Constitutive Production of Interleukin-6 and Tumor Necrosis Factor-α From Spontaneously Proliferating T Cells in Patients With Human T-Cell Lymphotropic Virus Type I/II

By Renu B. Lal and Donna L. Rudolph

The human T-cell lymphotropic viruses (HTLV) type I and type II are capable of inducing a variety of cellular genes, including many of the cytokines that regulate cell proliferation. To determine if the spontaneous proliferation of peripheral blood mononuclear cells from patients infected with HTLV-I and HTLV-II was related to coordinate expression of cytokines, we analyzed the levels of interleukin-1β (IL-1β), IL-2, IL-3, IL-4, IL-6, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in culture supernatants derived from spontaneously proliferating cells. Significantly elevated levels of IL-6 and TNF-α were present in culture supernatants from HTLV-I/II-infected individuals when compared with normal controls (P < .01). Kinetic experiments showed that both IL-6 and TNF-α were elevated by day 5. None of the other cytokines (IL-1β, IL-2, IL-3, IL-4, and IFN-γ) were detectable in any of the culture. These data suggest that release of IL-6 and TNF-α may regulate lymphocyte proliferation in HTLV-I/II-infected individuals.

MATERIALS AND METHODS

Study population. Sixteen HTLV-I/II seropositive (Serologicals Inc, Marietta, GA) and 16 seronegative healthy volunteers matched for age and sex were used in the study (Table 1). Heparinized (15 to 20 mL) blood specimens were collected and shipped to the Centers for Disease Control (Atlanta, GA) at room temperature within 24 hours of collection.

Serologic studies. Sera from all study subjects were screened for antibodies to HTLV-I/II and human immunodeficiency virus (HIV) using commercial enzyme-linked immunosorbent assay (ELISA) kits (Dupont Co, Wilmington, DE). ELISA-positive sera for HTLV were further confirmed by Western blot and immunoprecipitation analysis. Synthetic peptide-based immunomassays (Coulter Immunology, Hialeah, FL) and polymerase chain reaction (PCR) assays were used to distinguish between HTLV-I and HTLV-II infection as previously described.

Lymphocyte proliferation. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient (LSM; Bionetics/Laboratory Products, Charleston, SC) centrifugation at 400g for 30 minutes at room temperature. Cells from the interface were washed three times with RPMI 1640 medium and resuspended in medium supplemented with 10 mmol/L HEPES, 50 μg/mL gentamicin, and 10% heat-inactivated fetal calf serum (C-RPMI). Levels of endotoxin were approximately 20 pg/mL in C-RPMI and less than 25 to 50 pg/mL in medium containing human AB or autologous serum.

PBMC were cultured in round-bottomed microtiter plates at a concentration of 1 x 10⁶ cells/mL in 200 μL of C-RPMI either in the absence of exogenous signal or with phytohemagglutinin (PHA, 1 nmol/L).
was measured by liquid scintillation spectroscopy. Data are expressed as the mean ± for three separate experiments.

**IL1β**

Soluble HTLV antigen production was determined by using an antigen capture assay (Coulter Immunology) that detects an epitope of p24 antigen present on both HTLV-I and HTLV-II. In parallel, coculture was established with PHA-stimulated normal PBMC for optimal detection of HTLV antigen.

**Determination of soluble levels of cytokines.** The production of cytokines from unstimulated cultures was determined by commercial ELISA kits for IL-1β, IL-2, IL-4, IL-6 (Genzyme, Boston, MA), TNF-α (T cell Sciences, Cambridge, MA), and interferon-γ (IFN-γ) (Amgen, Thousand Oaks, CA).

**RESULTS**

**Spontaneous proliferation of PBMC.** Serologic analysis and PCR methods used to characterize the 16 HTLV-I/II-infected persons showed that four were infected with HTLV-I and 12 were infected with HTLV-II (Table 1). Cultured lymphocytes from these persons demonstrated significantly higher proliferation (9,312 ± 1,218 cpm) when compared with those of unstimulated normal controls (1,892 ± 699). No significant differences were seen with PHA-stimulated cultures (Table 1).

**Production of HTLV antigen.** To determine if spontaneous proliferation in persons infected with HTLV-I/II might be due to the mitogenic effect of HTLV antigen per se, we determined the levels of p24 antigen in culture supernatants. No detectable levels of p24 antigen were seen in any of the cultures; however, coculture of these patients’ PBMC with PHA-stimulated normal PBMC showed presence of HTLV antigen in most cultures by day 14 (data not shown).

**Cytokine production in cultured cells.** To analyze other soluble mediators of lymphoproliferation, culture supernatants derived from the spontaneously proliferating PBMC were analyzed for the presence of IL-1β, IL-2, IL-4, IL-6, TNF-α, and IFN-γ. No quantitative differences were observed in IL-1β, IL-2, IL-4, or IFN-γ in culture supernatants from HTLV-I/II-infected patients when compared with normal controls (Fig 1). However, a significant increase was observed in the levels of both IL-6 and TNF-α in HTLV-I/II-infected individuals (2,090 ± 352 and 650 ± 120 pg/mL, respectively) as compared with those of normal controls (Fig 1).

**Kinetics of IL-6 and TNF-α release.** Culture supernatants collected on day 2, day 5, and day 10 were analyzed for the levels of IL-6 and TNF-α. Levels of both IL-6 (Fig 2, top) and TNF-α (Fig 2, bottom) were significantly higher by day 5 in supernatants from patients infected with HTLV-I/II, and they remained elevated until day 10. Quantitative levels of IL-6 and TNF-α in the serum of these patients were not different from those of the normal controls (data not shown).

**DISCUSSION**

Various receptor ligand interactions between surface molecules on lymphocytes and other cells trigger the release of a variety of soluble mediators. Among such mediators are ILs, IFNs, and TNFs, all of which are important in the initiation and regulation of the immune response. In the present investigation, we analyzed the constitutive production of different cytokines from spont-

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Table 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HTLV Seropositive (n = 16)</th>
<th>Normal (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>12/5</td>
<td>10/6</td>
</tr>
<tr>
<td>Serologic test HTLV antibody†</td>
<td>16/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Synthetic peptide‡</td>
<td>12/16</td>
<td>0/16</td>
</tr>
<tr>
<td>HTLV-I</td>
<td>4/16</td>
<td>0/16</td>
</tr>
<tr>
<td>HTLV-II</td>
<td>12/16</td>
<td>0/16</td>
</tr>
<tr>
<td>PCR analysis§</td>
<td>12/16</td>
<td>ND</td>
</tr>
<tr>
<td>Lymphocytic proliferation</td>
<td>20,132 ± 2,312</td>
<td>22,648 ± 1,888</td>
</tr>
<tr>
<td>PHA</td>
<td>3,912 ± 1,218d</td>
<td>1,892 ± 699</td>
</tr>
</tbody>
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*Median (range). †Positivity based on p24 and gp46 reactivity. "Polymerase chain reaction using type-specific oligoprimer and oligoprobes. §Lymphocyte proliferation measured in the absence of exogenous stimuli. Cells, 1 x 10^6, were cultured in C-RPMI for 8 days and pulsed with 1 μCi/well of 'H thymidine. Results are expressed as counts per minute ± for three separate experiments. dP < .01 when compared with normal controls.

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Fig 1. Quantitative levels of various cytokines in the culture supernatants of unstimulated PBMC isolated from normal controls or from patients infected with HTLV-I/II. Values for IL-1β, IL-2, IL-4, IL-6, and TNF-α are expressed in picograms per milliliter. The asterisk represents a P value <.001 in HTLV-infected individuals when compared with normal donors.
CONSTITUTIVE EXPRESSION OF IL-6 AND TNF-α

Fig 2. Kinetics of IL-6 and TNF-α release in the cultures derived from normal controls (C) or from patients infected with HTLV-I/II (II). Culture supernatants were collected at time points indicated and assayed for IL-6 and TNF-α.

Consistently proliferating lymphocytes of patients infected with HTLV-I and HTLV-II.

In concordance with previous reports, we observed spontaneous proliferation in both HTLV-I- and HTLV-II-infected persons. That the HTLV antigen released in the culture supernatant might stimulate the proliferation is consistent with earlier reports of HTLV mitogenicity. However, absence of detectable levels of p24 antigen in culture supernatants from spontaneous proliferating cells argues against this possibility. Presence of other gene products, in particular tax, either alone or in synergy with cytokines, may be responsible for cellular proliferation.

Among the battery of cytokines evaluated, no differences in the levels of IL-1β, IL-2, IL-4, and IFN-γ were observed in the spontaneously proliferating cells. Conflicting reports of IL-2 in culture supernatants from spontaneously proliferating cells have been reported. Although Tendler et al reported significantly elevated levels of IL-2 in both asymptomatic and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients, Matsumoto et al found increased levels only in patients with HAM/TSP. Our inability to detect increased levels of IL-2 in spontaneously proliferating lymphocytes from HTLV-I/II-infected persons may be due to sequestration of IL-2 by its receptor. Indeed, elevated levels of soluble IL-2 receptor (IL-2Rα) were found in the culture supernatants from these patients (Lal RB, Rudolph DL, Rowe T, Folks TM: submitted for publication, April 1991).

Of greater significance is the finding that levels of IL-6 and TNF-α were significantly increased in the culture supernatants derived from HTLV-infected persons when compared with those of normal controls. Both IL-6 and TNF-α have pleotropic activities in vitro and in vivo, in particular stimulation of various immune effector functions. TNF-α can also induce its own secretion and serve as a cofactor in the production of several cytokines, including IL-6. Therefore, overall cytokine levels in culture supernatants may result from virus infection as well as the autocrine and paracrine induction of these cytokines in infected cells. With regard to the relationship of IL-6 with HTLV, long-term T-cell lines infected with HTLV have been shown to induce gene expression and secretion of IL-6. In addition, increased levels of IL-6 have been demonstrated in both the plasma and the cerebrospinal fluid of HTLV-infected persons. Both IL-6 and TNF-α have also been shown to regulate HIV replication at transcriptional and posttranscriptional levels.

The mechanism by which spontaneously proliferating cells induce the secretion of TNF-α and IL-6 might be mediated by transactivation through the HTLV regulatory gene product, tax. Tax has been shown to regulate in trans a variety of cellular genes, such as IL-2R, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and TGF-β and this transactivation is mediated via one or more transcription factors and cellular proteins. For example, studies on transcriptional regulation of IL-6 have identified sequence elements in its promoter that have the potential to bind cellular transcription factors (NF-IL-6) in response to tax. Similarly, TNF-α induces IL-2Rα gene expression via nuclear proteins that specifically interact with a nuclear factor enhancer element (NF-kB), which is capable of binding to the HTLV-LTR. TNF has also been shown to modulate growth and differentiation by inducing nuclear protooncogenes such as c-fos and c-jun. Both of these oncogenes encode for DNA binding proteins with a leucine zipper structure, that binds to the tax-responsive element within the HTLV-LTR. These transcription factors may function as intermediary transcriptional regulators in signal transduction, thereby sustaining the proliferation of lymphocytes from HTLV-I and HTLV-II infected individuals. Constitutive expression of both fos and jun transcripts have recently been observed in HTLV transformed long-term T-cell lines (Hooper CW, Rudolph DL, Laimore MD, Folks TM, Lal RB, manuscript in preparation, 1991). Further understanding of the role of IL-6 and TNF-α in the regulation of HTLV expression in infected cells may provide important insights into the basis of immune dysfunction in these patients.

REFERENCES


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RB Lal and DL Rudolph