Inhibitory Effect of Interleukin 4 on Production of Interleukin 6 by Adult T-Cell Leukemia Cells

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ABSTRACT

Freshly isolated leukemic cells from patients with adult T-cell leukemia (ATL) produce high levels of interleukin 6 (IL-6), which is suggested to play an important role in thrombocytosis, elevation of C-reactive protein, and hypercalcemia in ATL. In this study, we investigated the effects of T-cell growth factors such as interleukin 2 (IL-2) and interleukin 4 (IL-4) on IL-6 production by ATL cells in vitro. Although IL-2 and/or IL-4 enhanced the cell proliferation of freshly isolated ATL cells from seven of nine patients, IL-2 did not affect the IL-6 release in most cases. In contrast, another T-cell tropic factor, IL-4 markedly inhibited the release of IL-6 in the conditioned medium in all cases. This IL-4-mediated inhibition of IL-6 release was completely abrogated by the addition of anti-IL-4 monoclonal antibody. Time course experiments demonstrated that IL-4 reduced the secretion of IL-6 for a prolonged period of time (more than 72 h). By Northern analysis, IL-4 reduced the transcription level of IL-6 mRNA. Furthermore, by flow cytometry with the use of anti-human IL-4 receptor monoclonal antibody, ATL cells showed the significant level of IL-4 receptor on their cell surfaces without any stimulation. These data suggest that IL-4 may play an important regulatory role in the production of IL-6 in ATL.

INTRODUCTION

IL-4, a product of activated T-cells, was originally described as a B-cell growth and differentiation factor. IL-4 has been shown to possess a broader spectrum of biological activities affecting, among others, T-cells, monocytes, mast cells, and hematopoietic progenitor cells (1–4). Furthermore, it has been recently reported that IL-4 exerts suppressive effects on the spontaneous growth of CMMoL cells (5) and myeloma cells (6) through the inhibition of IL-6 production, which acts as an autocrine or paracrine growth factor of these cells. IL-6 was purified and its gene was cloned from HTLV-I-infected T-cell lines (7). ATL is causally associated with HTLV-I infection and ATL cells frequently produce cytokines such as IL-1 (8, 9), IL-6 (10), and IL-2 in some cases (11); also, ATL cells proliferate in response to IL-2 (11) and IL-4 (12, 13). In the preliminary experiments we also confirmed that serum IL-6 levels in patients with ATL increased significantly, as compared with those of either asymptomatic carriers or controls. These observations led us to investigate whether IL-4 affects the production of IL-6 by ATL cells. In this report, we show that IL-4 markedly inhibits IL-6 production by ATL cells.

MATERIALS AND METHODS

Patients. Nine patients with ATL in acute phase were studied. The age of the patients, five males and four females, ranged from 45 to 76 years old. The diagnosis of ATL was based on clinical features, morphological characteristics, cell surface phenotypes of leukemic cells, serum antibodies against HTLV-I-associated antigens, and the HTLV-I proviral genome in leukemic cells. WBC ranged from 9,800 to 242,900/μl. More than 85% of the peripheral WBCs were leukemic cells, as morphologically determined by Wright-Giemsa staining. CD4-positive (ATL) cells were more than 90% of the PMBC, as evaluated by flow cytometry.

Reagents. Recombinant human IL-2 and human IL-6 were provided by Shionogi Pharmaceutical Co., Ltd., Osaka, Japan, and Ajinomoto Central Research Laboratories Co., Inc., Yokohama, Japan, respectively. Recombinant human IL-4 and mouse MoAb against recombinant human IL-4 were supplied by Ono Pharmaceutical Co., Ltd., Osaka, Japan. Mouse anti-human IL-4R MoAb M57 (IgG1 isotype) was kindly provided by Dr. S. Gillis (Immunex, Seattle, WA). A cloned IL-6/BSF-2 complementary DNA (pBSF2.38) was provided by Dr. T. Taga (Osaka University, Osaka, Japan) (7).

Cell Separation and Cell Culture. PMBC were separated from heparinized blood of nine ATL patients by centrifugation on lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). Cells were further incubated at 37°C for 2 h in plastic culture dishes (Falcon 3002; Falcon Plastics, Oxnard, CA) to remove adherent cells. Furthermore, more enriched ATL cell preparations were obtained by CD4-conjugated immunomagnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway). After purification, the cells in suspension were used for the experiments described below. The percentage of contaminating monocytes was determined by flow cytometry; it was less than 2% of the obtained cells in all patients. Purity of T-cells as evaluated by flow cytometry of anti-CD4-stained cells was more than 95%.

ATL cells were cultured in 24-well culture plates (2 ml/well) at a concentration of 5 × 10^6 cells/ml in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% FCS (Grand Island Biological Co., Grand Island, NY) with or without various concentrations of IL-2 or IL-4 in the presence or absence of mouse MoAb against recombinant human IL-4. After culture for the indicated periods, culture supernatants were collected by centrifugation to measure the levels of released IL-6. The viability of the cells determined by trypan blue exclusion always exceeded 90%.

As a positive control for IL-6 mRNA expression, SALT-3 cells (HTLV-I-infected cell line, a gift from Dr. K. Sagawa, Kurume University, Kurume, Japan) were cultured in RPMI 1640 medium containing 10% FCS at 37°C.

Proliferative Response. ATL cells (1 × 10^6) from patients were incubated in 96-well flat bottomed microtiter plates in the presence of various concentrations of IL-2 or IL-4 in 200 μl of RPMI 1640 medium containing 10% FCS in triplicate for 72 h. The cells were pulsed with 0.5 μCi of [3H]Tdr for the last 24 h. [3H]Tdr incorporation was measured by liquid scintillation counting after harvesting the cells with a cell harvester (A bekagaku Co., Funabashi, Japan).

Detection of IL-6 in Culture Supernatant. IL-6 in the culture supernatant of ATL cells was measured by using the human IL-6 enzyme-linked immunosorbent assay kit (Toray-Fujix Bionix Inc., Tokyo, Japan). The sensitivity of this assay is 10 pg/ml of IL-6 and there is no cross-reactivity with other cytokines.

Northern Blot Analysis for IL-6 Gene Expression. Total cellular RNA was isolated from the cultured cells as previously described by using the single-step isolation procedure (14). Twenty-μg RNA samples were subjected to gel electrophoresis by using 1% agarose containing 6% formaldehyde and were blotted onto nylon membrane filters (Hybond-N, Amersham, Tokyo, Japan). Tapp-BanII fragment of pBSF2.38 complementary DNA was labeled with [32P]dCTP by using the multiprime DNA labeling kit (Amersham Japan).

Cytotoxic fluorescent Measurements of IL-4R Expression. Cells (1 × 10^6) were washed and resuspended in 100 μl PBA. For determination of IL-4R expression, cells were incubated for 30 min at 4°C with 1 μg mouse anti-human IL-4R MoAb M57, washed twice with 1 ml PBA, and further incubated for 30 min with 50 μl fluorescein isothiocyanate-conjugated goat anti-mouse...
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Fig. 1. Proliferative response of ATL cells to IL-2 and IL-4. [3H]Tdr incorporation of peripheral blood leukemic cells cultured with IL-2 or IL-4 was examined. Points, mean of triplicate cultures; bars, SE.

Table 1 Grouping of ATL cases by abilities to respond to IL-2 and IL-4

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient no.</th>
<th>Response to IL-2</th>
<th>Response to IL-4</th>
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<tr>
<td>1</td>
<td>1, 3, 5, 6</td>
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<td>4</td>
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IgG F(ab')2 (Immunotech, Marseille, France) diluted 1:50 in phosphate-buffered saline. After the second incubation, the cells were washed twice with 1 ml PBA and resuspended in 600 μl PBA. Cytofluorometric analysis was performed with FACScan flow cytometry (Becton Dickinson Immunocytometry Systems, Inc., Mountain View, CA). For controls, an isotype control antibody was used.

As a positive control for IL-4R expression, PBMC from normal donors were activated with PHA-P (Difco Laboratories, Detroit, MI) at 0.1% for 72 h.

RESULTS

Cell Proliferation of ATL Cells in Response to IL-2 and IL-4. As shown in Fig. 1 and Table 1, ATL cells from peripheral blood of 4 (patients 1, 3, 5, and 6) of 9 patients proliferated in response to both IL-2 and IL-4 in a dose-dependent manner. ATL cells from 2 patients (patients 2 and 4) responded to IL-2, but did not respond to IL-4. In contrast, ATL cells from 1 patient (patient 8) proliferated in response to IL-4, but did not respond to IL-2. In 2 patients (patients 7 and 9), ATL cells did not proliferate in response to either IL-2 or IL-4. The base-line values of [3H]Tdr incorporation varied from 398 to 8343 cpm. The levels of the spontaneous proliferation seem to bear some relationship to the clinical phase of the disease, as reported previously (13). The maximal response was obtained in most patients in the range of 1 to 10 ng/ml of either IL-2 or IL-4. As shown in Table 1, ATL cells from 6 of 9 patients responded to IL-2 and from 5 of 9 patients responded to IL-4. In agreement with a recent report by Sawada et al. (10), IL-6 itself did not show any effects on [3H]Tdr incorporation of ATL cells at all (data not shown).

Release of IL-6 into Medium. As shown in Fig. 2, culture supernatants of ATL cells sampled from nine patients and cultured for 72 h displayed quite high levels of IL-6 without any stimulation (83.5 to 20,600 pg/ml). Normal T-cells from healthy donors did not secrete IL-6 without stimulation (less than 10 pg/ml). Apart from one sample (patient 2), which was markedly enhanced for IL-6 by IL-2, IL-2 did not show any effects on IL-6 secretion (not statistically significant) (Fig. 2). In contrast, when ATL cells from nine patients were incubated with IL-4 for 72 h, the levels of IL-6 secretion were markedly decreased in all patients (P < 0.005; Wilcoxon test). This effect of IL-4 was observed in a dose-dependent manner. Three representative cases were shown in Fig. 3; other cases showed similar results (data not shown). Furthermore, the effect of IL-4 on IL-6 release was almost completely antagonized by anti-IL-4 MoAb. The representative case, No. 3, was shown in Fig. 4. The inhibition of IL-6 release by IL-4 was almost completely (95%) abrogated by the addition of anti-IL-4 MoAb. In other cases, anti-IL-4 MoAb also markedly reversed the inhibitory effect of IL-4 on IL-6 release (data not shown). These results suggest that IL-4 may markedly suppress the production and/or secretion of IL-6 by ATL cells.

Transcription of IL-6 mRNA. Next, we studied the effects of IL-2 and IL-4 on the transcription level of IL-6. As shown in Fig. 5, an HTLV-I-infected cell line, SALT-3, and freshly isolated ATL cells (patients 1, 3, and 6) showed high levels of IL-6 message without any stimulation. IL-4 also markedly inhibited the transcription levels of
positive cells were markedly increased up to 17% (Fig. 7B). ATL cells from patients showed surprisingly high levels of IL-4R expression without any stimulation. Two representative cases were shown in Fig. 7, C and D, and their IL-4R expression was 78 and 42%, respectively. Other cases also showed high IL-4R expression when compared with normal healthy donors (data not shown). These results suggest that ATL cells show quite high expression of IL-4R without stimulation.

**DISCUSSION**

IL-6 is one of the important and multifunctional cytokines that regulate host defense responses. For example, it is one of the major physiological stimulants for the hepatic production of acute phase proteins. We previously observed that ATL cells showed high expression levels of IL-6 transcripts, and these cells produced IL-6 when they were cultured with IL-2. In this study, we performed experiments to determine whether or not IL-4 can decrease the transcription levels of IL-6 message by ATL cells.

**Kinetics of IL-6 Secretion.** We performed time course experiments to characterize the kinetics of the effect of IL-4 on IL-6 secretion. As shown in Fig. 6, the release of IL-6 from ATL cells in the absence of IL-4 was markedly increased after 6 h in culture. However, it was markedly reduced by the addition of 10 ng/ml IL-4 at from 6 h to more than 72 h.

**Expression of IL-4R on ATL Cells.** Finally, we studied whether ATL cells have IL-4R without any stimulation. As shown in Fig. 7, the expression of IL-4R was examined by flow cytometry and by using a MoAb M57 directed against IL-4R. This antibody inhibited IL-4-dependent cell proliferation by PHA-activated human PBMC and could not bind to IL-4R when it was saturated with IL-4 (data not shown). Normal mononuclear cells from a healthy donor expressed only a very low level or almost none of IL-4R on their surface (2% positive in Fig. 7A). However, when cultured with PHA, IL-4R-expression was markedly increased up to 17% (Fig. 7B).

**Experimental Procedures.** ATL cells from patients were cultured with or without IL-4. The levels of IL-6 were measured by using an enzyme-linked immunosorbent assay. The amounts of IL-6 were expressed as fold changes compared with normal healthy donors.

**Results.**

1. **IL-6 Production by ATL Cells:**
   - ATL cells from patients showed high levels of IL-6 production without any stimulation. Two representative cases were shown in Fig. 7, C and D, and their IL-4R expression was 78 and 42%, respectively. Other cases also showed high IL-4R expression when compared with normal healthy donors (data not shown).

2. **Effect of IL-4 on IL-6 Production:**
   - IL-4 markedly inhibited IL-6 production by ATL cells. The inhibition was dose-dependent and the maximum inhibition was achieved at 10 ng/ml IL-4.

3. **Kinetics of IL-6 Secretion:**
   - The release of IL-6 from ATL cells in the absence of IL-4 was markedly increased after 6 h in culture. However, it was markedly reduced by the addition of 10 ng/ml IL-4 at from 6 h to more than 72 h.

4. **Expression of IL-4R on ATL Cells:**
   - ATL cells from patients showed high expression levels of IL-4R without any stimulation. Two representative cases were shown in Fig. 7, C and D, and their IL-4R expression was 78 and 42%, respectively. Other cases also showed high IL-4R expression when compared with normal healthy donors (data not shown).

**Discussion:**

IL-6 is one of the important and multifunctional cytokines that regulate host defense responses. For example, it is one of the major physiological stimulants for the hepatic production of acute phase proteins. We previously observed that ATL cells showed high expression levels of IL-6 transcripts, and these cells produced IL-6 when they were cultured with IL-2. In this study, we performed experiments to determine whether or not IL-4 can decrease the transcription levels of IL-6 message by ATL cells.
jugated goat anti-mouse IgG F(ab')2 was used as a secondary antibody to stain the cells. ATL cells and HTLV-I-infected cell lines spontaneously produce quite high levels of IL-6 production in ATL. The details of action and mechanisms of IL-4 on cell proliferation and IL-6 production were different among ATL cells. High responders in cell proliferation and IL-6 production such as in patients 1 and 3 expressed high levels of IL-4R on their surface (78% and 42% of IL-4R-positive cells, respectively). In addition, non- or low responders in cell proliferation or IL-6 production such as in patients 2 and 9 expressed lower levels of IL-4R (8.3% and 9.8%, respectively) than that of high responders. However, their (patients 2 and 9) IL-4R expression was still significantly high when compared with normal mononuclear cells (2% positive cells). In preliminary experiments, there seems to be a clear correlation between levels of IL-4R expression and cell proliferation in response to IL-4.

It has been reported that IL-2 and IL-4 mRNA expression was undetectable in leukemic cells from ATL patients (9). However, it is possible that ATL cells are regulated by IL-2 and IL-4 which are locally produced by activated T-cells present in lymphoid tissues in vivo. In fact, although IL-2 was not detected in cultures of PBMC isolated from one patient (patient 4) by immunosassay, we detected significant levels of IL-2 in cultures of mononuclear cells isolated from a lymph node of the same patient.

Several clinical features of ATL may be explained by up-regulated IL-6 production by ATL cells, as the frequently observed elevation of C-reactive protein, accelerated erythrocyte sedimentation rate, thrombocytosis, and hypercalcemia (15–17). We showed that IL-4 inhibits IL-6 synthesis by ATL cells at the mRNA and protein levels in a dose-dependent manner, but at the same time it enhances the cell growth of ATL cells. These characteristics are a remarkable contrast with CMMoL cells and myeloma cells. In these cells, IL-4 also inhibits IL-6 production but does inhibit the cell growth of CMMoL cells and myeloma cells (5, 6). This evidence suggests that differences may exist in the signals of IL-4 which induce cell growth and inhibit IL-6 production. Recently we demonstrated that IL-4 does not have an effect on the production of parathyroid hormone-related protein by ATL cells; however, IL-2 enhances cell growth and parathyroid hormone-related protein production in ATL cells (20). Thus, IL-4 seems to be not an enhancing factor in cytokines production in ATL. The details of action and mechanisms of IL-4 signals in cell growth and IL-6 production in ATL are currently unclear. However, this evidence strongly suggests that IL-4 may inhibit a signal involved in IL-6 gene expression and may activate another signal involved in cell proliferation in ATL. The mechanisms of up-regulated IL-6 production and inhibitory effect of IL-4 in ATL is currently under investigation in our laboratory. In this study, these observations suggest a possibility that cell growth and IL-6 production may not be correlated in ATL, even in vivo, and some mechanisms may exist for the explanation of up-regulated production of IL-6 in ATL.
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ATL. IL-4 may give us a new clue to study the mechanism of up-regulated IL-6 production in ATL.

REFERENCES


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