Lymphocyte protein synthesis is increased with the progression of HIV-associated disease to AIDS

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ABSTRACT

HIV infection has been shown to affect lymphocyte function and to reduce lymphocyte responsiveness in vitro to mitogenic stimulation, but little is known about lymphocyte metabolism in vivo and how it is affected during the course of the disease. This study investigated the metabolic activity of lymphocytes in vivo through the progression of HIV-associated disease. Lymphocyte protein synthesis was measured with L-[2H5]phenylalanine (45 mg/kg body weight) in healthy volunteers (n = 7), in patients who were HIV-positive (n = 7) but asymptomatic, and in patients with AIDS (n = 8). The rates of lymphocyte protein synthesis [expressed as a percentage of lymphocyte protein, i.e. fractional synthesis rate (FSR)] were not altered in HIV-positive patients compared with healthy controls (7.9 ± 1.28% and 9.1 ± 0.53%/day respectively), but were significantly elevated in AIDS patients (14.0 ± 1.16%/day; P < 0.05). The serum concentration of the cytokine tumour necrosis factor-α (TNF-α) increased with the progression of the disease, and TNF-α levels were significantly higher in AIDS patients (6.81 ± 0.88 ng/l) than in healthy controls (3.09 ± 0.27 ng/l; P < 0.05). Lymphocyte protein FSR was positively correlated with serum TNF-α concentration (r = 0.55, P = 0.009) and negatively correlated with CD4+ lymphocyte count (r = -0.70, P = 0.004). The elevation of lymphocyte protein synthesis in AIDS patients suggests a higher rate of turnover of lymphocytes. This may be associated with a generalized activation of the immune system, which is also reflected by the elevated serum TNF-α concentration in the late stages of HIV-associated disease.

INTRODUCTION

HIV infection is associated with the progressive impairment of immune function. Several different abnormalities of lymphocyte function have been described, affecting not only CD4+ T lymphocytes, which are the main target of the HIV virus, but also other lymphocyte subpopulations. These include decreased lymphocyte proliferation after mitogenic stimulation, reduced production of interleukin-2 and decreased expression of the interleukin-2 receptor [1–7]. However, many of the functional tests are carried out in vitro after isolating lymphocytes from whole blood, and the results might not be representative of lymphocyte metabolism in vivo. Little is known about lymphocyte metabolism in vivo and how it is affected in the different stages of HIV-associated disease.

The aim of the present study was to investigate lymphocyte metabolism in vivo during the course of HIV-associated disease, by assessing the rate of synthesis...
of lymphocyte protein. Lymphocyte protein synthesis is an index of cell metabolic activity, and has been shown to be stimulated by treatments that induce activation and proliferative responses of the immune cells, both in vitro [8,9] and in vivo [10]. Lymphocyte protein synthesis therefore represents an important metabolic marker of immune cell turnover and activation, and it can be safely measured in vivo with stable isotope techniques [10–12].

The effect of progression of HIV-associated disease was assessed by comparison of rates of lymphocyte protein synthesis in healthy subjects, patients with HIV infection alone and patients with AIDS. The relationship of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) to alterations in lymphocyte protein synthesis was also assessed.

A preliminary report of this study has been published previously in abstract form [13].

METHODS

Subjects

Seven healthy control subjects and 15 HIV-infected patients participated in the study. Controls were recruited by local advertisement. HIV-infected subjects were recruited from patients attending the AIDS Clinic at the University Medical Center, Stony Brook. HIV-infected subjects showing any signs of acute illness, opportunistic infection or metabolic abnormalities, or those with diagnosed malignancies or who had recently undergone surgery, were excluded.

HIV-infected patients were divided into two groups, HIV and AIDS. The HIV group (n = 7) included patients who were HIV-positive but asymptomatic, with a CD4+ cell count of ≥ 400/mm³ and no history of opportunistic infections. The AIDS group (n = 8) included patients with a CD4+ cell count of ≤ 200/mm³ or who had experienced an AIDS-defining illness [14]. Once patients were classified as having AIDS, they retained the classification even if CD4+ cell counts subsequently rose above 200/mm³. Consequently, at the time of the study two patients in the AIDS group had CD4+ cell counts between 200 and 400/mm³. In the AIDS group three patients experienced weight loss of > 10% compared with preillness body weight, and five had been treated previously for opportunistic infections.

The study was carried out in the period 1994–1996, before highly active anti-retroviral therapies (HAART) had become the standard care of treatment of HIV infection. Therefore none of the patients had been treated with HAART before participating in the study. However, the majority of the HIV-infected patients in both groups were taking medications, which were not discontinued before the study. These included antivirals (two HIV and eight AIDS patients), prophylactic antibiotics or antimycotics (one HIV and six AIDS patients), pain relievers (three HIV and two AIDS patients), and vitamin and mineral supplements (three HIV and four AIDS patients).

The study protocol was approved by the Committee on Research Involving Human Subjects at the State University of New York at Stony Brook. Informed written consent was obtained from all subjects involved in the study.

Protocol

The study was carried out at the Clinical Research Center (GCRC), University Hospital at Stony Brook. The subjects were admitted at 17.00 hours and assessed clinically. Lean body mass (LBM) was measured by dual-energy X-ray absorptiometry (DEXA, model DPX; Lunar Radiation, Madison, WI, U.S.A.). At 07.00 hours the next morning, with the subject in the post-absorptive state, two intravenous cannulas were inserted into contralateral forearm veins, and a blood sample was taken for measurement of TNF-α concentration and HIV load. Lymphocyte protein synthesis was then measured by injecting a 2% (w/v) solution of phenylalanine (45 mg/kg body weight) (Ajinomoto, Tokyo, Japan) containing 10% 1-[3H]phenylalanine (MassTrace, Woburn, MA, U.S.A.) over 10 min. Blood samples of 2 ml were drawn at various intervals over 90 min in order to measure changes in plasma 1-[3H]phenylalanine enrichment. A larger blood sample (30 ml) was then taken at 90 min to determine isotope incorporation into lymphocyte protein.

Isolation of lymphocytes

Blood for lymphocyte isolation was transferred into heparinized tubes containing cycloheximide (final concentration 0.5 mM) (Sigma, St. Louis, MO, U.S.A.) in order to prevent any further incorporation of the isotope into cell protein. Lymphocytes were then isolated with a modification of the method of Bøyum [15] which allowed purification of lymphocytes from phagocytic cells and platelets [16]. After sedimentation of red cells with 6% (w/v) dextran (Sigma) at room temperature for 30 min, the leucocyte-enriched plasma was depleted of platelets by addition of ADP (Sigma) [17]. Phagocytic cells were then removed by preincubating the leucocyte-enriched plasma with iron particles for 30 min at 37 °C. Lymphocytes were then isolated by density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) [15]. The lymphocyte layers were removed and washed several times with 0.9% NaCl solution and then stored at −70 °C until analysis.

Measurement of lymphocyte protein synthesis

Measurement of lymphocyte protein synthesis with 1-[3H]phenylalanine was similar to that described previously [11,12,16]. Briefly, plasma phenylalanine enrich-
ment was determined after cation-exchange chromatography by monitoring the ions at \( m/z \) 336 and 341 of the tertiary butyldimethylsilyl derivative on a VG MD 800 quadrupole gas chromatograph mass spectrometer (Fisons Instruments, Inc., Beverly, MA, U.S.A.).

Lymphocyte protein was precipitated with cold perchloric acid (20 g/l) and washed extensively before hydrolysis with 6 M HCl at 110 °C for 24 h. \( [\text{H}] \)phenylalanine enrichment in the hydrolysate was measured after enzymic conversion of phenylalanine into \( \beta \)-phenylethylamine, solvent extraction and derivatization \([18]\). The ions \( m/z \) 106 \((m+2)\) and \( m/z \) 109 \((m+5)\) of the heptafluorobutyryl derivative were monitored under selective ion-recording conditions on the same gas chromatograph mass spectrometer operated under electron-impact conditions in splitless mode \([19]\).

The lymphocyte protein fractional synthesis rate (FSR) was calculated from the \( 1-[\text{H}] \)phenylalanine enrichment in the lymphocyte protein and the area under the curve of the plasma free \( 1-[\text{H}] \)phenylalanine (precursor) enrichment, by using the formula \([20]\):

\[
\text{FSR (%/day)} = 100 \times \frac{P_r}{A}
\]

where \( P_r \) represents the enrichment of phenylalanine in protein at the end of the incorporation period and \( A \) is the area under the curve of precursor enrichment against time expressed in days. To the extent that protein synthesized by lymphocytes might have been secreted during the 90-min incorporation period, the rate of lymphocyte protein synthesis will have been underestimated.

**Measurement of TNF-\(\alpha\) and viral load**

The serum concentration of TNF-\(\alpha\) was determined on duplicate frozen samples by using an ELISA kit with a limit of detection of \( \leq 0.5 \) ng/l (Quantikine High Sensitivity; R&D Systems, Minneapolis, MN, U.S.A.). Viral load was measured by branched DNA hybridization (Quantiplex HIV-RNA assay; Chiron, Emeryville, CA, U.S.A.), as described previously \([19]\).

**Statistical analysis**

The data are expressed as means±S.E.M. Statistical differences between means were analysed by two-tailed Student’s \( t \)-tests for unpaired data. The correlation between two sets of data was determined by linear regression analysis. A \( P \) value of \( \leq 0.05 \) was considered statistically significant.

**RESULTS**

The characteristics and anthropometric measurements of the subjects are summarized in Table 1. The three groups were matched for age, which ranged between 25 and 48 years. The HIV-positive and AIDS groups had lower body mass indices than controls \((P < 0.003)\). The average weight and total LBM were also lower in the HIV-positive group than in controls \((P < 0.05)\), which might reflect the greater number of female subjects included in the HIV group (Table 1). However, the three groups showed no differences in LBM when expressed as a percentage of body weight (control, 76.5±2.2%; HIV, 75.1±2.0%; AIDS, 78.5±3.1%).

Table 2 lists values for viral load, CD4\(^+\) lymphocyte count and TNF-\(\alpha\) serum concentration. In four out of six patients in the HIV group and two out of eight in the AIDS group, the viral load was below the value of 10000 equivalents/ml of plasma, which represents the lower limit of sensitivity of the assay used. For statistical purposes, an arbitrary value of 10000 was therefore assigned to these patients. TNF-\(\alpha\) serum concentrations were significantly higher in the AIDS group \((P = 0.007)\) than in the controls.

Figure 1 shows the FSRs for lymphocyte protein in the control, HIV and AIDS groups. The lymphocyte protein FSR was 9.1±0.53%\%/day \((6.6–10.4\%\%/day)\) in the control group, which was similar to that found in the HIV-positive patients \((7.9±1.28\%\%/day; 5.6–15.3\%\%/day)\). The protein FSR in AIDS patients \((14.0±1.16\%\%/day; 8.8–19.1\%\%/day)\) was respectively 73% and 54% higher than that of HIV-positive patients \((P = 0.006)\) and controls \((P = 0.02)\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control ((n = 7))</th>
<th>HIV ((n = 7))</th>
<th>AIDS ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>7/0</td>
<td>3/4</td>
<td>7/1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35±3</td>
<td>33±3</td>
<td>37±2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74±2</td>
<td>61±2*</td>
<td>68±5</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.71±0.01</td>
<td>1.66±0.01</td>
<td>1.75±0.04</td>
</tr>
<tr>
<td>Body mass index (kg/m(^2))</td>
<td>25±1</td>
<td>22±1*</td>
<td>22±1*</td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>56.8±2.1</td>
<td>45.6±2.2*</td>
<td>52.5±4.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HIV</th>
<th>AIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4(^+) count (cells/mm(^3))</td>
<td>ND</td>
<td>647±112</td>
<td>134±45*</td>
</tr>
<tr>
<td>Viral load (equivalents/ml)</td>
<td>ND</td>
<td>12 100±1430</td>
<td>89 700±49 400</td>
</tr>
<tr>
<td>TNF-(\alpha) (ng/l)</td>
<td>3.09±0.27</td>
<td>4.94±1.23</td>
<td>6.81±0.88†</td>
</tr>
</tbody>
</table>
When each value of lymphocyte protein FSR from the three groups was plotted against the corresponding serum TNF-\(\alpha\) concentration, a significant positive correlation was found \((r = 0.55, P = 0.009)\) (Figure 2A). In HIV-infected individuals, both lymphocyte FSR and TNF-\(\alpha\) serum concentration were also negatively correlated with the CD4\(^+\) count (Figures 2B and 2C). No correlation was apparent between viral load and any of the other parameters.

**DISCUSSION**

Progression of HIV infection to AIDS is characterized by a depletion and dysfunction of CD4\(^+\) lymphocytes, which ultimately results in severe impairment of immune function. In the present study, lymphocyte metabolic activity was assessed *in vivo* by measuring the rate of lymphocyte protein synthesis using stable isotope methodology. The measurements were carried out in healthy volunteers, in patients with asymptomatic HIV infection, and in patients with AIDS. The results show that lymphocyte protein synthesis rates in asymptomatic HIV-infected individuals were comparable with those in healthy controls, but that lymphocyte protein synthesis was significantly increased in patients with AIDS, suggesting increased activity of lymphocytes with the progression of the disease.

The elevated rates of lymphocyte protein synthesis in AIDS are unlikely to have been due to drug treatment. Although there was some heterogeneity among patients with respect to drug treatment, there was no obvious relationship between lymphocyte protein synthesis rates and particular classes of drugs.

Previous studies have described several immunological abnormalities associated with HIV infection, such as impairment of lymphocyte proliferation *in vitro* after mitogenic stimulation and suppression of natural killer activity [2,3,5–7]. In contrast with the findings of our study, in which no change in lymphocyte activity was detected in HIV-positive asymptomatic individuals, defects in proliferative activity *in vitro* have been observed in stimulated lymphocytes from patients in the early stages of the disease [5,7]. These *in vitro* studies were performed during maximal stimulation of isolated lymphocytes with mitogens and, therefore, represent a measure of the maximal potential responsiveness of
circulating lymphocytes to immune challenges. By comparison, the rate of protein synthesis of lymphocytes assessed from in vivo labelling represents the prevailing level of metabolic activity.

The in vivo data from the present study would suggest that circulating lymphocytes in patients with asymptomatic HIV-associated disease are no more metabolically active than those in healthy, uninfected individuals. The rate of protein synthesis measured in the present study includes protein that is turned over (synthesized and degraded) within cells as well as protein synthesized for the production of new cells. Increased lymphocyte protein synthesis has been shown to be associated with elevated lymphocyte proliferation [10].

With progression to AIDS, the rates of protein synthesis in lymphocytes increased. This increase in metabolic activity is consistent with the conclusions of other studies examining the rate at which circulating levels of lymphocytes increase following HAART [21–23]. Because the CD4+ lymphocyte number increases rapidly following the initiation of HAART, the HIV pathogenesis of CD4+ depletion in AIDS has been viewed as being the result of elevated cell destruction by the virus and not of a failure of lymphocytes to regenerate [21–24]. Our study confirms the view of high lymphocyte metabolic activity and proliferative activity in the late stages of the disease.

Studies by McCune et al. [25] involving in vivo DNA-labelling of lymphocytes suggested that increased cell turnover of lymphocytes contributed to the observed increase in protein synthesis in patients with AIDS. The study of McCune et al. [25] also reported increased lymphocyte turnover in HIV-positive patients, which appears to conflict with the present data on protein synthesis. However, the criteria used to distinguish HIV-positive individuals from those suffering from AIDS were different in the two studies. In the McCune et al. study [25], five out of 11 HIV-positive subjects had CD4+ lymphocyte counts of < 200 cells/mm³; by this criterion these subjects would, in the present study, have been classified as having AIDS. In addition, based on CD4+ lymphocyte number and viraemia, HIV-positive patients in the study of McCune et al. [25] were at a later stage of disease than the HIV-positive patients in the present study.

There is a possibility that the higher rate of protein synthesis observed in AIDS patients may reflect a qualitative difference in the composition of the lymphocyte subpopulations in the peripheral blood with the advancement of the disease. For example, it has been observed that memory/effector-phenotype T-cell subpopulations have a higher turnover rate than naïve-phenotype T cells [25]. An increase in the proportion of memory/effector-phenotype T-cell subpopulations in the peripheral blood with the progression of the HIV infection may therefore contribute to the increased rate of turnover of the total lymphocyte population [25], which would also be reflected in a higher lymphocyte protein synthesis rate.

In addition to increased lymphocyte protein synthesis, increased rates of whole-body protein synthesis have also been reported in AIDS patients [26]. Assuming that the total mass of lymphocytic cells is approx. 1.5 kg, equivalent to 300 g of protein [27], it is possible to calculate the increase in whole-body protein synthesis that is due to the elevation in lymphocyte protein synthesis. The reported increase in whole-body protein synthesis in AIDS patients was approx. 27 μmol of leucine h⁻¹ kg⁻¹ [26]. Assuming body protein to be 8% leucine [28], the increase in lymphocyte protein synthesis could contribute approx. 20% of the increase in protein synthesis observed in the whole body.

The progression of HIV infection is commonly associated with a general activation of the immune system, which is reflected by elevated plasma levels of several cytokines and immune-activation molecules [29–33]. The higher rates of lymphocyte protein synthesis in AIDS patients might therefore be the result of this generalized activation of the immune system. As reported by other authors [34–36], the serum concentration of TNF-α was elevated in HIV-infected individuals. TNF-α levels increased with the progression of the disease, and were higher in AIDS patients than in healthy controls (Table 2).

The conclusion that elevated lymphocyte protein synthesis is the result of generalized activation of the immune system is supported by the highly significant inverse correlation between the rate of lymphocyte protein synthesis and the serum concentration of TNF-α (Figure 2A). It has been suggested that TNF-α might have an important role in the pathogenesis and progression of HIV-associated disease [37,38]. TNF-α is one of the cytokines that is produced by monocytes and macrophages in response to the HIV virus [39,40], but it has also been shown to enhance HIV replication in infected cells [41–43] and to be positively correlated with circulating HIV RNA levels in HIV-positive patients [32]. Dysregulated production of TNF-α could therefore reinforce a vicious circle, by activating the immune system on the one hand and enhancing HIV production and CD4+ lymphocyte destruction on the other, thus amplifying HIV infection and accelerating the clinical progression of the disease. Consistent with this hypothesis are the results of the present study showing that high TNF-α levels were associated with metabolic activation of lymphocytes in patients with progressed HIV-associated disease, and that both activation of lymphocyte protein synthesis and elevated circulating levels of TNF-α were associated with depletion of CD4+ cells (Figure 2).

In conclusion, the present study has investigated the metabolic activity in vivo of lymphocytes in subjects at
different stages of HIV infection. Lymphocyte protein synthesis rates in asymptomatic HIV-infected individuals were found to be comparable with those in healthy volunteers. In AIDS patients, however, rates of lymphocyte protein synthesis were increased by 54%. The enhancement of lymphocyte protein synthesis is consistent with a high rate of turnover of lymphocytes in AIDS patients. This might be associated with a generalized activation of the immune system, which is reflected in elevated plasma levels of pro-inflammatory cytokines, particularly TNF-α, in the late stages of HIV-associated disease.

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