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 thrombocytopenia, 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 PBMC, 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. Taka (Osaka University, Osaka, Japan) (7).

Cell Separation and Cell Culture. PBMC 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, 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 cytomtery; it was less than 2% of the obtained cells in all patients. Purity of T-cells as evaluated 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 x 10⁵) from patients were incubated in 96-well flat bottomed microtiter plates in the presence of 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.

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-Fujii 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 (Hybri-nabeads M-450; Dynal A.S., Oslo, Norway). After hybridization, the filters were washed twice with 1 ml of 10× SSC and twice with 1 ml of 0.1× SSC, and the filters were exposed to x-ray film overnight.

Cytotoxicity Studies. Cytotoxicity against leukemic cell lines was detected by using the trypan blue exclusion test.

Results

Expression of IL-6 in ATL Cells. IL-6 mRNA was detected in all ATL cell lines by Northern blot analysis. The expression level of IL-6 mRNA was higher in ATL cells than in normal cells.

IL-4 Inhibits IL-6 Production. In order to confirm the inhibitory effect of IL-4 on IL-6 production, we performed a time course experiment and Northern blot analysis. The results showed that IL-4 markedly inhibits IL-6 production by ATL cells.

Discussion

The results of this study suggest that IL-4 may play an important regulatory role in the production of IL-6 in ATL cells. Further studies are needed to clarify the mechanism of IL-4-mediated inhibition of IL-6 production.
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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.

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. Cytomfluorometric 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. The 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 IL-6.

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 show any effects on IL-6 secretion (less than 10 pg/ml). Apart from one sample (patient 2), which was markedly enhanced for IL-6 release 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.

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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<tr>
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<td>3</td>
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<td>4</td>
<td>7, 9</td>
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Fig. 2. Effects of IL-2 and IL-4 on IL-6 release from ATL cells. Cells were cultured for 72 h at 5 x 10⁶ cells/ml in RPMI 1640 medium containing 10% FCS. IL-2 and IL-4 (10 ng/ml) were added at the initiation of culture. IL-6 levels in the culture supernatants were measured by using an enzyme-linked immunosorbent assay. Points, means; bars, SE. N.S., not significant.
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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...
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 used in this study and the undetectable levels of IL-6 in the culture preparations because IL-4 also markedly inhibits the production of IL-6 in ATL cells cultured in the presence of IL-4, but further analyses are required to assess the relative contribution of changing rates of transcription initiation and/or mRNA stability to determine the influence of IL-4 on ATL cells steady-state mRNA levels.

In this study, we also found high expression of IL-4R on ATL cells without any stimulation. IL-4 mediates its action through the cell surface receptor of a single class of high affinity receptors for IL-4, as has been detected on hematopoietic cells (19). Indeed, the levels and magnitude of the effects 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 quite 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 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 immunoassay, 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, such as the frequently observed elevation of C-reactive protein, accelerated erythrocyte sedimentation rate, thrombocytosis, and hypercalcemia (15–17). We showed that IL-4 markedly 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 proteins including C-reactive protein (15). Also, IL-6 promotes the maturation of megakaryocyte (16) and it induces bone resorption (17). In ATL, several characteristic clinical features are frequently observed as following: high levels of C-reactive protein, accelerated erythrocyte sedimentation rate in the absence of anemia, thrombocytosis, hypercalcemia (more than 70% cases), generalized bone resorption, and markedly increased osteoclasts in bone. These clinical observations lead us to an idea that IL-6 may play an important role in ATL. In the preliminary experiments, we confirmed that the sera from ATL patients showed quite high levels of IL-6, and that ATL cells produce high levels of IL-6 into the culture medium.

In this report, we examined the effects of T-cell growth factors on IL-6 production by ATL cells. IL-4, in contrast to IL-2, markedly inhibited the production of IL-6 by ATL cells in all 9 patients. IL-4 also markedly inhibited the transcription levels of IL-6. These effects were completely abrogated by the addition of the anti-IL-4 MoAb. Furthermore, ATL cells showed quite high levels of IL-4R expression without any stimulation, in contrast to normal mononuclear cells.

IL-4 acts on many cell types by displaying either agonistic or antagonistic effects. IL-4 exhibits different effects on cells of a single lineage at different stages of differentiation. IL-4 either induces or inhibits expression of certain cytokines by various cell types. It has been recently demonstrated that IL-4 down-regulates clonalogenic growth of CMMoL cells (5) and myeloma cells (6) by antagonizing the growth stimulatory effects of endogenous IL-6. However, in ATL, IL-4 enhanced the cell proliferation of ATL cells from 5 of 9 patients examined. In addition, IL-6 did not show any effect on proliferation of ATL cells.

One may argue about the contamination of monocytes in ATL cell preparations because IL-4 also markedly inhibits the production of IL-1, tumor necrosis factor, and IL-6 by monocytes (18). Whereas monocytes cannot produce these cytokines without any stimulation, ATL cells and HTLV-I-infected cell lines spontaneously produce quite high levels of IL-6 (7, 10). Considering the high purity of ATL cells used in this study and the undetectable levels of IL-6 in the culture supernatants sampled from normal donors, the large amount of IL-6 detected in the culture supernatants sampled from ATL patients seemed to be produced by ATL cells themselves. On the other hand, IL-1α is known to be undetectable in the culture medium of normal human monocytes. Our preliminary studies indicated that IL-4 also inhibits the production of IL-1α in the ATL culture medium collected in this study. This information strongly suggests that IL-4 may inhibit IL-6 production by ATL cells.

The mechanism of action of IL-4 that interferes with IL-6 expression is not yet clear. Our studies indicate that IL-6 steady-state mRNA levels were reduced in ATL cells cultured in the presence of IL-4, but further analyses are required to assess the relative contribution of changing rates of transcription initiation and/or mRNA stability to determine the influence of IL-4 on ATL cells steady-state mRNA levels.

Fluorescence Intensity

Fig. 7. Assay of IL-4R on cell surfaces by flow cytometry. Cells were analyzed by cytofluorometry with MoAb M57 against human IL-4R. Fluorescein isothiocyanate conjugated goat anti-mouse IgG Fab' was used as a secondary antibody to stain the cells. Histograms of anti-IL-4R-stained cells (—) are superimposed over histograms of control cells (-----). A, unstimulated PBMC; B, PHA-activated PBMC; C, ATL cells from patient 1; D, ATL cells from patient 3.
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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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