Leu 11⁺ Tγ Cell Chronic Lymphocytic Leukemia With Partially Activated Natural Killer Function and Its Further Activation by Recombinant IL2 In Vitro

By Shinichi Tagawa, Yukihiro Tokumine, Etsuko Ueda, Koji Waki, Yoshio Kanayama, Nobuhiro Taniguchi, Toshihiro Nakanishi, Ryoichi Inoue, and Teruo Kitani

A patient with Tγ cell chronic lymphocytic leukemia with the Leu 11⁺ phenotype and novel function of activated natural killer cells is reported. The peripheral blood mononuclear cells of this patient showed large granular lymphocytes by May-Giemsa staining and lamellipodia by scanning electron microscopy. Tests on reactivity with monoclonal antibodies showed that most cells were Leu 11⁺, OKT3⁺/Leu 1⁺, OKT4⁺, OKT8⁺, Leu 7⁺, OKM1⁺, and Tac⁺. Freshly collected cells lysed only K562, which is highly sensitive to natural killer cells, but also Raji cells and Daudi cells, which are not. Leu 11⁺ cells were triggered by recombinant interleukin 2 (rIL2) to proliferate, produce γ-interferon (γIFN), and show enhanced HLA-DR antigen expression, and 30% of the Leu 11⁺ cells became positive for IL2 receptor antigen (Tac). The spectrum of cytolytic activity of these cells against target cells was extended by rIL2; after treatment with rIL2, the cells also lysed HeLa cells and even fresh cancer cells. This stimulation also increased the activities of acid phosphatase and tartrate-resistant acid phosphatase of the cells and resulted in the appearance of nonspecific esterase activity. The expanded cell population may represent a neoplasm, but these findings provide information on a novel differentiation stage of activated NK cells.

CHRONIC lymphocytic leukemia (CLL) involving Tγ cells with the morphology of large granular lymphocytes (LGL) has been described by many investigators and reviewed by Reynolds and Foon. Patients with this disease have persistently elevated numbers of Tγ cells, but their clinical courses are less severe than in other T cell malignancies, and often anemia or neutropenia is the only symptom.

In healthy persons, the Tγ cell population consists almost entirely of natural killer (NK) cells, which have the morphologic characteristics of LGL, produce lymphokines, and regulate hematopoiesis. Thus, study of patients with Tγ-CLL with NK activity should provide information on the origin of NK cells and their spectrum of lysis of specific targets, lymphokine production, and stage of differentiation.

Here we report detailed studies on cells of a patient with T cell leukemia of the Leu 11⁺ phenotype showing novel activated NK functions. Leukemic cells expressed the Leu 11 marker, an antigen associated with the Fc-IgG receptor of human NK cell. The cells of this patient mediated both strong antibody-dependent cell-mediated cytotoxicity (ADCC) and NK activity with an unusual specificity spectrum; freshly collected cells lysed not only K562, which is highly sensitive to NK cells, but also Raji and Daudi cells, which are relatively NK-insensitive B cell lines. Thus, the cells had the cytolytic specificity spectrum of the activated NK cells, but this spectrum was different from that of lymphokine-activated killer (LAK) cells because freshly collected cells did not lyse cultivated human cancer cells, HeLa cells, or fresh cancer cells. Thus, we called this cytolytic activity partially activated NK function. The activated NK features of leukemic cells of this patient suggest a novel differentiation stage of human NK cells.

MATERIALS AND METHODS

Case report. A 65-year-old man first noticed fatigue and malaise following exercise in March 1983. In June, 1983, his hemoglobin was 5.0 g/dL, his WBC count was 1 1,400 cells/μL, his platelet count was 19 × 10⁴/μL, and his lymphocyte count was 10,260 cells/μL. Physical examination in the same month revealed no abnormalities; liver and spleen were normal size, there was neither lymphadenopathy nor skin lesions. The delayed cutaneous hypersensitivity reaction to a purified protein derivative of tuberculin was positive. Serum lactic dehydrogenases was within the normal range. The serum Ig levels were 132 mg of IgM/dL, 1240 mg of IgG/dL, and 289 mg of IgA/dL. Bone marrow aspiration was done; leukemic cells were found to constitute 60% of the nuclear elements. Informed consent was obtained prior to this study. A diagnosis of CLL was made.

In July 1983, the patient was transferred to the Hospital of the Research Institute for Microbial Diseases at Osaka University. His serum was negative for antibodies to adult T cell leukemia-associated antigens (ATLA). The hematologic profile on July 22, 1983 is shown in Table I. Leukopheresis of 1 x 10⁷ cells was done once every 2 weeks with transfusion of 400 mL of blood; these procedures have been continued and, at present (February 1986), the condition of the patient is under control, with a WBC count of 13,300 cells/μL, a platelet count of 9.4 x 10⁴/μL, and a lymphocyte count of 12,635 cells/μL. Chromosomal study showed that the patient’s karyotype was normal.

Detection of ATLA antibodies. A test for detection of ATLA antibodies in the patient’s serum was done by enzyme immunoassay with nitrocellulose membrane strips blotted with partially purified ATLA-related proteins from an ATL/HTLV producing cell line, MT-2² (kindly provided by Dr I. Miyoshi, Koto Medical School, Japan).

Cells and cell fractionation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Paque density-gradient centrifugation. The cells were further separated into a fraction without cells positive for Leu 1 (Leu 1-depleted fraction) and a fraction without leukemic cells (Leu 11-depleted fraction) by complement-mediated lysis with the respective monoclonal antibodies. The extent of depletion of the respective cells was checked by indirect immunofluorescence. Leu 11⁺ cells were separated in a FACSV cell sorter (Becton Dickinson, Sunnyvale, Calif) for scanning electron microscopic examination.

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An assay was performed essentially as described. In brief, mixtures of appropriate concentration of rIL2, and pulsed with [3H]thymidine 6 hours before harvest. The stimulation index (SI) is the ratio of counts per minute of target cells sensitized with rabbit antibody (Ig G fraction) were used as targets for double staining of cells with Leu 11 and anti-Tac.

Surface markers. Test of formations of E rosettes and EAC rosettes and for the presence of Fcγ receptor (EAγ) and surface immunoglobulin were performed as described previously. Examination of the staining of the cells with the monoclonal antibodies OKT3, OKT4, OKT6, OKT8, OKM1, OKIa1, OKT11, Leu 1, Leu 7, Leu 10, Leu 11b, and anti-Tac (kindly provided by Dr T. Uchiyama, Kyoto, Japan) was done by the indirect immunofluorescence test. FITC-conjugated monoclonal antibody, Leu 11a, was used for double staining with Leu 11 and anti-Tac.

Tests of cytolytic activity. NK activity was measured at effector–target cell (E–T) ratios of 30:1 and 15:1, in a 4-hour 51Cr release assay. For examination of ADCC, 51Cr-labeled sheep erythrocytes sensitized with rabbit antibody (Ig G fraction) were used as targets in a 4-hour test, at E–T ratios of 1:1 and 2:1. For LAK assays, cells were cultured at 400 U/mL of rIL2 for 3 days. The target cells used were K562, Raji cells, Daudi cells, HeLa cells, and target cells in conjugates minus the percentage of spontaneous cell death was determined by counting the number of dead target cells in 100 conjugates. The background count of spontaneous cell death was determined by counting the number of dead target cells in control dishes. The percentage of lytic conjugate-forming cells (%LCFCs) was calculated as: the percentage of dead targets in conjugates minus the percentage of spontaneously dead targets x the percentage of dead targets in conjugates per 100. The percentage of active killer cells (%AKC) was calculated as %LCFCs x %LCFCs/100.

RESULTS

Morphology and cytochemistry. The cells were large (12.53 μm across), with a high ratio of cytoplasm to nucleus, and azurophilic granules in the cytoplasm. On cytochemical analysis, acid phosphatase in the cells was seen as scattered positive granules throughout the cytoplasm. The cells were weakly positive for tartrate-resistant acid phosphatase but weakly positive for tartrate-resistant acid phosphatase.

Table 2. Cell Surface Antigen Phenotype of Peripheral Blood Mononuclear Cells from Patient

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Patient (%)</th>
<th>Normal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>97</td>
<td>74.5 ± 5.6</td>
</tr>
<tr>
<td>EAγ</td>
<td>81</td>
<td>27.3 ± 5.6</td>
</tr>
<tr>
<td>OKT3</td>
<td>11</td>
<td>67.7 ± 8.7</td>
</tr>
<tr>
<td>OKT4</td>
<td>8</td>
<td>44.7 ± 7.0</td>
</tr>
<tr>
<td>OKT6</td>
<td>10</td>
<td>24.1 ± 6.5</td>
</tr>
<tr>
<td>OKT8</td>
<td>23</td>
<td>8.0 ± 4.1</td>
</tr>
<tr>
<td>OKIa1</td>
<td>82</td>
<td>73.5 ± 5.3</td>
</tr>
<tr>
<td>OKT11</td>
<td>11</td>
<td>9.4 ± 7.2</td>
</tr>
<tr>
<td>OKM1</td>
<td>12</td>
<td>71.0 ± 7.0</td>
</tr>
<tr>
<td>Leu 1</td>
<td>0</td>
<td>15.8 ± 3.9</td>
</tr>
<tr>
<td>Leu 7</td>
<td>81</td>
<td>15.4 ± 3.4</td>
</tr>
<tr>
<td>Leu 11b</td>
<td>0</td>
<td>5.6 ± 1.8</td>
</tr>
</tbody>
</table>

*Values are mean percentages of reactive cells ± SD (n = 10).
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of cells collected at four subsequent times were also examined. Results indicated that the activated NK function and the appearance of LAK activity against HeLa cells on stimulation with rIL2 were reproducible (Table 8). In addition, freshly collected cells mediated ADCC against antibody-coated sheep erythrocytes. No phagocytosis of these erythrocytes by lymphocytes was observed, however.

Studies on cytotoxic activity at the single cell level. As shown in Table 9, >50% of the peripheral lymphocytes of the patient bound not only to the NK-sensitive target cells K562, but also to the NK-resistant target, Raji and Daudi cells. After incubation for 3 hours, most CFCs had lysed the target cells. In contrast, 10% to 14% of the peripheral lymphocytes from healthy donors bound to these targets, and 29.2%, 13.1%, and 8.4% of the CFCs lysed K562, Raji, and Daudi cells, respectively.

The %AKCs among cells of the patient varied little with different targets, being 39.8, 34.3, and 36.6 with K562, Raji, and Daudi cells, respectively.

To detect the proportion of Leu 1 1 and 0KT3 phenotype from isolated cells, we found that 2 of 3 patients were Leu 1 1  (B73.1). Similarly, that cells from at least 2 of 11 patients were Leu 1 1  (B73.1). Thus, it is likely that many cases of Ty-LPD are Leu 1 1 . Cells from most cases with the Leu 1 1  phenotype were positive for OKT3, an antigen associated with the T cell antigen receptor. In contrast, cells from the present patient were Leu 1 1 , but OKT3 -. An interesting finding was that the freshly isolated cells with the Leu 1 1  and OKT3 - phenotype from this patient showed activated NK function. No leukemia cells with this function have been reported previously, and peripheral unstimulated NK cells from normal persons do not mediate this activated NK function.

In determining whether the cytotoxic spectrum characteristics of activated NK cells of this patient was attributable to the leukemic cells, which constituted most of the population of peripheral blood lymphocytes, or to the small proportion of residual normal lymphocytes, it was insufficient to consider only the overall cytotoxicity parameters. However, with an assay for detection of single cytotoxic NK cells, originally developed by Grimm and Bonavida, the overall cytotoxicity could be separated into its component parts of effector cell frequency and target cell lysis per effector. The assay showed

Table 6. Characterization of IFN Produced by Cells From Patient After Stimulation with rIL2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Residual IFN Activity (Percentage of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Anti-α-IFN</td>
<td>100</td>
</tr>
<tr>
<td>Anti-β-IFN</td>
<td>100</td>
</tr>
<tr>
<td>Anti-γ-IFN</td>
<td>0</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Table 7. Specificity Spectrum of Cytotoxic Activity of Patient’s Cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Treatment of Effector Cells</th>
<th>Target: K562</th>
<th>Treatment: Raji</th>
<th>Target: Raji</th>
<th>Target: Daudi Cell</th>
<th>Treatment: Daudi</th>
<th>Target: HeLa Cell</th>
<th>Treatment: HeLa</th>
<th>Target: Ovarian Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>None</td>
<td>70.4</td>
<td>67.4</td>
<td>63.0</td>
<td>55.2</td>
<td>89.7</td>
<td>87.3</td>
<td>8.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Patient</td>
<td>Cultured for 3 days with rIL2 (400 U/mL)</td>
<td>83.0</td>
<td>85.4</td>
<td>98.5</td>
<td>99.0</td>
<td>93.5</td>
<td>94.7</td>
<td>66.8</td>
<td>62.8</td>
</tr>
<tr>
<td>Patient</td>
<td>Cultured for 3 days with rIL2 (400 U/mL), then depleted of the Leu1- fraction</td>
<td>80.9</td>
<td>80.9</td>
<td>99.4</td>
<td>99.3</td>
<td>95.2</td>
<td>95.4</td>
<td>65.6</td>
<td>64.7</td>
</tr>
<tr>
<td>Normal donor</td>
<td>None</td>
<td>64.9</td>
<td>43.5</td>
<td>7.2</td>
<td>2.5</td>
<td>13.1</td>
<td>11.3</td>
<td>0.1</td>
<td>-1.2</td>
</tr>
<tr>
<td>Normal donor</td>
<td>Cultured for 3 days with rIL2 (400 U/mL)</td>
<td>58.3</td>
<td>51.8</td>
<td>52.6</td>
<td>24.3</td>
<td>91.8</td>
<td>63.5</td>
<td>98.0</td>
<td>68.6</td>
</tr>
</tbody>
</table>

Abbreviations as in Tables 4 and 5.

Results are means for triplicate determinations: SDs did not exceed 5% in each experiment. PBMCs from the patient depleted of macrophages and adherent cells were used as effector cells.

Table 8. Reproducibility of Data on Cytotoxicity of Cells From Patient

<table>
<thead>
<tr>
<th>Date of Sampling*</th>
<th>Percentage of Cytotoxicity of Fresh Cells</th>
<th>LAK (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/11/84</td>
<td>72.4</td>
<td>69.1</td>
</tr>
<tr>
<td>4/26/85</td>
<td>73.2</td>
<td>58.8</td>
</tr>
<tr>
<td>8/23/85</td>
<td>76.3</td>
<td>68.0</td>
</tr>
<tr>
<td>10/30/85</td>
<td>79.8</td>
<td>48.7</td>
</tr>
</tbody>
</table>

LAK, lymphokine-activated killer cells.

Cells were collected at four different times. The E:T ratio was 30:1 in all experiments. Results are means for triplicate measurements. SDs did not exceed 5%.

* Frozen cells were used in these experiments.
† For LAK assays, cells were cultured at 2 x 10⁸/mL with 400 U/mL of rIL2 for 3 days.

DISCUSSION

Phenotypic studies of cases of Ty lymphoproliferative diseases (Ty-LPD) by Rambaldi and colleagues showed that cells from at least 2 of 11 patients were Leu 1 1 (B73.1). Similarly, we found that 2 of 3 patients were Leu 1 1 . Thus, it is likely that many cases of Ty-LPD are Leu 1 1 . Cells from most cases with the Leu 1 1  phenotype were positive for OKT3, an antigen associated with the T cell antigen receptor. In contrast, cells from the present patient were Leu 1 1 , but OKT3 -. An interesting finding was that the freshly isolated cells with the Leu 1 1  and OKT3 - phenotype from this patient showed activated NK function. No leukemia cells with this function have been reported previously, and peripheral unstimulated NK cells from normal persons do not mediate this activated NK function.

In determining whether the cytotoxic spectrum characteristics of activated NK cells of this patient was attributable to the leukemic cells, which constituted most of the population of peripheral blood lymphocytes, or to the small proportion of residual normal lymphocytes, it was insufficient to consider only the overall cytotoxicity parameters. However, with an assay for detection of single cytotoxic NK cells, originally developed by Grimm and Bonavida, the overall cytotoxicity could be separated into its component parts of effector cell frequency and target cell lysis per effector. The assay showed
that AKCs in the cells of the patient exceeded 30%, whereas AKCs in cells from normal donors ranged from 1.1% to 2.2% with different targets. These findings support the hypothesis that the large population of Leu 11 cells among the leukemic cells of the patient, not the minor population of residual normal cells, mediated the activated NK function.

The lytic specificity spectrum of the cells of the patient is different from that of normal NK cells and the normal Leu 11 subset. The culture of normal Leu 11 cells with K562 or IL2 in vitro can give rise to NK cells with this spectrum of lytic activity. Phillips and Lanier designated NK cells with activated NK function as NK cells in a differentiation stage of NK cells. The Leu 11 leukemic cells of this patient may be due to clonal expansion of this novel differentiation stage of NK cells that has never been recognized in vivo before.

Four changes in parameters of the cells in response to IL2 stimulation were observed. One was expansion of the cytotoxic spectrum of the leukemic cells. The phenomenon has not been reported previously. A second was increase in the activities of enzymes in azurophilic granules. A third was expression of HLA-DR antigens on the surface of the cells. In macrophages, gamma IFN is reported to play a key role in the induction of HLA-DR antigens. In contrast, in the Leu 11 leukemic cells, we obtained evidence that the enhancement was due to direct effect of IL2. A fourth change was in Tac antigen expression. It is interesting that Tac antigens, which are now considered to be IL2 receptor molecules, were not expressed on freshly collected cells, but were expressed de novo on Leu 11 cells in response to IL2 in vitro.

There are at least two possible explanations for this finding. One is that the number of IL2 receptors expressed on one cell is too small to be readily detectable with anti-Tac monoclonal antibody by indirect immunofluorescence. Alternatively, cells may possess a distinct IL2 receptor that cannot react with antibody against the Tac epitope. The latter possibility is supported by reports that Tac-antigen negative cell subsets of normal peripheral lymphocytes such as LGL, Leu 11 cells, and T cells respond to IL2 and that anti-Tac antibody does not inhibit the increase of NK activity induced by IL2.

Unlike those in other reports, the cells of our patient produced gamma IFN. In contrast to the report of Hooks and colleagues, gamma IFN production by the cells was not spontaneous, but was regulated by IL2. Zoumbus and co-workers found that both alpha and gamma IFN are mediators of hematopoietic suppression in aplastic anemia. In patients with aplastic anemia, the IFN levels in the bone marrow are high, although circulating IFN is not always detected. Thus, in our patient, the gamma IFN produced by Leu 11 cells in the bone marrow may cause pancytopenia, although IFN was not detected in serum samples.

The cells of our patient had lamellipodia, which were previously reported to be characteristic of activated T cells. The leukemic cells with the phenotype of Leu 11 and Leu 7, which we reported previously, also had lamellipodia. These findings suggested that lamellipodia might be characteristic of Leu 11 cells. To investigate this possibility, we examined the morphology of normal Leu 11 cells obtained with a cell sorter. Scanning electron microscopy showed that ~10% of PBMCs had lamellipodia. Macrophages could be excluded in SEM studies, and most of the Leu 11 cells examined were found to have lamellipodia. Thus, we confirmed that the SEM feature of lamellipodia is commonly present on normal and leukemic Leu 11 cells (Inoue et al, manuscript in preparation).

Recently, DNA sequences coding for the beta chain of the T cell receptor (Tbeta) have been isolated. Waldmann and co-workers found that the patterns of Tbeta gene rearrangement can be used to identify the lineage and clonality of human lymphoid neoplasms. Among patients with Tgamma-LPD examined, T3-positive patients showed Tbeta gene rearrangement, whereas T3-negative patients did not. Moreover, Lanier and co-workers observed that the Tbeta gene was not rearranged in Leu 11 cells from normal PBMCs. Thus, the cell lineage of T3-negative cases such as that of the present patient requires further characterization.

Our patient with Tgamma-CLL had cells that showed many features of activated NK, and remained responsive to IL2. The pathogenesis of this lymphocytic disorder is unknown. This study provides more precise information than that which could be obtained from normal heterogeneous NK cells on a maturation stage of activated NK cells that has not previously been recognized in vivo.

ACKNOWLEDGMENT

We thank Dr T. Uchiyama, Kyoto University, for providing anti-Tac monoclonal antibody; Dr T. Abe, Kyoto Prefectural University of Medicine, for analyzing the chromosomes of the patient; H. Kozono, Osaka University, for providing anti-sheep erythrocyte rabbit IgG; and Dr E. Inada, Kyoritsu Hospital, for referring the patient to our hospital. We also thank Takeda Chemical Industries Ltd, for providing recombinant IL2 and Kyowa Hakko Kogyo Co., Ltd for providing recombinant gamma IFN.

Table 9. Capacity of Patient's Peripheral Lymphocytes to Bind to and Kill Targets

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>K562 Cells</th>
<th>Raji Cells</th>
<th>Daudi Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CFCs*</td>
<td>%CFCs†</td>
<td>%AKCs‡</td>
<td>%CFCs</td>
</tr>
<tr>
<td>Patient</td>
<td>52</td>
<td>76.5</td>
<td>39.8</td>
</tr>
<tr>
<td>Control</td>
<td>10.3 ± 1.7</td>
<td>20.2 ± 8.5</td>
<td>2.2 ± 1.4</td>
</tr>
</tbody>
</table>

*%CFCs, percentage of conjugate-forming cells. †%CFCs, percentage of lytic conjugate-forming cells. ‡%AKCs, percentage of active killer cells. §Results are means for triplicate determinations in one of three experiments that gave similar results. SDs were <5% of mean values. ||Mean percentages ± SD for three experiments on cells from different individuals.
LEU 11* Ty CELL WITH ACTIVATED NK FUNCTION

REFERENCES


37. Leonard LW, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC: A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature 300:267, 1982


Leu 11+ T gamma cell chronic lymphocytic leukemia with partially activated natural killer function and its further activation by recombinant IL2 in vitro

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