Mechanisms Accounting for the Defective Natural Killer Activity in Patients With Hairy Cell Leukemia

By Livio Trentin, Renato Zambello, Carlo Agostini, Achille Ambrosetti, Teodoro Chisesi, Roberto Raimondi, Pietro Bulian, Giovanni Pizzolo, and Gianpiero Semenzato

Natural killer (NK) cell activity is severely impaired in untreated patients with hairy cell leukemia (HCL). In an attempt to investigate whether this impairment is related to a defect at the target cell binding and/or at the post target cell binding level, we evaluated the peripheral blood mononuclear cells (PBMC) of HCL patients for their ability to: (1) bind and kill K-562 NK-sensitive targets at the single cell binding level; (2) release the NK cytotoxic factor (NKCF) under different in vitro stimuli, including K-562 and phytohemagglutinin; and (3) kill K-562 targets in a lectin-dependent cellular cytotoxicity (LDCC) assay. This study demonstrates that untreated HCL patients’ PBMC show a low ability to form conjugates with K-562 targets at the single cell binding level (5.7% ± 1.0%) with respect to patients studied after treatment (9.3% ± 1.3%) and controls (10.5% ± 4.0%); P < .05 and P < .001, respectively. A decreased ability to kill the bound target was demonstrated in untreated cases (1.2% ± 1.1%) versus patients studied after treatment and controls (12.3% ± 1.6%, 17.0% ± 3.1% respectively; P < .001 in both conditions. After activation of effector cells with interleukin-2 (IL-2) in vitro, an increase in the ability of PBMC to form conjugates with K-562 targets and kill the bound target was demonstrated in each group of patients. Moreover, IL-2 was able to increase the cytotoxicity against NK-sensitive targets in all patients tested. Evaluation of NKCF production showed that untreated patients release low levels of NKCF when PBMC were incubated in the presence of K-562 stimulators (1.8% ± 0.7%) with respect to patients after interferon-α (IFN-α) therapy (7.6% ± 2.1%) and controls (12.9% ± 2.2%); P < .02 and P < .001, respectively. When the recognition mechanisms were bypassed by triggering the cells with lectins in an LDCC assay, we demonstrated an increase of the lytic activity in both groups of patients with respect to the baseline values. However, the cytotoxic capacity observed in untreated patients was significantly lower than that observed in subjects after IFN-α therapy and controls (P < .001). These findings suggest that the impaired NK activity observed in patients with HCL is related to defects both at the target and posttarget cell binding levels.

Hairy Cell Leukemia (HCL) is a neoplastic disorder characterized by the proliferation of cells belonging to the B-cell lineage. The disorder is commonly characterized by a severe pancytopenia and several immunologic abnormalities. Among these, a defect of the natural killer (NK) cytotoxic function has been demonstrated in untreated patients. This in vitro function usually recovers after several months of interferon-α (IFN-α) therapy. Although it has been suggested that a defective availability of interleukin-2 (IL-2), possibly consequent to increased serum levels of soluble IL-2 receptor, accounts for the impairment of the cytotoxic cells in this disease, little is known about the mechanisms that regulate the different steps of this cell defect, either at the target binding level or after the target binding events.

NK cells are commonly defined as CD3 negative granular lymphocytes that express CD16 and CD56 surface markers and display a cytotoxic machinery that does not require a major histocompatibility complex (MHC) restriction. The ability of cytotoxic cells to kill the targets implies the capability of effectors to bind the targets and, as a further step, the property to kill the cells by releasing several cytotoxic factors. Although the nature of the receptor(s) used by NK cells for target cell recognition has not yet been characterized, more information is available now on the soluble molecules, which are released by effector cells at the postbinding step and are able to induce the cytolysis. The NK cytotoxic factor(s) (NKCF) is/are one of these molecules. It is released by human NK cells after recognition and binding of the target and it has been proved to be selectively cytotoxic for NK-sensitive tumor cells. Other molecules beside NKCF have been discovered to display a lytic activity against tumor cells, including lymphotixin and tumor necrosis factor-α.

This study is designed to determine the events taking place at the cell level that might account for the impaired cytotoxic function observed in HCL patients. To this end, we evaluated the ability of peripheral blood lymphocytes, taken from both untreated HCL patients and patients treated with IFN-α, to form conjugates with NK-sensitive targets, release NKCF under different in vitro stimuli, and kill the NK-sensitive targets in a lectin-dependent cellular cytotoxicity assay (LDCC).

MATERIALS AND METHODS

Patients. Nineteen patients (13 men and 6 women, aged from 40 to 68 years) were studied. Fourteen patients were studied at the time of diagnosis while five additional patients were evaluated after 9 to 12 months of IFN-α therapy. The diagnosis of HCL was based on clinical, morphologic, and cytochemical criteria.

From the Padua University School of Medicine, Department of Clinical Medicine, First Medical Clinic and Clinical Immunology Section; Hematology Section, University of Verona; and Department of Hematology, Hospital of Vicenza, Italy.

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Address reprint requests to G. Semenzato, MD, Istituto di Medicina Clinica dell’Università, di Padova, Clinica Medica 1°, Via Giustiniani 2, 35128 Padova, Italy.

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The treatment consisted of $2 \times 10^4$ IU/m$^2$ of recombinant (r) IFN-α (obtained from the Schering Corp, Kenilworth, NJ) injected subcutaneously three times a week for 12 months. Informed consent was obtained from all patients who had been advised as to procedure and attendant risks. Adverse reactions of moderate intensity consisting of fever were seen in eight patients and were controlled by acetaminophen. All patients had a partial remission, as determined by criteria currently used. In particular, a decrease $\geq 50\%$ in the bone marrow leukemic infiltrate (cellularity and percentage of hairy cells) for pretreatment values, restoration of the peripheral blood values, and $\geq 50\%$ reduction size of the spleen were observed.

Five normal volunteers with ages ranging from 35 to 60 years were used as controls.

**Preparation of effector cells.** Peripheral blood mononuclear cells (PBMC) were obtained from freshly heparinized blood by centrifugation on a Ficoll-Hypaque gradient. The cells were then washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (GIBCO, Paisley, Scotland). In patients who showed the leukemic form of the disease, samples were further enriched and depleted of hairy cells after removal of non-E-rosetting cells by neuraminidase (Sigma Chemical Co, St Louis, MO) treated sheep red blood cell rosetting technique according to the method previously described. More than 95% of the cells obtained using this procedure were viable when tested by the trypan blue exclusion test and expressed the CD2 antigen.

**Phenotypic evaluations.** Phenotypic analysis of effector cells was performed using fluorescein (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (MoAbs) and quantitated using a flow cytometer (Cytofluorograph II, Ortho Diagnostic, Raritan, NJ). MoAbs used were CD3 (OKT3), CD4 (OKT4), CD8 (OKT8) (Ortho Pharmaceutical), CD57 (anti-Leu7), CD56 (anti-Leu19), and CD16 (anti-Leu11a) (Becton-Dickinson, Sunnyvale, CA). The CD5 nomenclature was reported according to the Fourth Meeting on Leukocyte Typing. Predetermined optimum concentration of primary and secondary antibody was used in all cases. Briefly, cells at a concentration of $10^6$ mL were incubated with the above-quoted FITC- or PE-conjugated antibodies for 30 minutes in ice then centrifuged twice in cold PBS. For indirect fluorescence, an FITC-conjugated F(ab')2 goat anti-mouse immunoglobulin (Ig) (Techno Genetics, Turin, Italy) was added; cells were further incubated for 30 minutes at 4°C and washed twice. After these incubations, 10,000 gated cells were analyzed using the flow cytometer equipped with an argon laser operating at a wavelength of 488 nm to excite FITC and PE. Forward and 90° angle light scatter were used to discriminate viable cells from dead cells. FITC and PE mouse Ig of the same isotype (IgG1, IgG2a + b) were used as negative controls.

**Single cell binding and cytotoxic assay.** The method of Vargas-Cortez et al. was used. Briefly, effector and target conjugates were generated by incubating a 1:1 mixture of $1 \times 10^6$ effector and target cells in 200 mL RPMI medium final volume for 15 minutes at 37°C in 5% CO$_2$ atmosphere. The mixture was then centrifuged (200 g x 5 minutes) and the supernatant carefully removed. The pellet was gently resuspended in 500 mL of RPMI medium and plated on poly-L-lysine (molecular weight 70,000, Sigma) coated coverslips. The slides were incubated for 20 minutes at room temperature and then transferred to 37°C in a 5% CO$_2$ atmosphere. After a 4-hour incubation, the coverslips were rinsed with PBS, stained with 0.1% trypan blue, fixed in 1% formalin solution, and transferred to microscope slides to be scanned. Controls were prepared with target cells only.

The percentage of conjugates' formation was determined by counts, made in triplicate, of at least 300 conjugates, as follows: percent conjugates: (number of effector cells bound to targets) x $10^6$/100 effector cells. The percentage of target cell lysis was calculated as follows: percent target cell lysis: (number of conjugates with dead targets) x $100/100$ conjugate targets.

In some patients (4 untreated cases and 3 patients after IFN-α therapy), single-cell binding and cytotoxic assays were also performed after in vitro activation of effector cells ($1 \times 10^6$/mL) for 18 hours at 37°C in an atmosphere of 5% CO$_2$ with different concentrations (10, 100, 500 U/mL) of recombinant IL-2 (Glaxo Institute for Molecular Biology SA, Geneva, Switzerland). In these patients the cytotoxicity of resting and IL-2-activated effector cells was also determined in a $^{3}Cr$-labeled K-562 target cell assay. The tests were performed at different effector/target (E/T) ratios (40:1, 20:1, 10:1, 5:1) as reported below. Data are reported at the 40:1 E/T ratio.

**Production of NKCF.** The method of Wright and Bonavida, with minor modifications, was used to generate cytotoxic molecules. Briefly, PBMC from HCL patients or controls were used as effector (E) cells, washed with PBS and resuspended in serum-free Iscove's modified Dulbecco's medium (IMDM) (Flow Laboratories, Inc, Irvine, Scotland) supplemented with 1% penicillin-streptomycin. Subsequently effector cells were mixed with the K-562 cells used as stimulators to obtain an E/S ratio of 50:1. The final density of effector cells in all cultures was $2 \times 10^6$/mL. Controls were represented by supernatants devoid of cytokotic activity that had been obtained by stimulatory cells in the absence of effector cells (data not shown). NKCF release was induced by PHA by adding the lectin to effector cell cultures (5 µg/mL). After a 48-hour incubation at 37°C in 5% CO$_2$, cell-free supernatants were harvested, filtered through a 0.45-µm Millipore filter (Flow Pore Δ, by Sartorius, FRG), and tested for NKCF assay or stored at $-70°C$ until used.

**NKCF assay.** NKCF was assessed by measuring cytotoxicity against $^{3}Cr$-labeled K-562 target cells. Target cells (1×10$^6$/mL) were incubated overnight with 100 µCi/mL of Na$_2^{3}CrO_4$, (CEA IRE Sorin Biomedica, Saluggia, Italy, 1mCi/mL), washed three times, and resuspended at the final density of $2 \times 10^6$/mL in RPMI 1640 supplemented with 1% penicillin-streptomycin and 10% fetal calf serum (FCS) (Flow Laboratories). The assay was set up in triplicate in 96 wells, U-bottomed sterile microtiter plates. Fifty microliters of target cell suspension and 100 µL of cell-free supernatants were added to each well and the final volume was adjusted to 200 µL with IMDM plus 1% of penicillin-streptomycin. The final concentrations of FCS in each well was 2.5%. Wells for spontaneous and maximal $^{3}Cr$ release contained target cells plus 150 µL of IMDM.

After a 20-hour incubation at 37°C in 5% CO$_2$, 100 µL supernatants from each well was collected and radioactivity was measured using a γ-counter. PHA alone was not found to be cytotoxic for K-562 target cells. The mean percentage of lysis, in triplicate, was calculated as follows: percent of lysis (test cpm - spontaneous cpm) x $100/$(total cpm—spontaneous cpm).

In all experiments the percentage of spontaneous release was less than 15%.

**Leukot-dependent cellular cytotoxicity (LDC).** Effector cells were incubated in 96 wells, round-bottomed microtiter plates with $^{3}Cr$-labeled K-562 target cells at an E/T ratio of 40:1, both alone or with 0.2, 0.5, or 1 µg/mL of highly purified phytohemagglutinin (PHA-HP) (Flow Laboratories) in a final volume of 200 µL. The plates were then incubated for 4 hours at 37°C and 100 µL of supernatant was harvested and counted for radioactivity in a γ-counter. PHA alone was not found to be cytotoxic for K-562 target cells. The mean percentage of lysis, in triplicate, was calculated as follows: percent of lysis: (test release – spontaneous release) x $100/$(maximum release—spontaneous release).

In all experiments the percentage of spontaneous release was less than 10%.

**Statistical analysis.** Data are expressed as mean ± SEM, and comparison between values is made using Cochran-Cox analysis. A value of $P < .05$ was accepted as significant.
MECHANISMS ACCOUNTING FOR NK DEFECT IN HCL

The number of peripheral blood lymphocytes did not show any statistical differences between untreated HCL patients (2,432 ± 700/mm³) and patients after IFN-α therapy (2,257 ± 681/mm³), and between each group and controls (2,275 ± 520/mm³). Similarly, no statistically significant differences were observed in the percentage of CD3⁺, CD4⁺, and CD8⁺ cells between untreated and treated HCL patients and between the two groups of patients and controls (Table 1). Also, the phenotypic analysis of cytotoxic-related markers did not show any difference in the percentage of CD16⁺ and CD56⁺ cells between the different groups of patients and between patients and controls.

Figure 1 illustrates the results of the single cell binding assay in HCL patients. Untreated patients showed a low ability to form conjugates with K-562 target cells (5.6% ± 1.0%) with respect to treated patients (9.3% ± 1.3%) and controls (15.0% ± 4.0%) (P < .05 in both conditions (Fig 1A). The percentage of conjugates with dead targets were significantly lower (1.2 ± 1.1) in untreated patients as compared with treated patients (12.3 ± 1.6) and controls (17.0 ± 3.0; Fig 1B); P < .001 in both conditions. No statistical differences were observed between patients studied after IFN-α treatment and controls.

When effector cells were activated in vitro with different concentrations of IL-2 (Fig 2) an increase in the ability to form conjugates with K-562 targets (Fig 2A) and kill the percentage of conjugates with dead targets were significantly higher with treated patients (12.3 ± 1.6) and controls (17.0 ± 3.0; Fig 2B); P < .001 in both conditions. The possibility that the impairment of NK activity might be related to a defective presence of NK cells was ruled out by the evidence that the number of cells bearing NK-related markers is within the normal range, in both untreated cases and in patients after IFN-α treatment, suggests that the NK defect is not a quantitative phenomenon but rather related to a functional impairment of the cytotoxic compartment.

Table 1. Phenotypic Analysis of Peripheral Blood Lymphocytes Recovered From HCL Patients

<table>
<thead>
<tr>
<th>Groups</th>
<th>CD3 (%)</th>
<th>CD4 (%)</th>
<th>CD8 (%)</th>
<th>CD16 (%)</th>
<th>CD56 (%)</th>
<th>CD57 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n = 14)</td>
<td>72.8 ± 1.7</td>
<td>39.7 ± 3.6</td>
<td>29.6 ± 2.9</td>
<td>12.0 ± 2.9</td>
<td>16.5 ± 3.7</td>
<td>18.9 ± 4.6</td>
</tr>
<tr>
<td>Treated (n = 5)</td>
<td>81.0 ± 4.1</td>
<td>43.2 ± 5.7</td>
<td>29.5 ± 2.0</td>
<td>19.1 ± 4.4</td>
<td>15.0 ± 2.8</td>
<td>29.8 ± 8.7</td>
</tr>
<tr>
<td>Controls (n = 5)</td>
<td>75.0 ± 7.0</td>
<td>44.0 ± 6.1</td>
<td>27.3 ± 3.2</td>
<td>16.1 ± 3.3</td>
<td>14.4 ± 2.8</td>
<td>15.8 ± 1.2</td>
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DISCUSSION

Our study demonstrates that the impaired NK activity in untreated HCL patients is related to the defective abilities of peripheral blood lymphocytes to bind and kill NK-sensitive targets at the single cell binding level, and also to the inability to release NKCF after an appropriate in vitro stimulus. Bypassing the recognition step, ie, after in vitro triggering of the effector cells with PHA, a normal capacity to release NKCF was demonstrated in untreated patients. A recovery of all of the above functions has been observed in the same HCL patients after IFN-α therapy. Several explanations may be taken into account to interpret the defective activity, including a decreased number of effector cells, the inability of NK cells to recognize and/or interact with targets, the failure of the NK target to trigger and activate the NK effector to release NKCF, and eventually the failure per se of NK cells to release NKCF.23

The possibility that the impairment of NK activity might be related to a defective presence of NK cells was ruled out by the phenotypic analysis of PBMC in our patients. In fact, the evidence that the number of cells bearing NK-related markers is within the normal range, in both untreated cases and in patients after IFN-α treatment, suggests that the NK defect is not a quantitative phenomenon but rather related to a functional impairment of the cytotoxic compartment.
The next step was to investigate the cytotoxic function at the single cell level. The demonstration of a low conjugate formation with target cells in untreated HCL patients could be related to a functional defect of the putative receptor(s) involved in the mechanisms of cell binding or, alternatively, could be consequent to the activity of receptor blocking factors possibly present in the serum of these patients. In this regard, PBMC recovered from untreated patients with low NK in vitro function were able to display a partial recovery of their lytic ability when cultured in medium supplemented with 10% FCS without any other stimulus for 24 to 48 hours. However, the serum recovered from untreated HCL patients was unable to affect the cytotoxic function of purified normal CD16+ NK cells (data not shown). The possibility that the increase of cytotoxic function of NK cells might be related to a displacement of monomeric IgG from the Fc (CD16) receptor must be taken into account to explain these findings. Alternatively, the presence of the p55 chain of IL-2 receptor on the membrane of hairy cells coupled to the demonstration of high levels of soluble IL-2R in the serum of these patients, which is actively shedded by leukemic cells, suggests that the p55 chain of IL-2 receptor might indirectly behave as an inhibitory factor. Binding to IL-2 efficiently, the soluble IL-2R could induce a decreased availability of endogenous IL-2 ultimately leading to a hyporesponsiveness of NK cells. According to this interpretation, we found that IL-2 is able to induce an increase in the percentage of conjugates with K-562 cells at single cell binding level and an increase of the killing of target cells both at single cell binding level and in the Cr-labeled target assay (Figs 2 and 3). These data confirm and extend previous results reported by Hooper et al. To verify the possibility that the defect of the cytotoxic machinery lies at the level of cytotoxic molecule production, we investigated the ability of NK cells to release NKCF. We demonstrated that NK cells from untreated patients were not able to release NKCF, whereas NK cells from IFN-α treated patients were able to do so (Fig 4).
inducible to release NKCF when cultured in the presence of K-562 stimulators, thus suggesting that the abnormality might take place at the level of the trigger of cytotoxic molecules or, alternatively, that these cells are intrinsically defective. To differentiate these two possibilities, we evaluated the role of PHA in the induction of NKCF. Despite the poor NKCF production observed on stimulation with target cells in untreated patients, effector cells from both untreated patients and patients after IFN-α therapy were able to release NKCF after in vitro activation with PHA. This finding suggests that cytotoxic cells in untreated HCL patients have the ability to produce and/or release NKCF, and also supports the notion that, rather than an intrinsic abnormality of the cytotoxic cell per se, the defect observed in these patients primarily lies at the level of the activation signal provided by stimulatory targets, which is mandatory for NK activity.

Because we used PBMC unfractionated populations, the influence of PHA on target cell lysis, production of NKCF, and LDCC function might also be related to the effect of PHA on T lymphocytes either directly or indirectly as a result of their release of immunomodulatory molecules, such as IL-2 and γ-IFN. This is consistent with the finding that in using the LDCC assay in untreated patients, an increased killing of K-562 cells in the presence of PHA with respect to the baseline values was demonstrated. Although the significance of the LDCC assay is not completely understood, it has been demonstrated that PHA is able to induce cytotoxicity in CD57+ T lymphocytes while the NK function mediated by CD16+ cells is unaffected by the presence of PHA. The evidence that PHA triggers the cytotoxic function in our patients favors the hypothesis that other cells, MHC-unrestricted cytotoxic T lymphocytes (notably CD57+ cells), might be involved as effectors of the nonspecific cytotoxicity displayed by PBMC in the presence of PHA. Further studies using purified NK cells from HCL patients aimed to evaluate the adhesion molecules, including those recognized by CD2, CD16, CD56, and CD18 related MoAbs, and their role in the cytotoxic events might contribute to specify the precise level of cytotoxic defect.

The demonstration we provided of a greater release of NKCF by PBMC from patients after IFN-α therapy with respect to untreated HCL patients deserves some comment on the biologic effect of IFN-α therapy. Interferons have been reported to play an important role in the regulation of NK cells both in experimental models and in humans, and notably in HCL. As far as the cytotoxic events we have been dealing with in this report are concerned, Wright and Bonavida demonstrated that fibroblast interferon induces an increase in the release of NK cytotoxic factors in mice. Similar effects have been observed in humans, in that IFN-α has been demonstrated to be involved in the modulation of NKCF production by NK cells both in normal conditions and in malignancy. Besides providing new proof of the nature of the NK defect in HCL patients at the single cell level, our results extend the above-mentioned observations to HCL, supporting the concept that IFN-α plays a pivotal role in modulating the function of NK cells, possibly exerting a multistep action at the cell level along the different pathways of the cytotoxic machinery.

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