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 Umadome, 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(+)+CD8(−) 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.

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 nucleoli 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 had been alive for 60 months with increased WBC count ranging up to 2 × 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 I (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 μg/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

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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.19

**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 clone18 were provided by Drs Naoko and Ken-ichi Arai (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, Calif). Cells (1 × 10⁶) were plated in a 96-well microtiter plate (Falcon Labware, Oxnard, Calif) and cultured in 200 μL of RPMI 1640 medium containing 10% FCS and 20 μg/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 μg/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/mmol; Amersham Japan, Tokyo) during the last six hours.

**Southern and Northern blot analyses.** Southern blot hybridization was performed as previously described.30 Total cellular RNA was extracted30 and poly(A)+ RNA was purified by oligo(dT)-cellulose column chromatography. Poly(A)+ RNA (5 μg) was denatured with glyoxal and dimethyl sulfoxide, electrophoresed, transferred to a nitrocellulose filter, and hybridized.30 As probes, 2.6 kilobase pairs (kbp) HindIII-EcoRI fragment of the human T-cell receptor β-chain gene containing a constant region and 456 base pairs (bp) SmaI-SacI 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); Hinfl fragment of IL-2 cDNA, provided by Dr T. Taniguchi (Osaka University, Osaka, Japan); and pcD-hIL-4 containing human IL-4 cDNA,19 provided by Dr K. Arai (DNAX Research Institute of Molecular and Cellular Biology); 2 kbp BamHI-PvuII fragment of human pseudo-β actin gene, provided by Dr T. Kakunaga (Osaka University) were nick-translated using α-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(+)3(+)4(+)8(−) 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 β-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 × 10⁻¹² mol/L) binding sites per cell and 6,750 low-affinity (Kd = 4.4 × 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>
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<tr>
<th>Table 1. Cell Surface Phenotype of Peripheral Blood Mononuclear Cells of the Patient</th>
<th>Positive Cells (%)</th>
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<tbody>
<tr>
<td>CD2 (T11)</td>
<td>97.5</td>
</tr>
<tr>
<td>CD3 (Leu4)</td>
<td>94.1</td>
</tr>
<tr>
<td>CD4 (T4)</td>
<td>86.4</td>
</tr>
<tr>
<td>CD5 (T1B)</td>
<td>87.4*</td>
</tr>
<tr>
<td>CD8 (OKT8)</td>
<td>6.0*</td>
</tr>
<tr>
<td>CD16 (Leu11)</td>
<td>3.2</td>
</tr>
<tr>
<td>CD19 (Leu12)</td>
<td>3.9</td>
</tr>
<tr>
<td>Leu7</td>
<td>8.2</td>
</tr>
<tr>
<td>OKIa1</td>
<td>5.0*</td>
</tr>
<tr>
<td>slg</td>
<td>5.3</td>
</tr>
<tr>
<td>Tac</td>
<td>55.6*</td>
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*Indirect immunofluorescence.
T-CLL CELLS RESPOND TO IL-2 AND IL-4

![Graph](Image)

**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 pcDNA 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 = 10) 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 in response to IL-2 than the leukemic cells. The maximal response was 39,759 ± 11,782 cpm (n = 9) and required higher doses of IL-2 (2.5 nmol/L in four samples, 12.5 nmol/L in four samples, and 62.5 nmol/L in one sample). On the other hand, the leukemic cells also proliferated in response to Cos cell culture supernatants containing human IL-4, but not to control culture supernatants (Fig 2B). Their proliferation was not inhibited by the anti-Tac monoclonal antibody. [3H]Tdr uptake of normal fresh PBMC was 66 ± 27 cpm (n = 10) when cultured without IL-4, and the maximal [3H]Tdr uptake in the presence of IL-4 was 151 ± 74 cpm (n = 10) at the concentration of 74 U/mL. PHA-blasts proliferated in response to IL-4, and their maximal response (13,307 ± 5,447 cpm, n = 9) was one-half to one-fourth of the maximal response to IL-2. In contrast, the maximal response of the leukemic cells to IL-4 (10,784 ± 519 cpm) was comparable to their maximal response to IL-2 (11,486 ± 1,202 cpm). When both IL-2 and IL-4 were present in the culture, they exerted a synergistic effect on the DNA synthesis of the leukemic cells. Furthermore, even in the presence of low doses of IL-2 and IL-4, the leukemic cells 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 cyt centrifuged 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.

**Table 2.** Synergistic Effect of IL-2 and IL-4 on the Proliferation of Leukemic Cells

<table>
<thead>
<tr>
<th>[3H]Thymidine Incorporation (cpm)</th>
<th>0 mol/L</th>
<th>0 U/mL</th>
<th>IL-4* Added 4.8 U/mL</th>
<th>74 U/mL</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2 added</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.01 nmol/L</td>
<td>7,730 ± 91</td>
<td>18,137 ± 997</td>
<td>21,056 ± 1,105</td>
<td>ND</td>
</tr>
<tr>
<td>0.5 nmol/L</td>
<td>8,664 ± 562</td>
<td>18,137 ± 997</td>
<td>21,056 ± 1,105</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Culture supernatants of Cos-7 cells transfected with a cDNA clone encoding human IL-4 were used as the source of IL-4.

†All cultures were carried out in triplicate and mean ± SD are presented.
Fig 3. Northern blot analyses of IL-2 mRNA and IL-4 mRNA expression in fresh and PHA-stimulated PBMC of the patient.

Poly(A) + RNA was purified from the following sources: lane 1, fresh PBMC of the patient; lane 2, PBMC of the patient stimulated with 0.1% PHA-P for 24 hours; lane 3, HPB-ALL; lane 4, normal PBMC stimulated with 0.1% PHA-P and 10 ng/mL TPA for 12 hours. Poly(A) + RNA (5 μg) was denatured, fractionated on 1% agarose gel, and transferred to a nitrocellulose filter. After being baked, the filter was hybridized with probes of the (A) human IL-2, (B) IL-4, and (C) pseudo-β actin genes. (C) The expression of the β-actin gene as a control for the amount of mRNA loaded in each lane is shown.

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 11,484 ± 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-4 receptor system is involved in the pathogenesis of this leukemia. 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 an autocrine mechanism when the other growth factor is produced by normal cells with appropriate stimulation.

Recently, Mosmann et al described two types of murine helper T-cell (Th0) clones, one type (Th01) produced IL-2, IL-3, interferon-γ (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF); the other type (Th02) 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 Th0 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 Th0 cell clones is applicable to human peripheral helper T cells. On the other hand, Spits et al 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, Th-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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T-CLL cells respond to IL-2 and IL-4

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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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