyophilized. Five-microliter aliquots of ADA reaction mixture containing 0.26 mmol/L [8,14C]-adenosine (specific activity, 20 mCi/mmol; ICN Pharmaceuticals, Irvine, Calif), 0.5% BSA, 0.8 μg/mL streptomycin, 0.8 μg/mL penicillin in 0.17 mmol/L Tris-HCl buffer (pH 7.4) (GIBCO, Grand Island, NY), and PNP reaction mixture containing 0.17 mol/L Tris-HCl, pH 7.0; 0.5% BSA; 0.8% Pen-strep; 3.3 mmol/L EDTA; 4.7 mmol/L phosphate; and 0.26 mmol/L [3-3H]-inosine (Amersham, Arlington Heights, Ill; specific activity, 50 mCi/mmol) were added to each cell extract. Plates were incubated for 30 minutes (ADA) or two hours (PNP) in high humidity at 37 ℃. From these assay conditions, the amount of product formed was linear with respect to cell number and length of incubation. From 10% to 15% of substrate was converted to product over the specified time intervals. After incubation, plates were placed in ice, and 1-μL aliquots were removed and spotted on α-cellulose-coated thin-layer chromatography (TLC) plates (Anatech, Inc, Newark, Del). Plates were incubated for two hours in ADA solvent (butanol: NH₄OH:H₂O = 100:16:5) or PNP solvent (ethanol: NH₄Ac = 4:1) and dried. Products were identified by ultraviolet (UV) fluorescence and were scraped off and added to 5-mL plastic counting vials (Curtin Matheson Scientific, Houston) along with 3 mL aquasol (Packard Instruments, Downers Grove, Ill). Radioactivity in cpm was measured using a β-scintillation counter (Packard Instruments) and the percentage of substrate that converted to product was calculated. Results were expressed as nmol of converted substrate/10⁶ lymphocytes/h.

**RESULTS**

**Percent Yield and Purity of Lymphocyte Subpopulations**

The average yield of nylon wool column-purified lymphocytes expressed as a percentage of the total number of Ficoll-Hypaque density gradient-purified mononuclear cells obtained from 60 mL of whole blood was significantly lower in ARC (49% ± 3%; mean ± SD) and AIDS patients (53% ± 6%) compared with controls (71% ± 10%; P < .005 and P <
The percentage of yield of null cell-enriched lymphocytes obtained after neuraminidase-treated SRBC separation was significantly higher in ARC (37% ± 31%) and AIDS (22% ± 17%) patients as compared with controls (10% ± 8%; \( P < .005 \) and \( P < .025 \), respectively). The percentage of yield of T cell-enriched lymphocytes in both ARC and AIDS was significantly lower than in controls (62% ± 9%; \( P < .005 \) and \( P < .001 \), respectively). No differences in yield of lymphocyte subpopulations were observed between controls and asymptomatic homosexuals.

In contrast to the above, the yield of nonadherent lymphocytes obtained following panning (method 2) was similar in ARC patients (68% ± 9%), AIDS patients (58% ± 27%), and asymptomatic homosexuals (69% ± 10%) as compared with controls (70% ± 9%). The yield of null cell-enriched cells following ox erythrocyte rosetting remained significantly higher in ARC patients (23% ± 5%) and AIDS patients (30% ± 8%) compared with controls (16% ± 3%; \( P < .025 \) and \( P < .005 \), respectively). There were no differences, however, in the yield of T cell-enriched lymphocytes between groups. Hence, overall yield was lower following the nylon wool column procedure, most likely because of a loss of adherent T cells on the column. Despite this, the purity of null and T lymphocyte subfractions with respect to monocyte and B cell contamination as well as T cell enrichment was similar regardless of the separation procedure used (Table 1).

**ADA and PNP Activity in Null Cell- and T Cell-Enriched Subpopulations**

Both ADA and PNP activity expressed as nmol/10^6 lymphocytes/h are shown in Table 2. Mean ADA activity was slightly higher in null lymphocytes from controls (\( P = .051 \)) and markedly higher in null lymphocytes from all homosexuals combined (\( P < .001 \)) than in T lymphocytes from each group respectively. ADA activity in null cell-enriched cells from AIDS patients was significantly elevated (161 ± 8; \( P < .025 \)) as compared with controls (127 ± 8; \( P < .025 \)) and asymptomatic homosexuals (114 ± 11; \( P < .025 \)). There were no significant differences in ADA activity in null cells between patients with AIDS and patients with ARC. Neither was ADA activity significantly increased in patients with ARC compared with controls. PNP activity was increased in null cells from ARC patients (84 ± 11; \( P < .025 \)) and most significantly in AIDS patients (96 ± 10; \( P < .005 \)) as compared with controls (61 ± 5). Significant differences in enzymes were not seen in the T cell fractions of all homosexual groups compared with controls, irrespective of the separation procedures used.

Enzyme activity per milligram of protein was not determined on all patient cells because of the low lymphocyte yields. However, in three AIDS patients and three controls in whom protein determinations were performed, the mean milligram of protein per 10^6 null cells in AIDS (30.1 mg) did not differ from controls (30.6 mg). This indicates that the elevated enzyme activity in AIDS null cells was probably not caused by the presence of large blastlike cells with high protein content, but rather was increased on an individual cell basis. T cells also had similar protein content (AIDS, 34.1 mg; controls, 41.5 mg).

**TdT, PNA, and Surface Marker Expression in Null Cell- and T Cell-Enriched Lymphocytes**

We and others have previously determined that patients with AIDS have significantly elevated percentages of lymphocytes expressing the surface antigens T10 and HLA-DR along with a decreased percentage and absolute number of OKT4-positive lymphocytes.14,15 Because the highest enzyme levels were noted in the null cell-enriched cells from AIDS

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**Table 1. Relative Purity of Lymphocyte Subsets in Homosexuals v Controls**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Null Cell-Enriched</th>
<th>T Cell-Enriched</th>
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</thead>
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<tr>
<td></td>
<td>Control Homosexuals</td>
<td>Control Homosexuals</td>
</tr>
<tr>
<td>NWC/E*</td>
<td></td>
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</tr>
<tr>
<td>Esterase +</td>
<td>6 ± 2.0†</td>
<td>8 ± 2.0</td>
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<tr>
<td>Slg +</td>
<td>2 ± 0.8</td>
<td>2 ± 0.4</td>
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<tr>
<td>E4 +</td>
<td>7 ± 2.0</td>
<td>8 ± 0.8</td>
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<tr>
<td>Panning/OR†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase +</td>
<td>7 ± 0.9</td>
<td>6 ± 0.6</td>
</tr>
<tr>
<td>Slg +</td>
<td>2 ± 0.7</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Anti-Leu 1/T3+</td>
<td>17 ± 3.0</td>
<td>11 ± 5.0</td>
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</tbody>
</table>

*NWC/E = lymphocytes purified by nylon wool followed by E-rosetting.
†Mean percentage ± SEM.
‡Panning/OR, lymphocytes purified by panning followed by ox erythrocyte rosetting.

**Table 2. ADA and PNP Activity in Null Cell- and T Cell-Enriched Lymphocytes**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Null T</th>
<th>PNP T</th>
<th>Null T</th>
<th>PNP T</th>
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<tr>
<td>Controls</td>
<td>23</td>
<td>127 ± 8*</td>
<td>107 ± 6†</td>
<td>61 ± 5</td>
<td>62 ± 5</td>
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<tr>
<td>Homosexuals</td>
<td>30</td>
<td>146 ± 8</td>
<td>109 ± 5‡</td>
<td>85 ± 7§</td>
<td>68 ± 3</td>
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<tr>
<td>Asymptomatic</td>
<td>7</td>
<td>114 ± 11</td>
<td>82 ± 10</td>
<td>53 ± 13</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>ARC</td>
<td>7</td>
<td>139 ± 16</td>
<td>104 ± 5</td>
<td>84 ± 11§</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>AIDS</td>
<td>16</td>
<td>161 ± 125</td>
<td>121 ± 7</td>
<td>96 ± 10</td>
<td>72 ± 4</td>
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</table>

*Activity expressed as nmol/10^6 lymphocytes/h; mean ± SEM.
†Control T cell-enriched v control null cell-enriched; \( P = .051 \).
‡Homosexual T v null; \( P < .001 \).
§Homosexuals v controls; \( P < .025 \).
|| \( P < .005 \).
patients, we measured the percentages of cells expressing the immature surface antigens, T10, and HLA-DR as well as Leu 1, PNA receptor, and the enzyme TdT in both null cell- and T cell-enriched subpopulations in 15 controls and in 12 of the 16 patients with AIDS (Table 3). In controls, a higher percentage of OKT10- and HLA-DR–positive lymphocytes were found in the null fraction (OKT10, 24 ± 6; HLA-DR, 11 ± 2) compared to the T fraction (OKT10, 8 ± 2; HLA-DR, 5 ± 1), whereas AIDS patients had similar percentages of OKT10- and HLA-DR–positive lymphocytes in each fraction. However, AIDS patients had significantly elevated percentages of HLA-DR–positive (P < .025), TdT-positive (P < .0001), and PNA-positive (P < .0001) lymphocytes in the null fraction as compared with controls. Significantly elevated percentages of OKT10- and anti–HLA-DR positive cells were also observed in the T cell-enriched fraction of AIDS patients compared with the T cell-enriched fraction of controls. No TdT-positive or PNA-positive lymphocytes were seen in the T cell-enriched fraction of any group studied.

As seen in Figs 1 and 2, the TdT-positive cells observed in the null fraction of AIDS patients were generally large lymphocytes (ie, greater than 12 μm) with an increased nuclear to cytoplasmic ratio. Only cells with both cytoplasmic and nuclear staining for TdT were counted.

**DISCUSSION**

Although the pathophysiology of AIDS is not entirely clear, the findings of this report shed additional light on several hypothetical considerations. ADA and TdT are elevated in human and rat cortical thymocytes. Activity of both enzymes decreases pro-

<table>
<thead>
<tr>
<th>Marker</th>
<th>Group</th>
<th>Controls (15)</th>
<th>AIDS (12)</th>
<th>P Value*</th>
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<tr>
<td></td>
<td>Null cell-enriched</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OKT10</td>
<td>24 ± 6.0†</td>
<td>42 ± 10</td>
<td>.10</td>
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<tr>
<td>Anti-HLA-DR</td>
<td>11 ± 2.0</td>
<td>31 ± 4</td>
<td>&lt;.001</td>
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<tr>
<td>Anti-TdT</td>
<td>0 ± 6</td>
<td>2 ± 2</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>PNA</td>
<td>1 ± 0.4</td>
<td>11 ± 3</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>Anti-Leu 1</td>
<td>14 ± 3.0</td>
<td>11 ± 5</td>
<td>NS</td>
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<tr>
<td>T cell-enriched</td>
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<td></td>
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<td></td>
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<tr>
<td>OKT10</td>
<td>8 ± 2.0</td>
<td>22 ± 6</td>
<td>&lt;.025</td>
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<tr>
<td>Anti-HLA-DR</td>
<td>5 ± 1.0</td>
<td>34 ± 6</td>
<td>&lt;.001</td>
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<tr>
<td>Anti-TdT</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>NS</td>
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</tr>
<tr>
<td>PNA</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>OKT3</td>
<td>93 ± 3.0</td>
<td>93 ± 2</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant.

*Student’s t test; controls v AIDS.
†Expressed as mean percentage of positive cells ± SEM.
gressively as cortical thymocytes mature into medullary thymocytes and peripheral T cells.‡ Hence, it is possible that an increase in ADA activity in null cells from AIDS patients, along with an increase in the percentages of TdT-positive lymphocytes, could reflect abnormalities in T cell differentiation. This hypothesis is attractive in light of recent evidence that demonstrates thymic atrophy in AIDS patients along with elevated levels of thymosin-α.

ADA levels in immature thymocytes have been reported to be five- to tenfold higher than in peripheral blood T cells.‡ Because ADA levels in null lymphocytes were only 1.3-fold higher than T cells in this study, it is most likely that we are dealing with a very small subset of immature and/or activated T cells within the null cell fraction. An alternative explanation for the increase in enzyme activity in AIDS might be the selective loss of the OKT4-positive subpopulation that could have very low ADA and PNP activity. However, this is unlikely in view of similar percentages of contaminating anti-Leu 1/OKT3-positive lymphocytes in the null fraction of both AIDS patients and controls (17% ± 3% vs 11% ± 5%, respectively). Moreover, in preliminary studies, we have measured ADA and PNP levels in OKT4-positive cells in both AIDS patients (ADA, 55 ± 37; PNP, 26 ± 11) and controls (ADA, 36 ± 31; PNP, 27 ± 11) without noting significant differences.

Unlike ADA and TdT, PNP levels are low in cortical thymocytes and increase with subsequent maturation.§ PNP is elevated over ADA in bone marrow null cells as well as various T, B, and null lymphoblastoid cell lines. Therefore, a more likely explanation for the findings observed could be that a normal compensatory bone marrow response is occurring secondary to thymic, lymph node, or peripheral destruction of helper T cells. The presence of immature lymphocyte subsets expressing “activation” antigens T10 or DR in association with thymic atrophy has also been observed in disease states in which regeneration of the lymphoid system is occurring, such as following bone marrow transplantation. Hence, this hypothesis is also feasible and would explain the occasional appearance of TdT- and PNA-positive stem cells in the peripheral blood. Also, in preliminary experiments, we have not seen increased percentages of OKT9-positive or OKT6-positive lymphocytes in AIDS, which suggests that changes are occurring at the prethymic level.

Both ADA and TdT levels have been shown to be transiently increased in mitogen-activated lymphocytes.° The increased percentage of large, blastlike cells seen in AIDS could reflect T cell tropism secondary to viral infection. In the last several years, considerable evidence has been presented linking the cause of AIDS to infection by a retrovirus of the human T cell leukemia family, HTLV III. A virus of similar serologic reactivity designated LAV has also been isolated from AIDS and pre-AIDS patients in France. HTLV III is cytopathic for helper (OKT4) lymphocytes; T cell lines that have been infected with virus and grown in the presence of interleukin 2 transform into large cells with basophilic cytoplasm and indented nuclei. Occasional multinucleated giant cells and numerous cells in mitosis are also seen. In addition, from 35% to 45% of infected lymphocytes collected from sperm samples in AIDS patients express HLA-DR antigens and demonstrate reverse transcriptase activity. Hence, the increases in enzymes along with the presence of occasional TdT- and PNA receptor-positive cells in the null cell fraction of AIDS patients could be a direct result of infection by virus. It is also possible that an association could exist between T cell infection by HTLV III and thymic dysplasia. For example, recent evidence demonstrates the presence of an antigen (P19) on thymic epithelium which cross-reacts with an internal core protein of HTLV. Significantly elevated percentages of OKT10- and HLA-DR-negative lymphocytes were also noted in the T lymphocyte fraction of AIDS patients as compared with controls (Table 3), yet significant increases in enzymes, TdT-positive or PNA receptor-positive lymphocytes were not observed. These findings could most likely represent a small subset of T cells which are coexpressing activation antigens. Nicholson et al have reported an increase in Ia-positive cells in ARC patients that were of the suppressor (OKT8) phenotype. Additional studies using double immunofluorescent labeling techniques are needed to confirm these observations.
In summary, the findings in this study confirm that the cellular immunodeficiencies manifested in AIDS are the result of, or are associated with, abnormal lymphocyte differentiation or activation rather than due to deficiencies of either enzyme. Additional studies should be directed toward elucidating the interactions between cytopathic viral infections in this disease, and their subsequent influence on thymic and bone marrow physiology.

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