Soluble Interleukin-2 Receptors in the Sera of Patients With Hairy Cell Leukemia: Relationship With the Effect of Recombinant α-Interferon Therapy on Clinical Parameters and Natural Killer In Vitro Activity


In this study we provide evidence that the sera of patients with hairy cell leukemia (HCL) contain a factor that can prevent the binding of a monoclonal antibody specific for interleukin-2 receptor (IL-2R) to its target. This factor corresponds to the soluble form of IL-2R (sIL-2R), as assessed by a specific enzyme-linked immunosorbent assay test, and appears to be released by neoplastic hairy cells. The serum sIL-2R levels were very high at diagnosis and significantly reduced during recombinant α-interferon (rIFNα2) therapy. Values of sIL-2R appeared to be inversely related to the natural killer in vitro function displayed by peripheral blood mononuclear cells from the same patients. The presence of sIL-2R in the serum of patients with HCL might be involved in the impairment of cell-mediated immunity observed in these patients and could represent a valuable marker for monitoring different phases of the disease and for modulating IFN therapy. 

© 1987 by Grune & Stratton, Inc.

Hairy cell leukemia (HCL) is a chronic lymphoproliferative disorder characterized by splenomegaly and pancytopenia. The bone marrow is infiltrated by atypical cells showing a peculiar hairy morphology that can express the interleukin-2 receptor (IL-2R) on their membranes. This molecule plays a crucial role in the surface of activated T and B cells. It has been recently demonstrated in both human and experimental animals that their membranes. This molecule plays a crucial role in the proliferation of HC in vitro. There are some data suggesting the possibility that impairment of the IL-2/IL-2R mechanism might be related to immunologic abnormalities observed in other lymphoproliferative disorders.

A severe impairment of natural killer (NK) activity has been extensively demonstrated in HCL. The defect of cytotoxic function could contribute to the increase susceptibility of HCL patients to infections, which represent the primary cause of mortality in this disease. One interesting possibility in HCL is that the IL-2R might adsorb and block IL-2, thereby making it unavailable for stimulating NK cells. Such a mechanism would be greatly facilitated by the release of sIL-2R from HC and its diffusion through the plasma and internal milieu. To test this hypothesis, we looked for the presence of sIL-2R in the sera of patients with HCL by using two different techniques: a competitive binding immunocytochemical assay and a recently standardized enzyme-linked immunosorbent assay (ELISA) test. We tested eight patients with HCL at diagnosis and during their clinical and hematologic improvement after therapy with α-interferon (rIFNα2), which is highly effective in HCL. The findings have been compared with the analysis of NK cell in vitro function.

MATERIALS AND METHODS

Patients and sera. Eight patients with HCL, one female and seven males (average age, 62 years, Table I), were studied. The main clinical and laboratory data of patients at diagnosis and their variations during rIFNα2 therapy are shown in Fig 1. The diagnosis of HCL was based on clinical, morphological, cytochemical, and immunophenotypic criteria. In all patients bone marrow involvement was confirmed by the immunohistochemical demonstration of HC. All patients had severe neutropenia (<0.5 × 10⁹/L) and entered a therapeutic protocol with rIFNα2 (Schering Corp, Kenilworth, NJ). None of the patients had splenectomy nor underwent any other treatment before rIFNα2 therapy. Treatment started in November 1984 for the first three patients, whereas the other patients started the therapeutic regimen later. The protocol consisted of 2 × 10⁹ IU/m² of rIFNα2 injected subcutaneously three times a week for 12 months. Informed consent was obtained from all patients, who had been advised of procedures and attendant risks. Samples of serum were collected from all patients at diagnosis and at different times during rIFNα2 therapy and evaluated for the presence of sIL-2R in conjunction with clinical and phenotypic assessment of the disease status. Sera from 40 normal age-matched subjects were used as control.

Competitive binding immunocytochemical assay to detect sIL-2R. The exquisitely sensitive alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used to detect the specific immunostaining of anti-IL-2R+ target cells and reveal the inhibitory capability of sera. Highly enriched T cells obtained by rosetting sedimentation from the peripheral blood mononuclear cells (PBMC) of normal donors were activated in vitro for 72 hours with 2 μg/mL Phytohaemagglutinin (PHA-M) (Difco Detroit) and used as standard IL-2R+ targets. The anti–IL-2R monoclonal antibody (MoAb) was purchased from Becton Dickinson, Sunnyvale, CA (lot no. H0716).
SOLUBLE IL-2R IN HCL

Table 1. Semiquantitative Evaluation of the Inhibitory Capability of Patients' Sera on the Binding of Anti-IL-2R Antibody to Activated T Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>Dilutions of Patient's Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:1</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
<td>F</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>71</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>57</td>
<td>M</td>
<td>+</td>
</tr>
</tbody>
</table>

*One microliter anti-IL-2R antibody (1:100 dilution) was mixed with different volumes of patient's serum (1 μL, 2 μL, 5 μL, 10 μL), the final volume adjusted to 10 μL with PBS.

Patients' sera were obtained at diagnosis. + complete inhibition of anti-IL2R binding to activated T cells.

Anti-IL-2R antibody was first diluted 1:100 in phosphate-buffered saline (PBS). One microliter of this solution was then added to 10 μL PBS (obtaining the 1:1,000 final working dilution used to detect positive staining) or to 10 μL of sera at different dilutions (1:1, 1:2, 1:5, 1:10, 1:20). The mixtures containing antibody and sera were allowed to incubate for 20 minutes at room temperature before the immunocytochemical staining.

Acetone-fixed cytocentrifuged preparations of activated T cells were incubated for 30 minutes with anti-IL-2R antibody diluted 1:1,000 in PBS or sera at various dilutions. After washing, the preparations were reacted for 30 minutes with a goat antimouse Ig "bridge" antibody before the incubation with the APAAP complex (purchased from Dako, Copenhagen). Finally, alkaline phosphatase was revealed cytochemically by means of naphthol AS-BI phosphate (Sigma Chemical Co. St Louis) as the substrate and hexazotized neufuchsin (Serva, Heidelberg, FRG) as the chromogen.

The immunostained preparations were evaluated as positive or negative by direct microscope observation and semiquantitative scoring of the number of IL-2R+ cells in the preparations. Preparations were considered negative when less than 1% of the cells showed a faint positivity.

Additional IL-2R+ targets were cryostat sections of HCL bone marrow trephine biopsy material and samples of Hodgkin's disease-involved lymph nodes. Both presented large numbers of IL-2R+ cells, HC, and Reed-Sternberg cells, respectively.

Controls. Three different types of controls were performed. First, the possible inhibition of binding of MoAbs other than anti-IL-2R (Leu-4, Leu-1, and anti-HLA-DR, from Becton Dickinson) by normal and patient's sera was investigated in parallel tests. The antibodies used as controls were either of the same class as anti-IL-2R (IgGl, Leu-4) or of different classes (IgG2a, anti-HLA-DR and Leu-1).

Second, the possibility that the preincubation of target cells with the sera could prevent the binding of anti-IL-2R antibody was checked to rule out the presence in HCL sera of autoantibodies specific for IL-2R, as found in systemic lupus erythematosus. These antibodies could produce similar inhibition of anti-IL-2R binding to target cells in the same system. Finally, the sera were dialyzed overnight in buffered saline by using standard Visking tubes before testing for immunocytochemical inhibition.

ELISA test for the determination of sIL-2R. Two MoAbs recognizing different epitopes of the IL-2R molecule were used in an ELISA test (Cellfree, T Cell Science, Cambridge, MA). Briefly, sIL-2R available in the samples or standards bind to the polystyrene microtiter wells previously incubated with 100 μL anti-IL-2R MoAb (1 μg/mL). A horseradish peroxidase-conjugated anti-IL-2R MoAb directed against a second epitope on the IL-2R molecule binds to the IL-2R captured by the first antibody and completes the sandwich. After washing to remove the unbound enzyme-conjugated anti-IL-2R MoAb, a substrate solution is added to the wells. The reaction is then stopped and the absorbance determined at 490 nm. A standard curve was prepared by using different sIL-2R concentrations defined in arbitrary units per milliliter (considering 1,000 U/mL the amount of sIL-2R present in 0.1 mL of a T Cell Science reference preparation of supernatant from phytohemagglutinin-stimulated lymphocytes).

Demonstration of sIL-2R release by HC in vitro. HC-enriched populations were obtained in five patients in which, at the time of diagnosis, the frequency of HC was highest. HC-enriched cell suspensions were obtained after removal of T cells according to the method previously described in detail and never contained >10% T cells or monocytes as detected by indirect immunofluorescence with CD3 MoAb and α-naphthyl acetate esterase. The frequency of anti-Tac and tetrarotate-resistant acid phosphatase–positive cells was >77% and >86%, respectively.

The HC-enriched populations (1 × 10^7/well) were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS, Microbiological Products, Wakersville, MA), penicillin, streptomycin, and L-glutamine in 96-well U-bottomed microtiter plates in a final volume of 200 μL. They were cultured for three or seven days in a 5% CO2 atmosphere in the presence of medium alone or 12-O-tetradecanoylphorbol-13-acetate (TPA 10 ng/mL/1 × 10^5 cells; Sigma) and/or BCGF, 20% vol/vol 1 × 10^5 cells; Chemicon, El Segundo, CA). All cultures were performed in triplicate. After the incubation period, the plates were centrifuged, and culture supernatants were harvested to determine the presence of sIL-2R as described earlier. Cellular viability was always >95%.

Evaluation of NK activity. PBMC were obtained from freshly drawn heparinized peripheral blood by centrifugation on a Ficoll/Hypaque gradient. The cells were washed three times and resuspended in RPMI 1640. Adherent cells were removed by two sequential incubations in plastic Petri dishes for 45 minutes at 37°C in an atmosphere of 95% air and 5% CO2.

NK activity was assessed by lysis of 3HCr-labeled K-562 target cells as previously described. Briefly, target cells were labeled overnight at 37°C in 5% CO2 with 100 μCi Na 3HCrO4 (CEA, Saluggia, Italy; IRE Sorin, Biomedica), extensively washed three times before use, and then adjusted to a final concentration of 10^4 cells/mL in RPMI 1640 supplemented with 10% FCS. To perform the test, 100 μL labeled targets (10^3 cells) was cocultured with effector cells for four hours at 37°C in 5% CO2 at different dilutions to final ratios between effectors and target cells (E/T) of 5:1, 10:1, 20:1, 40:1, and 80:1 in triplicate round-bottomed wells (Limbro Scientific Co, Hamden, CT). Triplicate wells with target cells in medium alone and in detergent were assessed to determine spontaneous and maximum release, respectively. After this incubation, supernatants were harvested and counted in a gamma counter. The mean value of triplicate assays was used to calculate the percentage of cytotoxicity according to the following formula: percent cytotoxicity = (cpm release in test – cpm spontaneous release) × 100/(cpm maximum release – cpm spontaneous release). Spontaneous release from the target cells was always <8%. Nonadherent PBMC from 20 age-matched healthy volunteers were used as control. Data are presented at the standard 80:1 E/T ratio as previously described in detail.

NK cell activity after in vitro incubation with IL-2. The effect of IL-2 on the cytotoxic activity was determined after in vitro boosting. Effector cells (1 × 10^5/mL) were cultured in RPMI 1640
medium supplemented with 10% FCS, 4 mmol/L glutamine, penicillin (100 U/mL), and streptomycin (50 mg/mL) and that contained 20% vol/vol lectin-free IL-2 (Lymphocult-LF, Biotest, Frankfurt, FRG) for 72 hours at 37°C in a 5% CO2 atmosphere. After incubation with IL-2, the cells were washed, resuspended in fresh medium, and immediately tested for cytotoxicity as described earlier. These doses and times were previously found to give the highest increase of NK activity on normal peripheral blood lymphocytes (data not shown). In all experiments effector cells cultured in the same conditions, but without IL-2, were used as controls. To rule out the possibility of a stimulatory effect of the FCS present in the culture medium, appropriate controls with medium alone were performed. In these experiments no appreciable cytotoxic activity was detected. Results are expressed as percentages of 51Cr release (at the 80:1 E/T ratio) by cells cultured with IL-2 with respect to the cells cultured in the same experimental conditions but without IL-2.

RESULTS

When cytospin preparations of activated T cells were stained with anti–IL-2R antibody at a 1:1,000 dilution, the APAAP technique revealed membrane staining in 25% to 50% of the cells. The reactivity was not inhibited by normal sera (ten cases) added in the highest concentrations used in our system (1 μL prediluted anti–IL-2R antibody plus 10 μL serum; final concentration, 1:1,000). On the other hand, all the sera of HCL patients at diagnosis completely inhibited the anti–IL-2R staining (Table 1). The inhibition was seen at 1:1, 1:2, and 1:5 dilutions, and a positive staining appeared again only when anti–IL-2R antibody was mixed with HCL sera at the highest dilutions. Most cases (5/8) showed inhibitory capability at a 1:5 serum dilution, one case at 1:10, and two cases at 1:2 (Table 1).
confirmed by using the enzyme immunoassay (Table 2). In all cases the values were very high (mean ± SD = 11,846 U/mL; range, 13,370 to 48,090 U/mL) when compared with those observed in age-matched normal controls (mean ± SD = 232 ± 118 U/mL; range, 75 to 496 U/mL).

Controls. The sera of patients with HCL did not inhibit the immunostaining of MoAbs other than anti-IL-2R (anti-HLA-DR, Leu-4, and Leu-1) in an identical system using the same (1:1,000) antibody dilution. The binding of anti-IL-2R antibody was not prevented by preincubation of activated T cells with the sera of patients affected with HCL. Overnight dialysis of sera did not modify their inhibitory capability.

ELISA test for the determination of sIL-2R. The presence of sIL-2R in the sera of HCL patients at diagnosis was confirmed by using the enzyme immunoassay (Table 2). In all cases the values were very high (mean ± SD = 25,858 ± 11,846 U/mL; range, 13,370 to 48,090 U/mL) when compared with those observed in age-matched normal controls (mean ± SD = 232 ± 118 U/mL; range, 75 to 496 U/mL).

Detectable levels of sIL-2R were found to be released in the supernatants of cultured HC after stimulation up to seven days. sIL-2R were undetectable in all cases at day 0, ranged between 73 and 1,300 U/mL at day 3, and between 132 and 2,600 U/mL at day 7. Spontaneous sIL-2R release was documented without stimulation in two cases.

sIL-2R serum levels after rIFNa2 therapy. A consistent reduction of sIL-2R levels was observed after rIFNa2 therapy. This was demonstrated by the decrease and eventually the disappearance of the inhibitory effect of sera in the cytotoxic competitive assay (Fig 1) as well as by the progressive reduction of values obtained with the ELISA test (Table 2). sIL-2R levels started to rise again after discontinuation of the rIFNa2 therapy as shown in the patients with the longest follow-up (Fig 1 and Table 2). In only one patient who received rIFNa2 for 1 year (case no. 2) the sIL-2R levels only partially reduced during the course of therapy. This same patient experienced a rather modest clinical response (only a moderate increase of granulocytes and a small reduction of HC levels in the bone marrow). In the other patients the progressive loss of sIL-2R in the serum correlated with clinical improvement, as judged by the decrease of neoplastic cells in peripheral blood and marrow, the reduction of the spleen size in patients with enlarged spleens, and the increase in granulocytes and hemoglobin levels (Fig 1).

Relationship between sIL-2R levels and NK in vitro activity. As shown in Fig 1, the NK in vitro function was severely impaired before therapy in our patients. The cytotoxic ability of patients' mononuclear cells started to increase in the third month of rIFNa2 therapy and reached normal values after 6 months. In the three patients who had already discontinued therapy, rIFNa2 interruption was followed by a new decrease in NK function (Fig 1). Interestingly, the NK function of the HCL patients appeared inversely related to the levels of sIL-2R (Fig 1). Notably, when the sIL-2R levels were high, the NK function was low; when the sIL-2R levels reached their nadir, the cytotoxic ability began to increase.

In vitro effect of IL-2 on NK activity. The stimulatory effect of exogenous IL-2 on the NK in vitro function of PBMC from HCL patients is shown in Table 3. At the time of diagnosis before any treatment, exogenous IL-2 was able to provide a dramatic increase in the cytotoxic in vitro function in all tested patients. It is worth mentioning that at this time the highest levels of sIL-2R were also observed in the patients' serum.

After a 6-month period of therapy with rIFNa2 when sIL-2R levels were significantly decreased, the NK in vitro function was only slightly susceptible to further increase upon IL-2 stimulation in all cases but one (case no. 2). This pattern was similar to that observed in normal subjects.

The immunophenotypic evaluation of NK-related cells when using Leu-7 and Leu-11 MoAbs showed comparable figures at diagnosis and after 6 months of rIFNa2 therapy (Leu-7, 0.311 x 10^9/L ± 0.169 v 0.237 x 10^9/L ± 0.207; Leu-11, 0.127 x 10^9/L ± 0.089 v 0.107 x 10^9/L ± 0.071)

### Table 2. Quantitative Evaluation of sIL-2R in the Sera of HCL Patients at the Time of Diagnosis and During rIFNa2 Therapy

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Before Therapy</th>
<th>3 mo</th>
<th>6 mo</th>
<th>12 mo</th>
<th>15 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sIL-2R* (U/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>37.980</td>
<td>5.400</td>
<td>1.880</td>
<td>1.452</td>
<td>10.220</td>
</tr>
<tr>
<td>3</td>
<td>13.370</td>
<td>1.935</td>
<td>1.112</td>
<td>0.958</td>
<td>2.194</td>
</tr>
<tr>
<td>4</td>
<td>20.500</td>
<td>1.885</td>
<td>0.981</td>
<td>0.690</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18.100</td>
<td>ND</td>
<td>2.748</td>
<td>1.656</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>48.090</td>
<td>8.095</td>
<td>3.660</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>16.080</td>
<td>2.232</td>
<td>1.294</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>26.070</td>
<td>4.875</td>
<td>2.748</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

*Age-matched normal controls: mean ± SD = 232 ± 118 U/mL; range, 75 to 496 U/mL.
†Months after the beginning of rIFNa2 therapy.

### Table 3. In Vitro Effect of IL-2 on the NK Activity of PBMC From HCL Patients Before and During rIFNa2 Treatment

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Before Therapy Baseline Value</th>
<th>After IL-2 Activation 36.0</th>
<th>Variance</th>
<th>After IL-2 Activation 21.8</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.6</td>
<td>36.0</td>
<td>30.4</td>
<td>21.8</td>
<td>27.0</td>
</tr>
<tr>
<td>3</td>
<td>6.8</td>
<td>39.5</td>
<td>32.7</td>
<td>17.3</td>
<td>4.3</td>
</tr>
<tr>
<td>4</td>
<td>3.7</td>
<td>26.2</td>
<td>21.5</td>
<td>12.0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>8.0</td>
<td>57.3</td>
<td>49.3</td>
<td>42.5</td>
<td>8.2</td>
</tr>
</tbody>
</table>

Controls (n = 5) 44.6 ± 8.6* 57.7 ± 6.4* 13.1

For NK activity, a standard assay was performed at an 80:1 E/T ratio.
SD.
(mean ± SD). This finding suggests that the variations in NK activity are not primarily produced by fluctuations in NK cell numbers.

**DISCUSSION**

In this paper we provide evidence that the serum of patients with HCL contains high levels of sIL-2R. This was suggested by the selective inhibition of anti-IL-2R antibody binding to activated T cells that was exerted by patients' sera and confirmed by a specific ELISA test. The inhibitory factor corresponding to the sIL-2R should be released in vivo by HC, as suggested by experiments performed in vitro. T cells have been demonstrated to secrete sIL-2R after activation in vitro and might then represent an alternative or supplementary source of sIL-2R in vivo in our patients. Nevertheless, no evidence has been reported of increased T cell activation in HCL. In addition, the sIL-2R levels rarely exceed 2,000 to 4,000 U/mL in other diseases where T cell activation is commonly present such as viral hepatitis, infectious mononucleosis, sarcoidosis, and Hodgkin's disease.

Using both methods we could demonstrate that the sIL-2R levels in our patients' sera dramatically decreased after a few months of rIFNa therapy. In most patients the decrease in sIL-2R levels corresponded to the clinical recovery and the improvement of erythromyelopoiesis. However, a small number of residual HC could be detected in the bone marrow of all patients, even at the maximum therapeutic effect of improvement of erythromyelopoiesis. Nevertheless, no evidence has been reported of increased T cell activation in HCL. In addition, the sIL-2R levels rarely exceed 2,000 to 4,000 U/mL in other diseases where T cell activation is commonly present such as viral hepatitis, infectious mononucleosis, sarcoidosis, and Hodgkin's disease.

Using both methods we could demonstrate that the sIL-2R levels in our patients' sera dramatically decreased after a few months of rIFNa therapy. In most patients the decrease in sIL-2R levels corresponded to the clinical recovery and the improvement of erythromyelopoiesis. However, a small number of residual HC could be detected in the bone marrow of all patients, even at the maximum therapeutic effect of rIFNa therapy (Fig 1). This discrepancy may be tentatively explained by differences in IL-2-binding capacity of soluble receptors and/or by a direct activation of NK cells by interferons independent of IL-2 availability. This latter possibility, however, is unlikely because exogenous IL-2 also produced a significant stimulation of in vitro NK activity observed in HCL after a-IFN therapy represents the effect of a nonspecific enhancement of the host immune system.

The only exception in our study was represented by patient no. 2 in whom high serum levels of sIL-2R were detectable during the entire follow-up of the treatment. A limited clinical response was obtained in this patient, but NK activity significantly recovered during rIFNa therapy. The observed lack of timing between the restoration of NK activity and HC decrease in treated patients does not support this latter possibility. This is also suggested by the recent data on the absence of cytotoxic activity of NK cells from HCL patients on their own neoplastic cell population. All these findings favor the hypothesis that the increase in cytotoxic activity observed in HCL after alpha-IFN therapy represents the effect of a nonspecific enhancement of the host immune system.

The authors wish to thank the Essex Italia (Schering Corp, Kenilworth, NJ) for providing the rIFNa2. We thank Professor G. Janossy, London, for advice and criticism and L. Montagna for her skillful technical assistance.

**REFERENCES**

6. Baran D, Moreau JL, The'ze J: New radioimmunoassay for the detection of free IL2 receptor in supernatants of murine helper
Soluble interleukin-2 receptors in the sera of patients with hairy cell leukemia: relationship with the effect of recombinant alpha-interferon therapy on clinical parameters and natural killer in vitro activity

M Chilosi, G Semenzato, G Cetto, A Ambrosetti, L Fiore-Donati, G Perona, G Berton, M Lestani, A Scarpa and C Agostini