Lymphokine Induction of NK-Like Cytotoxicity in T Cells From B-CLL

By Melchor Alvarez de Mon, Juan Casas, Rafael Laguna, Maria Luisa Toribio, Manuel O. de Landázuri, and Alberto Durántez

T cells from patients with B cell chronic lymphocytic leukemia (B-CLL) exhibit defective natural killer (NK) activity. In this study, we have analyzed the cytotoxic-inducer effects of gamma interferon (γ-IFN) and supernatants containing interleukin 2 (IL 2 sup). T cells from patients with B-CLL were incubated with γ-IFN or IL 2 sup. γ-IFN did not modulate the very low or undetectable levels of NK activity present in the T cell population. However, IL 2 sup induced a potent cellular cytotoxicity against NK-sensitive and NK-resistant tumoral target cells. This cytotoxic inducer effect (a) was present in lectin-free IL 2 sup and in a 15,000- to 20,000-dalton molecular weight fraction obtained by gel filtration chromatography of this supernatant; (b) was directed against NK-sensitive and NK-resistant target cells; (c) was not correlated with the basal levels of NK activity; and (d) was not associated with the development or augmentation of the proportion of lymphocytes with classic NK cell phenotype. Taken together, these results demonstrate that unstimulated T cells from B-CLL patients, incubated briefly (18 hours) with IL 2 sup but not γ-IFN, have strong NK-like cytotoxicity, despite the lack of classic NK activity.

B CELL CHRONIC lymphocytic leukemia (B-CLL) is characterized by the monoclonal expansion and accumulation of B cells, which are arrested in their differentiation pathway at an early stage. There are indications that not only B cells are affected in B-CLL, but that T cells from these patients also show functional and phenotypic abnormalities. In addition, it has been shown that E rosette-positive cells from B-CLL patients mediate defective natural killer (NK) cytotoxicity and antibody-dependent cellular cytotoxicity. The defective NK activity present in these patients may play a role in the development of this disease, since NK activity is thought to be involved in the surveillance against tumors and in the control of both normal hematopoiesis and viral infection.

It has been demonstrated that classic NK cells, defined by their ability to lyse a number of tumor cell line targets without prior activation, are modulated by interferon (IFN) and interleukin 2 (IL 2). There are also reports describing the induction of killer cells that lyse tumor cell line targets during long-term culture with lymphokine-conditioned medium. This study examined the in vitro effect of IL 2 supernatants (IL 2 sup) on T cells from B-CLL patients in the induction of cytotoxic activity against tumor target cells. Our results demonstrate that short-term (18 hours) incubation with IL 2 sup can induce strong NK-like activity in unstimulated T cells from B-CLL patients that lack classical NK activity.

MATERIALS AND METHODS

Patient population. Twenty-six previously untreated patients with clinically, histologically, and immunologically defined B-CLL (according to the Chronic Leukemia Myeloma Task Force 1973) were studied. Their median age was 68 ± 9. Controls were 14 healthy, age- and sex-matched individuals.

Culture medium. RPMI 1640 (Microbiological Assoc, Walkersville, Md) supplemented with 1% L-glutamine (Flow Lab, Irvine, UK), 0.5% HEPES (Flow Lab), and 1% penicillin streptomycin (Difco Lab, Detroit) was used for cultures. This will be referred to as complete medium.

Isolation of lymphoid cells. Peripheral blood mononuclear cells (PBMs) were obtained from the heparinized venous blood of the subjects by Ficoll-Hypaque gradient centrifugation. T lymphocytes were separated by their capacity to form stable rosettes with neuraminidase-treated sheep red blood cells (SRBCs). Briefly, E-SRBCs (T cells) were obtained after incubation of PBMs with neuraminidase-treated SRBCs for two hours at 4°C and centrifugation on a Ficoll-Hypaque gradient. The interface, containing mainly B cells, null cells, and macrophages, is referred to as non-T cells. A T cell purity of 90% to 94% (51% ± 10% OKT4, 41% ± 10% OKT8) in the control group and 78% to 94% (36% ± 5% OKT4, 34% ± 4% OKT8) in the B-CLL group was obtained after subjecting pellets to hypotonic lysis in order to eliminate SRBCs.

Production of IL 2 supernatants. IL 2 sups were obtained from human tonsil lymphocytes stimulated with 1% phytohemagglutinin (PHA) (Difco Lab) for 24 hours, as previously described. The culture supernatants were concentrated by double-step precipitation with (NH₄)₂SO₄ and dialyzed against phosphate-buffered saline (PBS), pH 7.4. The remaining PHA was removed by chicken red blood cell absorption, as reported elsewhere. Partially purified IL 2 was obtained by gel filtration chromatography. The IL 2 activity was recovered in fractions corresponding to a molecular weight (mol wt) of 15,000 to 20,000 daltons.

IL 2 was also obtained from a tumor T cell line. The Gibbon lymphosarcoma cell line, MLA-144, provided by Dr H. Rabin (Frederick Cancer Research Center, Frederick, Md), spontaneously releases a factor that is biologically and biochemically similar to human IL 2. MLA-144 cells were cultured at 2 × 10⁶ cells per milliliter in complete medium without serum for 48 hours. After the culture period, supernatants were collected and concentrated as previously described.

To study the production of IL 2 by the T cells from either patients or controls, 5 × 10⁶ cells from each subject and control were resuspended in 1 mL of complete medium supplemented with 1% fetal bovine serum (FBS) and cultured for 24 h in the presence of 1% PHA in round-bottom culture tubes (Falcon, Oxnard, Calif). At the end of the culture period, the supernatants were collected and stored at −20°C until tested. IL 2 assays were performed as previously described.

PHA-stimulated cultures. Purified T cells were cultured in 200 μL of complete medium with 10% FBS and 1% PHA, at a density of...

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1.5 x 10^5 cells per well, in triplicates in 96-well plates (Dynatech, Billingshurst, UK) for three days. Plates were incubated at 37 °C in a 5% CO2 humid atmosphere. On day 2, 1 μCi of [3H]-thymidine was added, and 24 hours later, cultures were harvested (Skatron, A.S., Lysbyen, Norway) and counted in a liquid scintillation counter.

**Cell cultures with IL 2 sup.** Cell cultures were set up in macrowells (1.6 x 10^5 cells per milliliter) in complete medium supplemented with 10% FBS and in the presence or absence of different concentrations of IL 2 sup. The cultures were carried out for variable periods.

**Cells cultured with γ-IFN.** As previously described, effector cell populations were cultured in macrowells (1.6 x 10^5 cells per milliliter) in complete medium supplemented with 10% FBS for 18 hours. In the last two hours, we added 1,000 IU/mL of human γ-IFN (specific activity 2 x 10^7 IU/mg protein HEM) (Research Inc, Rockville, Md).

**Cytotoxicity assays.** Cytotoxicity was quantified by a ^51^Cr-specific release assay using the following tumor target cells: K-562, Chang, MT-1, Alab, Raji, Molt-4, Molt-4-Thioguanin resistant, and Jurkat. Assays were performed at effector-target ratios of 50, 25, and 12:1 in 96-microwell, round-bottom plates. After a four-incubation, 100 μL of supernatant from each well was collected and analyzed in a gamma counter. The specific lysis (SL) and lytic units (LU) were calculated as previously described.

**Cell surface markers.** Indirect immunofluorescence (IF) with the monoclonal antibodies (OKT4, OKT8 [Ortho, Raritan, NJ], and Leu 11b [Becton Dickinson, Mountain View, Calif]) was performed as described. Samples were prepared as described for IF and analyzed with an EPICS-C Flow Cytometer (Coulter Electronics, Hialeah, Fla).

**RESULTS**

**NK activity of PBMs, T, and non-T cells.** PBMs, T, and non-T cells from 26 patients with untreated B-CLL and from 13 normal controls were separated to study their NK activity. As can be observed in Fig 1, E rosette-positive cells from B-CLL showed a significantly depressed NK activity when compared with normal controls (P < .0001, Mann-Whitney U test). Neither PBMs nor non-T cells from B-CLL mediated any significant cytotoxic activity (data not shown). The lack of NK activity present in the T cell-enriched population was not due to inhibition by the small number of contaminating non-T cells (range, 6% to 22%), since we observed in mixing experiments that non-T cells from B-CLL patients did not inhibit the cytotoxic activity of normal T cells (data not shown).

**Modulation of the cytotoxic activity against K-562 target cells by γ-IFN and IL 2 sup.** In preliminary experiments, we investigated whether IL 2 sup could induce cytotoxic activity in T cells from B-CLL patients. To this end, the T cells were incubated for two or 18 hours with IL 2 sup, and subsequently, their cytotoxic activity was tested against K-562 target cells. This revealed an increase in their lytic capacity that was maximal after incubation for 18 hours (lytic units at time 0 < 4 LU x 10^-4 cells; after two hours = 20 LU x 10^-4; and after 18 hours, 40 LU x 10^-4). Therefore, in the following experiments, this period of incubation was routinely used.

To compare the cytotoxic-inducer effect of IL 2 with respect to γ-IFN, T cells from 12 cases of B-CLL with very low or absent levels of NK activity and T cells from controls with normal NK activity were incubated for 18 hours with these lymphokines (Table 1). After incubation with 1,000 IU/mL of γ-IFN, there was a significant increase in the cytotoxic activity of T cells in all the normal controls (P < .05, Wilcoxon signed-ranked test). In contrast, T cells from B-CLL patients were either unresponsive to γ-IFN (patients I, II, IV, and VI) or minimally so (rest of the patients). When the data of all 12 patients were analyzed together, the increments observed in NK activity after γ-IFN treatment were not statistically significant (P > .05, Wilcoxon signed-ranked test). On the other hand, when all the data from either patients or controls were examined, we did observe a significant correlation (P < .05, least squares method) between the levels of cytotoxicity observed after γ-IFN treatment and the basal values of NK activity (Fig 2).

The incubation with IL 2 sup of T cell from each of the B-CLL patients induced a significant increase in their ability to lyse K-562 target cells, which proved significant when all the cases were analyzed together (P < .01, Wilcoxon signed-ranked test). We also observed that the highest levels of cytotoxicity induced by IL 2 sup were obtained precisely in those patients who did not show spontaneous cytotoxic activity and did not respond to γ-IFN. We did not observe any correlation, either in B-CLL patients or in normal controls, between the basal levels of NK activity and the killing activity after incubation with IL 2 sup (Fig 2).
This finding led us to study the possibility that the T cells from B-CLL patients could be deficient in their synthesis of IL-2. We found that IL-2 production by PHA-stimulated T cells from nine patients were similar to that of six normal controls (Fig 3). However, the proliferative response to PHA stimulation of the T cells from these B-CLL patients was significantly lower (Table 1).

**Table 1. Effects of γ-IFN and IL-2 Sup on Cytotoxic Activity of T Cells From B-CLL Against K-562 Target Cells**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Basal</th>
<th>After Treatment with γ-IFN</th>
<th>After Treatment with IL-2 Sup</th>
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<tbody>
<tr>
<td></td>
<td>50/1</td>
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<td>XII</td>
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</tbody>
</table>

Patients (x ± s) | 11 ± 6 | 6 ± 4 | 2 ± 3 | 18 ± 13 | 12 ± 9 | 6 ± 6 | 50 ± 14 | 36 ± 15 | 23 ± 13 |

Controls (x ± s) | 45 ± 18 | 36 ± 15 | 25 ± 17 | 72 ± 15 | 61 ± 9 | 45 ± 22 | 76 ± 20 | 66 ± 25 | 51 ± 26 |

T cells from 12 B-CLL patients and six normal controls were incubated for 18 hours either with complete medium; complete medium for the first 16 hours, γ-IFN (1,000 IU/mL) being added in the last two hours; or complete medium plus 20% IL-2 sup. After this period of time, the cultures were analyzed for cytotoxic activity against K-562 target cells. The data are given as the percentage of specific lysis at effector-target cell ratios of 50:1, 25:1, and 12:1.

This finding led us to study the possibility that the T cells from B-CLL patients could be deficient in their synthesis of IL-2. We found that IL-2 production by PHA-stimulated T cells from nine patients were similar to that of six normal controls (Fig 3). However, the proliferative response to PHA stimulation of the T cells from these B-CLL patients...
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(34,407 ± 15,709 cpm/1.5 x 10^6 cells) was significantly decreased (P < .01, Mann-Whitney U test) when compared with that of the normal controls (87,082 ± 21,179 cpm/1.5 x 10^6 cells).

We also ruled out the possibility that the enhancement of cytotoxic activity by IL 2 sup was due to activation of the contaminating non-T cells, since we did not observe any cytotoxic activity when non-T cells from B-CLL patients were incubated with IL 2 sup (data not shown). To investigate whether the observed effects were due to PHA that could still be present in the IL 2 sup, we used supernatants from the T cell line, MLA-144, which have been shown to produce IL 2 constitutively. As can be seen in one representative experiment (Fig 4), MLA-144 sup induced an increase in the cytotoxic activity of T cells from B-CLL patients similar to that of IL 2 sup. Finally, and to further delineate the molecules responsible for the enhancement of the cytotoxic activity, we used a semipurified preparation of IL 2 obtained by gel filtration chromatography (15,000 to 20,000 mol wt). As can be seen in Fig 4, this preparation also induced strong cytotoxic activity in T cells from B-CLL patients.

We investigated the target cell specificity of the T cells from B-CLL patients after incubation with IL 2 sup. As can be seen in Fig 5, short-term incubation with IL 2 sup induced lytic activity against both NK-sensitive and NK-resistant target cells.

Finally, since the previous results demonstrated that IL 2 sup induced strong NK-like activity in the T cells from B-CLL patients, we studied whether this cytotoxic-inducer effect could be explained by an increase in the percentage of lymphocytes with the classic NK cell phenotype. In five experiments we observed that incubation of the IL 2 sup did not modify the low percentage of Leu I + cells (<1%) present in the T-enriched populations from the B-CLL patients.

**DISCUSSION**

This paper, above all, poses an intriguing question regarding the nature of the IL 2 sup-induced cytotoxicity generated in the T cells obtained from untreated B-CLL patients. The origin of these effector cells may be explained by (a) the activation of already mature NK cytotoxic cells; (b) the differentiation of pre-killer NK cells into effective cytotoxic cells; or (c) the recruitment of a different cell population not related to the NK pathway. Our data suggest that this last possibility is the most likely. The following reasons support this contention. First, there was no correlation between the basal levels of NK activity and the cytotoxicity acquired after incubation with IL 2 sup. Second, mature NK cells responded to IFN with an increment in their lytic activity, but using T cells from B-CLL patients, we did not observe this enhancement. (Other authors have also reported this observation.45) Third, the cytotoxicity generated by the IL 2 sup was active against both NK-sensitive and NK-resistant target cells. Finally, there was no induction of cytotoxic effector lymphocytes with phenotype of classic NK cells after the incubation with IL 2 sup.

Furthermore, recent reports in the literature support the possibility that, in some systems, the effector cells generated by IL 2 are not typical mature NK cells. We have observed that human thymocytes cultivated in the presence of IL 2 sup acquire de novo cytotoxic activity against tumor target cells.15 Grimm et al10 showed that PBM(s) incubated with IL 2 acquire strong cytotoxic activity against NK-resistant target cells. Although the mechanisms underlying these observations remain to be determined, the effector cells in these models have several similarities to those described by us in this report. For example, these effector cells can be generated by IL 2 in populations devoid of spontaneous NK activity and have neither the specificity nor the phenotypic markers of mature NK cells. The relationship of these effector cells with classic NK cells is unknown.

Several pathological conditions in addition to B-CLL have
been found to have similar immunological abnormalities, including an inverted T4/T8 ratio, a poor response to T cell mitogen, and a loss of NK activity.\textsuperscript{20-22} In the acquired immunodeficiency syndrome, Rook et al\textsuperscript{19} have recently shown that the depressed NK activity present in PBMs of these patients cannot be restored by \gamma-IFN, but can be raised to normal levels by treatment with IL-2. These results are identical to those obtained by us with B-CLL patients. Thus, it is possible that the defective NK activity present in B-CLL and other diseases could be overcome by in vitro treatment with IL-2.

Further studies are needed in order to understand (a) the relationship between the effector cells mediating NK activity and those generated in the presence of IL-2; (b) the significance of the T cell abnormalities in B-CLL; and (c) the potential immunotherapeutic use of lymphokines in this group of diseases.

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