Production and Regulation of Interleukin-2 in Human Lymphoblastic Leukemias Studied With T-Cell Monoclonal Antibodies

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Human leukemias are illnesses of hemopoietic stem cells that go through processes of self-replication and partial differentiation under the control of as yet largely unknown growth and differentiation factors. IL-2 is a powerful factor controlling proliferation of normal T cells. We report that acute lymphoblastic leukemias of T and non-B, non-T phenotypes produce a growth factor after mitogen stimulation. This factor is able to support the proliferation of human and murine IL-2-dependent cytotoxic cells, has a mol wt of 26,000 daltons by gel filtration, an isoelectric point of 6.6, and its biologic activity is inhibited by an anti-IL-2 monoclonal antibody. This factor is, therefore, by all parameters studied very similar to IL-2 produced by normal lymphocytes. A recently developed monoclonal antibody, Pan T2, binds to normal T cells, renders T cells responsive to IL-2, and induces the release of IL-2, which in turn provides the second signal for T-cell proliferation. Mononuclear cells from acute lymphoblastic leukemia do not respond to the addition of this monoclonal antibody unless cocultured with irradiated Daudi cells. Since normal T cells do not require Daudi to produce IL-2 and since Daudi cells do not produce IL-2 under any conditions, we conclude that the cell responsible for IL-2 production in acute lymphatic leukemia is a leukemic T cell with an altered mechanism of IL-2 production at the level of the Pan T2 binding site.

HUMAN LEUKEMIAS, or at least a large majority of them, are illnesses of early progenitor cells of the hemopoietic system. The classic example for the transformation of an early stem cell is chronic myeloid leukemia (CML). The knowledge of the regulatory mechanisms underlying this process of growth and differentiation is crucial for understanding the pathophysiology of leukemias and for their therapeutic control.

Recent developments permit the study of such mechanisms. The mixed colony assay of Fauser and Messner offers the opportunity to study stem cells that can differentiate along the myeloid, erythroid, and megakaryocytic lineages and, according to recent results, into lymphoid cells as well. Immunologic reagents have been developed that permit the unequivocal phenotypic characterization of hemopoietic cells. At the same time, the purification and characterization of lymphokines, and of other regulatory factors, have progressed rapidly, offering powerful biologic tools for the investigation of growth regulatory requirements of both normal and leukemic cells. One well studied lymphokine is Interleukin-2 (IL-2) or T-cell growth factor (TCGF). IL-2 was discovered by Morgan, Ruscetti, and Gallo in 1976.

The growth of leukemic cells appears to be dependent on as yet undefined factors. IL-2, with its growth stimulatory capacity for lymphoid cells, has the characteristics required to fit into such a group of factors. This laboratory has reported preliminary results showing that peripheral blood lymphocytes (PBL) from terminal deoxynucleotidyl-positive (TdT +) acute lymphoblastic leukemia (ALL) patients produced a factor with biologic characteristics similar to those of IL-2. We now present data showing that leukemic cell populations, characterized by morphological, biochemical, and immunologic markers, produce a factor which, based on its biochemical, biologic, and immunologic characteristics, is IL-2.

We have also studied a new monoclonal antibody, Pan T2, with respect to its biologic properties. This monoclonal antibody binds to a receptor present on normal T cells, is mitogenic, and induces IL-2 production in normal PBL. We show that the expression and function of the receptor recognized by this monoclonal antibody is altered on leukemic cells and, therefore, that ALL cells have an altered mechanism of IL-2 production.

MATERIALS AND METHODS

Cells

Peripheral blood mononuclear cells from ALL patients or normal donors were separated from heparinized blood on Ficoll-Hypaque and were used either directly or after cryopreservation. The clone of the Jurkat line (originally designated JM) used in this study was obtained from Dr. P. Ralph (Sloan-Kettering Institute) and originally from Dr. U. Schneider. The murine cytotoxic lymphoid line (CTLL-1) was isolated by Dr. S. Gillis (Fred Hutchinson Cancer Center, Seattle, Wash.), while the human CTLL, CI 3.3, was obtained from Drs. N. Flomenberg and J. Kornbluth (Sloan-Kettering Institute).
Kettering Institute) after it had been maintained in culture for over 4 mo. Both cell lines require IL-2 for survival and proliferation. PBL and human lines were grown in RPMI supplemented with 10% heat-inactivated (30 min at 56°C) fetal calf serum (FCS), glutamine 2 mM, penicillin 50 U/mL, and streptomycin 50 μg/mL. Marine IL-2-dependent CTLL were grown in Click’s medium (Altick Associates, Hudson, Wisc.) with the above supplements plus 25 mM HEPES buffer, 16 mM NaHCO3, and 3 U/mL of either human or rat IL-2. Rat IL-2 was purchased from Bethesda Research Laboratories (Gaithersburg, Md.), while human IL-2 was produced in this laboratory (see below). C1 3.3 was grown in RPMI containing 15% heat-inactivated human AB serum with the supplements reported above and 3 U/mL of human IL-2.

Marker Analysis

The Leu system of monoclonals (Becton and Dickinson, Sunnyvale, Calif.) used is comprised of Leu-1, which binds to 95% of thymocytes and peripheral blood T cells, Leu 2a, directed against cytotoxic/suppressor T cells, and Leu-3a, which recognizes the helper T cells.26 Other monoclonals used were Leu-4, a T-cell-specific monoclonal, and Leu-6, a thymocyte-specific monoclonal, kindly provided by Dr. R. Evans (Sloan-Kettering Institute). Pan T2, a T-cell-specific monoclonal, and anti-Ia were isolated by one of us (C.Y.W.). The binding of these monoclonal antibodies was studied by indirect immunofluorescence.

Sheep red blood cell (SRBC) rosettes were prepared according to Hoffman and Kunkel.27 Terminal deoxynucleotidyl transferase (TdT, EC2.7.7.31) was determined biochemically and by immunofluorescence,21,24 while the ploidy of the cells established by cytofluorographic analysis,25 kindly performed by Dr. M. Andreeff (Sloan-Kettering Institute).

IL-2 Production, Purification, and Assay

Normal or leukemic PBL were plated in RPMI supplemented with 0.25% bovine serum albumin (BSA, Sigma, Saint Louis, Mo.) and 0.5% phytohemagglutinin (PHA, M form, GIBCO, Grand Island, N.Y.) and incubated at 37°C in 5% CO2. The conditioned medium (CM) was harvested 48 hr later.

IL-2 to be used for culture studies was precipitated from CM with (NH4)2SO4 (557 g/liter) and purified by passage through DEAE cellulose (DE-52, Whatman, England). For biochemical studies, IL-2 was then applied on an isoelectrofocusing column (IEF), high performance liquid chromatography (HPLC, Varian, Palo Alto, Calif.), or AcA 44 Ultragel (LKB, Rockland, Md.). The conditions used during the purification procedures have been described.26

For the IL-2 assay,27 4000 murine CTLL cells were grown in the presence of log dilutions of putative IL-2 containing medium in 96-well microtiter plates (Costar, Cambridge, Mass.). Twenty-four hours later, 0.5 μCi of 3H-TdR (specific activity 20 Ci/m mole, New England Nuclear, Boston, Mass.) were added to each well. After 4 hr, the cells were harvested on glass fiber strips and 3H-TdR incorporation measured in a liquid scintillation counter (Packard, Downers Grove, Ill.). The IL-2 concentration in the experimental sample was then calculated by probit analysis27 using a standard containing 2 U of IL-2. One unit per milliliter of IL-2 was defined as the quantity of IL-2 released in 48-hr culture medium conditioned by rat spleen cells (10⁷/ml) stimulated by Con-A (5 g/ml).27 The standard error for replicate determinations was less than 15%.

RESULTS

Marker Profile of Normal and Leukemic PBL

Ficoll-Hypaque-separated mononuclear cells from normal donors, three ALL patients, and two continuous cell lines, Jurkat and Daudi, were studied for their capacity to form rosettes with sheep red blood cells, for binding to monoclonals against Ia-like and T-cell antigens, for presence of the enzyme terminal deoxynucleotidyl transferase (TdT), and for DNA content. Three of the four leukemias studied had high white cell counts (Table 1).

ALL-4 had the characteristics of a non-T, non-B ALL, since of all markers studied, only TdT and Ia were positive (Table 1). ALL-5 was a T-cell leukemia, TdT-negative, with the majority of cells forming SRBC rosettes and a very low reactivity with the anti-Ia monoclonal. The leukemic clone of this leukemia was hypodiploid, as determined by flow cytometry, expressed the suppressor phenotype according to its high binding of Leu-2a, and presented a deranged expression of differentiation markers, since SRBC receptor, Leu-1, and Pan T2 receptors were coexpressed in normal PBL but not in ALL-5 (Table 1). ALL-8 and ALL-IP showed an intermediate composition.

| Table 1. Marker Profile of ALL Cells, Human Cell Lines, and Normal PBL* |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                            | Cells           | PBL             | ALL-4           | ALL-5           | ALL-8           | ALL-IP          | Jurkat          | Daudi           |
| Surface marker (% positive cells) | | | | | | | | |
| SRBC 4°C                    | 80             | 15              | 78              | 3.5             | 17              | 50              | 0              | 0              |
| Ia                          | 16             | 62              | 4               | 19              | 76              | <ND>            | <ND>            | <ND>            |
| Leu-1                       | 70             | 3               | 13              | 18              | 6               | 61              | 0              | 99              |
| Leu-2a                      | 20             | 0               | 0               | 0               | 0               | 0               | 0              | 0               |
| Leu-3a                      | 60             | 3               | 7               | 36              | 0               | 20              | 0              | 0               |
| Leu-6                       | 0              | 2               | 1               | 48              | 0               | 45              | 0              | 0               |
| Pan T2                      | 70             | 0†              | 6†              | 7               | 9               | 0               | 0              | 0               |
| Ploidy                      | 2.0            | 2.0             | 1.8             | >2.0            | 2.3             | ND              | ND              | ND              |

*For methods see Materials and Methods section.
†The microscopic analysis showed a very weak fluorescence very close to the background level on 10% of ALL-4 and 20% of ALL-5 cells; 6% of ALL-5 cells were clearly positive.

ND, not determined.
Production of IL-2 by Normal and Leukemic Cells

Normal mononuclear PBL were plated in the presence of 0.5% PHA. Culture medium was harvested daily and tested for IL-2 activity in the IL-2 microassay. At the same time points, the rate of proliferation of PBL was determined by \(^3\)H-TdR incorporation.

Figure 1A shows that the addition of PHA to normal PBL induced peak IL-2 production on day 1, which was followed 24 hr later by a rise in cell proliferation. When ALL-4 cells were used (Fig. 2), IL-2 production was again induced by PHA stimulation, but continued to increase over the 4 days tested and was not followed by a rise in cell proliferation. This was in marked contrast to the response of PBL to PHA stimulation (Fig. 1A). This pattern of response was common to ALL with different phenotypic characteristics, since ALL-5, a T-cell leukemia, produced IL-2 (Fig. 3) in the same fashion as ALL-4 and with no increase in cell proliferation (not shown).

The Factor Produced by the Leukemic Population is IL-2

In order to show that the factor produced by the leukemic cell populations was indeed IL-2, we tested if the ALL-4 factor was able to support the growth of the human cytotoxic cell line, C13.3, which requires IL-2 for survival and proliferation. Figure 4 shows that the factor produced by ALL-4 and IL-2 purified from normal PBL supported the growth of C13.3 equally well.

IL-2 from normal PBL has been purified in this laboratory and shown to have molecular heterogeneity depending on the experimental conditions used for factor production. In our experimental conditions, normal IL-2 elutes from DEAE cellulose with 0.03 M salt, has a mol wt of 26,000 as determined by gel filtration and high performance liquid chromatography (HPLC), and an isoelectric point (pI) of 6.7. The IL-2 activity present in ALL-4 conditioned medium elutes with 0.03 M salt from DE 52 and with a pattern identical to that of normal IL-2 (not shown), appears to have a mol wt of 26,000 according to gel filtration

Figure 1. IL-2 production and cell proliferation in PBL after PHA or Pan T2 stimulation. PBL were plated in 96-well microtiter plates at 4 x 10^5/ml in RPMI supplemented with 5% heat-inactivated FCS plus or minus 0.5% PHA (A) or Pan T2 diluted 1:20 (B). Every day thereafter, 100 µl were harvested from each well and 0.5 µCi of \(^3\)H-TdR added to measure DNA synthesis. IL-2 concentration was then determined in the harvested culture medium. Each point is the average of a triplicate determination. IL-2 production (-); PBL proliferation (O-O) in presence of medium alone; IL-2 production (A-A) and PBL proliferation (O-O) in presence of PHA (A) or Pan T2 (B).
Fig. 4. Growth of a human CTLL, C13.3, in IL-2 produced by normal PBL or ALL-4 cells. C13.3 in resting phase was diluted to 1 cell/mL in RPMI plus 15% heat-inactivated human AB serum and plated in presence of 3 U/mL of DE 52 purified normal IL-2 or 5 U/mL ALL-4 IL-2 (Δ-Δ). Each point is the average of a triplicate determination.

Mitogenesis and Induction of IL-2 by Pan T2

Pan T2 is a monoclonal antibody produced against human T cells. This monoclonal specifically binds to T cells and recognizes 70%–90% of peripheral T lymphocytes. The addition of this monoclonal to normal PBL induced a very strong proliferative response that was preceded by release of IL-2 into the culture medium. The kinetics of IL-2 production and proliferative response induced in PBL by Pan T2 is shown in Fig. 1B. The culture supernatant of the Pan-T2-producing hybridoma was mitogenic at the highest dilution tested (1:2000). We tested if Pan T2 was active also on leukemia cell populations. Figure 2 shows that Pan T2

Fig. 5. Gel filtration of ALL-4 IL-2. CM harvested from PHA-stimulated ALL-4 cells was applied to an AcA 44 Ultrogel column equilibrated in phosphate-buffered saline (PBS). The column was then eluted with PBS containing 0.1% polyethylene glycol 6000 and 7.5-ml fractions were collected. The following mol wt markers were used: ovalbumin, 45000, chymotripsinogen, 25000, and ribonuclease A, 13700. Absorption at 280 nm (○-○), IL-2 U/mL (Δ-Δ).
was unable to induce either proliferation or IL-2 production in ALL-4. The monoclonal had only a minimal effect on IL-2 production (Fig. 3) and cell proliferation (not shown) in ALL-5. This pattern of response was markedly different from the effect of PHA on leukemic cells or Pan T2 on normal cells (Figs. 1B and 2).

Jurkat, a cell line derived from an ALL, is TdT+, Ia−, E+, Leu-1+ (Table 1) and, after PHA stimulation, produces IL-2 but does not proliferate11 (Table 2). We studied the effect of Pan T2 on this line and found that this monoclonal antibody does not induce IL-2 production or stimulate cell proliferation (Table 2). Therefore, Jurkat and fresh ALL cells have the same pattern of IL-2 production and proliferation after PHA or Pan T2 stimulation (Table 2 and Fig. 2).

**Rescue of the Response to Pan T2 by Cocultivation of ALL Cells or Jurkat With Daudi**

The B-lymphoblastoid line Daudi increases IL-2 production in PHA-stimulated PBL26,12,31 and, if added to resting PBL, induces factor production (Table 2). We tested if the addition of Daudi was able to rescue the response of ALL cells to Pan T2 and induce IL-2

![Graph showing pH and IL-2 levels](image-url)

**Table 2. IL-2 Production by Jurkat, Daudi, and Cells From 4 Patients With ALL**

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*For culture conditions see Fig. 7. These additions had no effect on the proliferation of Jurkat, Daudi, or ALL cells, but DNA synthesis in 48-hr-old culture of PBL was stimulated 80-fold by PHA, 66-fold by pan T2 plus Daudi, and 11-fold by Daudi alone.*
production. We could not detect any effect of Daudi alone on IL-2 production by any of the ALL cells and Jurkat (Fig. 7 and Table 2). Figure 7 shows that the addition of this B-cell line to ALL-4 restored the capacity of Pan T2 to induce IL-2 production to levels comparable to that obtained by the addition of PHA. A similar response was obtained when cells from the other ALL patients or Jurkat cells were costimulated by the monoclonal and Daudi (Table 2). The addition of Daudi, however, did not rescue the proliferative response of ALL cells or Jurkat to PHA or Pan T2.

**DISCUSSION**

Tumor cells are characterized by the lack of or their unresponsiveness to "brakes" that control the growth of normal cells. Tumor cells, however, frequently are still responsive to differentiation stimuli.1,34

The differentiation destiny of normal precursor cells depends on the interplay of growth and differentiation factors, which are being recognized in low number because adequate test systems are not currently available. One of the systems in which such factors have been better studied is the hematopoietic system.35 Agonist and antagonist factors have been identified, as well as positive and negative feedback mechanisms by which terminally differentiated cells act on the proliferative state of their precursors.

We have studied the capacity of leukemic cells to produce and respond to IL-2, a growth regulator of normal T cells. The leukemic cells studied were either non-T, non-B ALL or T-cell ALL. In every case, after appropriate stimulation, the cells produced a large quantity of a factor that supported the growth of a cloned murine cytotoxic line as well as that of an IL-2-dependent human cytotoxic line. This factor had physicochemical characteristics identical to that of normal IL-2, with a native mol wt of 26,000, elution with 0.03 M salt from DE 52, and reacted with a monoclonal antibody directed against normal IL-2. These data therefore strongly suggest that the factor produced by ALL cells is identical or at least closely related to IL-2.

It is possible that residual normal T cells are responsible for the production of IL-2 in ALL. There are, however, several lines of evidence suggesting that IL-2 is in fact produced by leukemic cells. First, after mitogenic stimulation, normal cells release IL-2 and proliferate, whereas ALL cells release IL-2 but do not proliferate (Fig. 2). IL-2 production by normal cells peaks during the first 24 hr after stimulation and then quickly falls. ALL cells continuously release IL-2 into the culture medium (Fig. 2). The leukemic populations studied (ALL-4, ALL-5) could not have had more than 1%-1.5% normal cells. The leukemic clone present in ALL-5 was hypodiploid as determined by flow cytometry, showing that at least 98%-99% of the ALL-5 cells were hypodiploid. The normal cell responsible for IL-2 production is E+,14 and these cells represented not more than 1%-1.5% in the ALL-4 population, assuming that all the E+ cells present in ALL-4 were normal. Since the amount of IL-2 produced by ALL cells was comparable and frequently higher than that of normal PBL, which have about 80% E+ cells, it is unlikely that the IL-2 produced by the ALL cells was derived from normal diploid E+ cells. If the producer cells were normal, then IL-2 production by a very small population of normal cells (1.5%) would have to be greatly stimulated in ALL.

Pan T2 is a monoclonal antibody that recognizes an antigenic determinant present on the surface of T cells.36 We have shown this monoclonal to be mitogenic for PBL and that it does so by inducing IL-2 production (Fig. 1B). This is a highly specific phenomenon, since after a literature search and after testing several other monoclonal antibodies produced against T cells, we have only found two other antibodies, OKT3 and Leu-4, which are mitogenic37 (Evans R., unpublished) and induce IL-2 production from normal PBL.37 Pan T2 alone does not cause the release of IL-2.
from ALL cells (Figs. 2 and 3, Table 2). However, the Pan T2 activation “pathway” is not constitutively repressed in ALL cells, since it can be activated (Fig. 7) by costimulating ALL cells with Pan T2 and Daudi. Daudi cells do not produce IL-2 under any circumstances (Table 2) and are not required for the Pan T2 stimulation of IL-2 production from normal cells (Fig. 1). Furthermore, Daudi cells are mitogenic and induce IL-2 production by normal PBL, but fail to induce either response in ALL cells. Therefore, the pattern of response of ALL cells to Pan T2 and Daudi documents that the IL-2 producer cell in the ALL population is not normal.

This conclusion is supported by the study on Jurkat, an ALL cell line. The characteristics of IL-2 production in this clonal population of leukemic cells were found to be similar to that of ALL cells: PHA alone induced IL-2 production, while Pan T2 required the presence of Daudi for IL-2 production (Table 2).

The role of IL-2 production in ALL remains to be determined. It appears unlikely that the release of this factor, critical for the proliferation of cells of T lineage, is only an epiphenomenon in ALL, irrelevant for the expansion of the leukemic clone. Recently, a clonal assay system permitting the growth of blast cell progenitors in non-T, non-B ALL has been developed. This assay is similar to those previously described for malignant B lymphocytes and AML blasts. In all these assays, factors present in PHA CM are required for the successful growth of the leukemic stem cells. Also, in other systems, tumor cells have been shown to release a variety of growth factors. Rubin reported in 1970 that Rous sarcoma virus transformed fibroblasts produce a factor that stimulates the growth of normal cells. Todaro and his coworkers have demonstrated the presence of transforming factors in the culture fluid of tumor cells of different histiogenetic origin (reviewed in ref. 39). These factors appear to be related to growth factors produced by normal cells. Recently it has been shown that the promyelocytic cell line HL-60 stimulates its growth by producing a factor that can be isolated from HL-60 conditioned medium. These and our own data clearly establish that the production of growth factors is a characteristic shared by many tumors and therefore should be of significance for the regulation of tumor growth.

The target cell, if any, for IL-2 in ALL remains to be established. One possibility is that IL-2 acts on the producer cell, stimulating its proliferation. This does not appear to be the case for the following reasons. Leukemic stem cells go through self-replication and differentiation. Studies performed in the nude mouse have shown that the T-cell capacity to produce IL-2 is acquired by a subset of T cells after thymus-dependent maturation steps, demonstrating that IL-2 production is a late differentiation marker. We have shown that leukemic cell populations express this post-thymic differentiation marker. IL-2 production, while Izaguirre et al. have previously found that the leukemic blast progenitors require normal PHA CM to proliferate. Since (A) IL-2 is present in PHA CM, (B) IL-2 produced by leukemic and normal T cells appears to be identical (Figs. 4–6), and (C) peripheral blast populations of ALL do not proliferate in response to IL-2 (Fig. 2), as measured by $^3$H-TdR incorporation, we hypothesize that IL-2 is a factor (or one of the factors) produced by partially differentiated leukemic cells and required for the replication of the leukemic stem cells. Since the leukemic stem cell represents only a small percentage of the total leukemic population, its proliferation cannot be shown in the assays used here. The clonal assay should be able to clarify this important point, and appropriate experiments are in progress. Positive feedback mechanisms are not unusual in the hematopoietic system: e.g., colony-stimulating factor (CSF), produced by macrophages stimulates the granulocyte-macrophage precursor cells (GM-CFU).

Since the factor produced appears to be identical in normal and leukemic cells (Figs. 4–6), the leukemic transformation could be linked to the appearance of an IL-2 receptor not present, or having a lower binding capacity, in normal cells and resulting in a proliferative advantage of leukemic cells over normal stem cells. In this context, it is of interest that the virus identified by Gallo and coworkers in cutaneous T-cell lymphoma could induce the expression of IL-2 receptors in the infected cell. Still another possibility is that leukemic stem cells have a specific way to turn on the IL-2 receptor not present, or having a lower binding capacity, in normal cells and resulting in a proliferative advantage of leukemic cells over normal stem cells. This hypothesis would require that in partially differentiated leukemic cells, the transformation induces the expression of a receptor that responds to a stimulun presented by the leukemic stem cells only. We have shown that normal cells have receptors for OKT3 and Pan T2, which induce IL-2 production and cell proliferation. We have further demonstrated that the receptor for Pan T2 is altered in ALL cells (Figs. 2 and 7, Table 2). These data strongly suggest a very specific alteration of the membrane of ALL cells at the level of a receptor whose stimulation induces production of IL-2 (and possibly of other factors).

Ia-like molecules and Fc receptors are present on Daudi cells, (Table 1) and have been shown to regulate IL-2 production. We are investigating the role of these two membrane components on Daudi cells and their capacity to activate IL-2 production from Pan-T2-stimulated ALL cells.
ACKNOWLEDGMENT

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Production and regulation of interleukin-2 in human lymphoblastic leukemias studied with T-cell monoclonal antibodies

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