Leukemic Cells From a Chronic T-Lymphocytic Leukemia Patient Proliferated in Response to Both Interleukin-2 and Interleukin-4 Without Prior Stimulation and Produced Interleukin-2 mRNA With Stimulation

By Hiroshi Umamoto, Takashi Uchiyama, Rie Onishi, Toshiyuki Hori, Haruto Uchino, and Naofumi Nesumi

Recently, interleukin-4 (IL-4) has been clarified as having T-cell growth factor activity; therefore, it becomes of interest whether IL-4, as well as interleukin-2 (IL-2), affects the proliferation of leukemic cells derived from mature T cells. In the present study, we describe a case of chronic T-lymphocytic leukemia (T-CLL) with monoclonal proliferation of human T-lymphotropic retrovirus (HTLV)-I or HTLV-II negative CD3(+)/4(+)8(-) T cell expressing IL-2 receptors without stimulation. Radiolabeled IL-2 binding assay revealed 750 high-affinity and 6,750 low-affinity binding sites per cell. In accordance with the expression of high-affinity IL-2 receptors, the leukemic cells proliferated in response to exogenous IL-2 without prior stimulation. In addition, exogenous IL-4 also induced their proliferation. Moreover, IL-2 and IL-4 exerted a synergistic effect on the leukemic cell proliferation. Although the expression of IL-2 or IL-4 mRNA was not detected in fresh leukemic cells, the expression of IL-2 mRNA, but not IL-4 mRNA, was induced by phytohemagglutinin stimulation, and the leukemic cells proliferated. These findings suggest that not only IL-2, but also IL-4 are involved in the proliferation of leukemic cells of T-CLL.

SINCE THE REPORTS that oncogene products showed homology with growth factors or their receptors, the possibility that growth factors and/or their receptors are involved in oncogenesis has been studied extensively. We previously proposed that interleukin-2 (IL-2) and its receptor system are involved in the leukemogenesis of adult T-cell leukemia (ATL) and some cases of chronic T-lymphocytic leukemia (T-CLL). Normal mature T cells activated by appropriate antigens or lectins produce and secrete IL-2, which interacts with its receptors expressed on the activated T cells. Recent studies using cross-linking reagents have demonstrated another IL-2 binding protein (p75) in addition to Tac peptide, and IL-2 binding studies have revealed IL-2 receptors with at least three different affinities to IL-2. IL-2-driven T-cell proliferation has been considered to be mediated mainly by high-affinity IL-2 receptors expressed on the cell surface. Thus, leukemic cells of mature T-cell origin have a potential to proliferate by an autocrine mechanism; IL-2 is possibly an autocrine growth factor involved in the leukemogenesis of T-cell malignancies.

Recently, mouse and human interleukin-4 (IL-4; B-cell stimulatory factor 1 [BSF-1]) cDNAs have been cloned and the studies using a recombinant product have shown that IL-4 exerts multiple biologic effects on several kinds of hematopoietic cells, including T-cell growth factor activity. Since IL-4 is also produced by activated T cells and enhances their proliferation, the proliferation of normal T cells may be mediated by IL-4 through an autocrine mechanism. IL-4 is another putative autocrine growth factor involved in the leukemogenesis of T-cell malignancies.

On the other hand, whether the clonal expansion of T cells is mediated by IL-2, IL-4, or both is unknown. Since most of the human T-cell clones reactive with specific antigens were selected and cultured using IL-2 and since normal peripheral blood T cells are a mixed cell population, the studies of leukemic cells derived from monoclonal proliferation of a T-cell clone might give us clues to answer this question.

In this context, it is of interest to study the effect of these growth factors on the proliferation of leukemic cells of mature T-cell origin. In addition, it is necessary to characterize IL-2 receptors expressed on leukemic cells and the production of these growth factors.

MATERIALS AND METHODS

Patient. A 53-year-old man was hospitalized on November 1, 1982, because of common cold-like symptoms and general lymph node swelling. Physical examination revealed neither skin eruption nor hepatosplenomegaly. The serum antibodies to ATL-associated antigen (ATLA) were negative. A blood count on admission showed no anemia and an increased WBC count (29,200/μL) with 56% abnormal lymphocytes, which had clear nuclei but no deformity of the nuclei. A bone marrow aspirate revealed that 27.6% of nucleated cells were lymphoid cells, 19.2% of which were morphologically abnormal cells. Biopsy of a neck lymph node revealed diffuse infiltration of leukemic cells. The patient was treated with prednisolone and cyclophosphamide or vincristine when peripheral WBC count progressively increased and has been alive for 60 months with increased WBC count ranging up to 2 x 10^9/μL since the initial diagnosis.

Cell separation and culture. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll-Conray density gradient centrifugation. PBMC and HPB-ALL, human T-lymphotropic retrovirus type 1 (HTLV-I)-positive MT-1, and HTLV type II (HTLV-II)-positive Mo cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, North Ryde, Australia) and 20 ng/mL tobramycin. PBMC from the patient were cultured for 24 hours with 0.1% phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit) for the study of the expression of IL-2 and IL-4 mRNA.

PBMC from normal volunteers were stimulated with 0.1% PHA-P and 10 ng/mL 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co, St Louis) for 12 hours as a positive control of IL-2 and IL-4 mRNA expression. PBMC from normal volunteers and those cultured with

From the First Division of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto, Japan, and the First Division of Internal Medicine, Kansai Denryoku Hospital, Osaka, Japan.

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Address reprint requests to Takashi Uchiyama, MD, The First Division of Internal Medicine, Faculty of Medicine, Kyoto University, 54 Shogoin-Kawaramachi, Sakyo, Kyoto 606, Japan.

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0.1% PHA-P for seven days (PHA-blasts) were used to study the proliferative response to IL-2 and IL-4.

Monoclonal antibodies. The OKT series (Ortho Diagnostic Systems Inc, Raritan, NJ), the Leu series (Becton Dickinson & Co, FACS Systems, Sunnyvale, Calif), and the T series (Coulter Immunology, Hialeah, Fla) monoclonal antibodies were purchased. Cell surface IL-2 receptors were detected by anti-Tac monoclonal antibody.

Analysis of cell surface markers. Cell surface antigens were stained with the direct or indirect immunofluorescence method and detected by flow cytofluorometry as previously described.6

Radiolabeled IL-2 binding assay. Radiolabeled IL-2 binding assay was performed as previously described.6

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of IL-2 receptors was performed as previously described.6

Chemical cross-linking of 125I-labeled IL-2. Cross-linking study of IL-2 receptors was performed as described.11

Proliferative response to exogenous IL-2 and IL-4. PBMC from the patient, normal volunteers, and normal PHA-blasts were studied for their proliferative response to IL-2 and IL-4. Recombinant IL-2 was provided by Takeda Chemical Industries (Osaka, Japan). Supernatants from Cos cell cultures transfected with the pcD vector containing the human IL-4 cDNA clone19 were provided by Drs Naoko and Ken-ichi Arai (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, Calif). Cells (1 x 10^9) were placed in a 96-well microtiter plate (Falcon Labware, Oxnard, Calif) and cultured in 200 microliter RPMI 1640 medium containing 10% FCS and 20 micrograms/mL of tobramycin in the presence of various concentrations of recombinant IL-2 and/or culture supernatants containing human IL-4 with or without 100 micrograms/mL of anti-Tac monoclonal antibody at 37°C in a humid atmosphere of 5% CO2 for 72 hours. Proliferation was measured by the incorporation of 3H-thymidine (3H-TdR) (2 Ci/mol; Amersham Japan, Tokyo) during the last six hours.

Southern and Northern blot analyses. Southern blot hybridization was performed as previously described.20 Total cellular RNA was extracted and poly(A)+ RNA was purified by oligo(dT)-cellulose column chromatography. Poly(A)+ RNA (5 micrograms) was denatured with glyoxal and dimethyl sulfoxide, electrophoresed, transferred to a nitrocellulose filter, and hybridized.26 As probes, 2.6 kilobase pairs (kbp) HindIII-EcoRI fragment of the human T-cell receptor beta-chain gene containing a constant region and 456 base pairs (bpa) SmaI-SalI fragment of HTLV-I genome containing long terminal repeats (LTR) region, which were provided by Dr T. Honjo (Kyoto University, Kyoto, Japan); LTR region of HTLV-II genome, provided by Dr K. Shimotohno (National Cancer Center Research Institute, Tokyo); HindIII fragment of IL-2 cDNA, provided by Dr T. Taniguchi (Osaka University, Osaka, Japan); and pcD-hIL-4 containing human IL-4 cDNA,18 provided by Dr K. Arai (DNAX Research Institute of Molecular and Cellular Biology); 2 kbp BamH1-PvuII fragment of human pseudo-beta actin gene, provided by Dr T. Kakunaga (Osaka University) were nick-translation using alpha-32P-dCTP (3,000 Ci/mmol; Amersham Japan).

RESULTS

Characterization of the leukemic cells. Table I shows the cell surface phenotype of PBMC of the patient when this study was performed. The leukemic cells were CD2(+)(+)(+)(+)(+)(+/-) and classified as a helper-inducer subset. In addition, the leukemic cells expressed IL-2 receptors without stimulation. These phenotypic features have not changed since the beginning of the disease. Southern blot hybridization analysis showed no integration of HTLV-I or HTLV-II provirus DNA, but it showed the rearranged T-cell receptor beta-chain gene (Fig 1), which indicated monoclonal proliferation of HTLV-I or HTLV-II negative T-lymphocyte.

IL-2 receptors expressed on the leukemic cells. The leukemic cells expressing IL-2 receptors without stimulation were studied by radiolabeled IL-2 binding assay. From Scatchard plot analysis, the leukemic cells expressed 750 high-affinity (Kd = 6.25 x 10^-12 mol/L) binding sites per cell and 6,750 low-affinity (Kd = 4.4 x 10^-8 mol/L) binding sites per cell. The ratio of high- and low-affinity binding sites was comparable to that of normal PHA-activated lymphocytes (approximately 1:10). SDS-PAGE of IL-2 receptors expressed on the leukemic cells revealed no difference in their apparent molecular weights (mol wts) compared with those expressed on normal PHA-activated lymphocytes (data not shown). A cross-linking study using radiolabeled

Table 1. Cell Surface Phenotype of Peripheral Blood Mononuclear Cells of the Patient

<table>
<thead>
<tr>
<th>Positive Cells (%)</th>
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<tr>
<td>CD2 (T11) 97.5</td>
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<tr>
<td>CD3 (Leu4) 94.1</td>
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<tr>
<td>CD4 (T4) 86.4</td>
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<tr>
<td>CD5 (T18) 87.4*</td>
</tr>
<tr>
<td>CD8 (OKT8) 6.0*</td>
</tr>
<tr>
<td>CD16 (Leu11) 3.2</td>
</tr>
<tr>
<td>CD19 (Leu12) 3.9</td>
</tr>
<tr>
<td>Leu7 8.2</td>
</tr>
<tr>
<td>OK1a1 5.0*</td>
</tr>
<tr>
<td>slg 5.3</td>
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<tr>
<td>Tac 55.6*</td>
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*Indirect immunofluorescence.

Fig 1. Southern blot analyses of HTLV-I and HTLV-II provirus integration and T-cell receptor beta-chain gene rearrangement. High-mol wt DNA (5 micrograms) was digested with restriction enzyme EcoRI, electrophoresed, and transferred to nitrocellulose filters. After being baked, the filters were hybridized with probes of (A) HTLV-I, (B) HTLV-II, and (C) T-cell receptor beta-chain. Arrowheads indicate the germline bands. Origin of each DNA sample was as follows: lane 1, PBMC of the patient; lane 2, MT-1; lane 3, Mo; lane 4, human placenta.
T-CLL CELLS RESPOND TO IL-2 AND IL-4

Fig 2. Proliferative response to IL-2 and IL-4 of PBMC of the patient and its inhibition by anti-Tac monoclonal antibody. (A) PBMC were cultured with various concentrations of recombinant IL-2 in the absence (●) or presence (○) of anti-Tac monoclonal antibody (100 µg/mL) for 72 hours, and [3H]thymidine incorporation during the last six hours was determined. Each point represents the mean of triplicate cultures. (B) PBMC were cultured for 72 hours with various dilutions of supernatants from Cos cell cultures transfected with the pcCMV vector containing the human IL-4 cDNA clone in the absence (●) or presence (○) of anti-Tac monoclonal antibody (100 µg/mL), or various dilutions of control supernatants from mock-transfected Cos cell cultures (▲). [3H]Thymidine incorporation during the last six hours was determined. Each point represents the mean of triplicate cultures. The data with control supernatants were plotted at the concentrations of IL-4 with the same dilutions.

IL-2 showed the expression of the p75 IL-2 binding peptide, as well as the p55 Tac peptide on the leukemic cells without stimulation (data not shown), which is consistent with the expression of high-affinity IL-2 receptors on the leukemic cells.

Proliferative response of PBMC of the patient to exogenous IL-2 and IL-4. As shown in Fig 2A, the leukemic cells proliferated significantly in the presence of IL-2. Their proliferation was induced at the concentration of 0.01 nmol/L, and the maximal response was seen at 0.02 nmol/L. Anti-Tac monoclonal antibody partially inhibited their proliferation at low concentrations of IL-2. Fresh PBMC from normal individuals also proliferated in response to IL-2, but the maximal response (4,471 ± 1,845 cpm, n = 9) was lower than that of the leukemic cells and required higher concentrations of IL-2 (62.5 nmol/L in nine of ten samples and 12.5 nmol/L in one sample). Normal PHA-blasts incorporated much more [3H]Tdr than expected from the effect induced by each growth factor alone (Table 2). In contrast, although normal PHA-blasts proliferated in response to both IL-2 and IL-4, the maximal proliferation of PHA-blasts in the presence of both was less than the level seen in response to IL-2 alone (data not shown). The leukemic cells proliferated when cultured with 0.1% PHA-P (25,571 ± 834 cpm).

The mRNA expression of IL-2 and IL-4 by the leukemic cells. Although the fresh leukemic cells expressed IL-2 receptor mRNA (data not shown), we failed to detect mRNA expression of IL-2 or IL-4 without stimulation. After stimulation with PHA-P, the cells expressed IL-2 mRNA, but not IL-4 mRNA (Fig 3).

DISCUSSION

We have described a case of monoclonal proliferation of CD3(+)4(+)8(-) T cells that expressed functional IL-2 receptors and proliferated in response to exogenous IL-2 and IL-4 without stimulation. Since we analyzed the PBMC from the patient as a leukemic cell population, which included normal T cells, we must take into consideration the effects of these residual normal T cells. However, leukemic cells accounted for more than 80% of the PBMC when the cytocentrifuge specimens were morphologically examined, and more than 50% of the PBMC expressed IL-2 receptors. This indicates that more than 30% of leukemic cells expressed IL-2 receptors. Furthermore, although Spits et al. reported that normal peripheral lymphocytes proliferated in response to IL-4 when cultured for six days, in our experiment, normal PBMC cultured for three days in the presence of IL-4 did not proliferate in response to IL-4 (the maximal [3H]Tdr uptake was 151 ± 74 cpm, n = 10). From this data, it is unlikely that residual normal resting T cells proliferated...
in response to IL-4. On the other hand, although normal PBMC proliferated in response to IL-2, the maximal proliferative response was 4,471 ± 1,845 cpm (n = 10) at 12.5 nmol/L or 62.5 nmol/L IL-2 when cultured for three days. In contrast, the maximal 3H-TdR uptake by PBMC from the patient was 11,486 ± 1,202 cpm, which was induced by a lower dose (0.02 nmol/L) of IL-2. These results suggest that this proliferative response to IL-2 was not due to the normal residual T cells.

Previously, we reported that leukemic cells from CD4(+) T-CLL responded to IL-2 and suggested that the IL-2/IL-2 receptor system is involved in the pathogenesis of this leukemia.7 In the present study, we studied the IL-2 receptors expressed on the leukemic cells and found that they expressed high- and low-affinity IL-2 receptors and the p75 non-Tac peptide as well as the p55 Tac peptide. These results coincided with the finding that leukemic cells proliferated in response to IL-2. Furthermore, we studied the expression of IL-2 mRNA. Although we did not detect IL-2 mRNA expression in the fresh leukemic cells, IL-2 mRNA expression was induced by stimulation with PHA, suggesting that the leukemic cells can proliferate through an autocrine or paracrine mechanism when stimulated by the appropriate antigens or stimulants. In addition, the leukemic cells proliferated in response to IL-4, but IL-4 mRNA expression was not detected in fresh or PHA-stimulated leukemic cells. However, the expression of IL-4 mRNA, if it had occurred, may have been too weak to be detected in our study. Therefore, we cannot rule out the possibility that IL-4 is involved in the leukemic cell proliferation through an autocrine or paracrine mechanism. Since IL-2 and IL-4 exerted synergistic effects on the proliferation of the leukemic cells even at low concentrations, one of these growth factors might be involved in the leukemic cell proliferation through paracrine mechanism when the other growth factor is produced by normal cells with appropriate stimulation.

Recently, Mosmann et al22 described two types of murine helper T-cell (Th) clones, one type (Th1) produced IL-2, IL-3, interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF); the other type (Th2) produced IL-4, as well as IL-3 and GM-CSF. Thus, IL-2 and IL-4 are not produced by the same type of Th cell clone. Our finding that the leukemic cells of the present case expressed IL-2 mRNA, but not IL-4 mRNA, is consistent with their finding, although it is not determined whether their subtyping of murine Th cell clones is applicable to human peripheral helper T cells. On the other hand, Spits et al23 reported that most human T-cell clones selected by IL-2 responded to IL-4, as well as IL-2. In contrast, our study on the effects of IL-2 and IL-4 on the proliferation of leukemic cells from ATL (eight cases, Uchiyama et al., manuscript submitted for publication), T-CLL (one case), and LGL leukemia (five cases) patients disclosed no other cases in which leukemic cells obviously responded to both IL-2 and IL-4. The following explanations are possible for these inconsistent results: leukemic cells might have a tendency to lose the responsiveness to these growth factors, T-cell clones, which respond only to one of them, may have a greater chance to transform than other clones, and alternatively, a culture using IL-2 may preferentially select T-cell clones that respond to both IL-2 and IL-4. In any case, studies on leukemic cells expressing functional receptors for IL-2 and IL-4, and analysis of the interaction of these two lymphokine pathways at the single cell level or population level, might clarify the significance of these two T-cell growth factors.

Finally, further studies of more T-CLL cases are necessary to clarify the role of these growth factors in the leukemogenesis of T-CLL, since the number of T-CLL cases we have studied is limited and the case shown in the present study might be rare.

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Leukemic cells from a chronic T-lymphocytic leukemia patient proliferated in response to both interleukin-2 and interleukin-4 without prior stimulation and produced interleukin-2 mRNA with stimulation

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