Prolonged Defects of Interleukin-2 Production, Responsiveness, and Receptor Expression in Patients With Acute Lymphoblastic Leukemia

By Michael S. Borzy and Derry Ridgway

The proliferative responsiveness to, production of, and the expression of cell-surface receptors for interleukin-2 (IL-2) are increased in patients with acute lymphoblastic leukemia. The increased susceptibility to infection is present for the duration of chemotherapy, and may persist for a period after the cessation of all therapy. These long-lasting defects of the IL-2 system are most likely a late effect of chemotherapy and may result in increased complications in some long-term survivors of ALL.

PATIENTS WITH acute lymphoblastic leukemia (ALL) receiving maintenance chemotherapy are at an increased risk to develop serious, life-threatening infections. The increased susceptibility to infection is present for the duration of chemotherapy, and may persist for a period following the cessation of chemotherapy. In addition to infectious complications of chemotherapy, second neoplasms and virus-induced lymphoproliferative disease have been reported in survivors of ALL, suggesting a long-lasting cellular immunosuppression. The exact mechanism(s) that accounts for this prolonged therapy-induced cellular immunosuppression is unknown.

Interleukin-2 (IL-2) is a T lymphocyte-derived growth factor that plays a central role in maintaining normal T-cell immunity by mediating the clonal expansion of mature T cells through its proliferative capacity, as well as effecting the functional maturation of cytotoxic T cells. IL-2 is also responsible for the expansion and functional maturation of natural killer cells and lymphokine-activated killer cells. Decreased production of and proliferative responsiveness to IL-2 has been documented in a number of congenital immunodeficiency diseases as well as the acquired immunodeficiency syndrome. Similarly, a prolonged deficiency in the production of, and the response to, IL-2 has been reported in recipients of allogeneic bone marrow transplantation and likely is an important factor in the posttransplant immunodeficiency seen in these patients. Likewise, defective IL-2 production and responsiveness has been observed in nonmalignant T cells from patients with chronic lymphocytic leukemia of B cell origin.

However, little is known concerning the status of the IL-2 system in children with ALL. Previous studies, which focused on adult patients with a variety of treated and untreated solid tumors, have produced conflicting results, with decreased IL-2 production reported in some studies but not others. Therefore, this study was undertaken to examine the IL-2 system in children with ALL during and after maintenance chemotherapy in an attempt to characterize the magnitude and duration of their cellular immunodeficiency. Significant and long-lasting defects in the production of, proliferative responsiveness to, and the expression of cell-surface receptors for IL-2 were found both in patients receiving maintenance chemotherapy and in patients off all therapy for an average of 3 years.

MATERIALS AND METHODS

Subjects. Fourteen children with ALL, 3 to 18 years of age (mean, 10.2 years), were studied while in continuous first remission and receiving maintenance chemotherapy. The patients had been in complete remission for 6 to 35 months (mean, 19.4 months) at the time of the study. All patients were receiving daily oral 6-mercaptopurine, weekly oral methotrexate, and monthly pulses of intravenous vincristine and oral prednisone. Blood was drawn on the day of vincristine therapy, before treatment. Initial induction and consolidation treatment for five of these patients included five drugs (vincristine, prednisone, L-asparaginase, 6-mercaptopurine, and intrathecal methotrexate for 2 months). The Children's Cancer Study Group modification of the Berlin-Frankfurt-Münster (BFM) protocol was used for nine patients.

Nineteen patients with ALL, 6 to 20 years of age (mean, 12.5 years), were studied in continuous first remission after elective discontinuation of chemotherapy. The patients had been on all chemotherapy for 2 to 138 months (mean, 40.3 months). Of these patients 12 had been treated with five-drug initial therapy and 24 to

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36 months of maintenance therapy as described above; four patients had received the Children's Cancer Study Group modification of the BFM protocol, and three patients received five-drug initial treatment and the maintenance therapy described above plus intravenous cytoxan, intravenous cytosine arabinoside, and intravenous adriamycin for 34 to 36 months (cyclic intensive maintenance).

Studies were performed on 15 normal, control children 5 to 15 years of age (mean, 9.9 years).

This study was conducted over a 14-month period. The study protocol was reviewed and approved by the Human Subjects Committee of the Oregon Health Sciences University. Written, informed consent was obtained from all study participants, or their parent, if a minor.

Separation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from fresh, heparinized (10 U/mL), venous blood by Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation.

PBMC were used for analysis of T lymphocytes and T-lymphocyte subsets; proliferative response to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM); production of IL-2; and quantitation of cell-surface IL-2 receptor (IL-2R) expression.

Analysis of T-lymphocyte phenotypes. T-lymphocyte phenotypes were determined by direct immunofluorescence staining of aliquots of PBMC (4 x 10^6 cells/mL) in phosphate-buffered saline with appropriate dilutions of fluorescein-isothiocyanate (FITC) conjugates of OKT3 (CD3), OKT4 (CD4), and OKT8 (CD8) monoclonal antibodies (Ortho Diagnostic Systems, Raritan, NJ). Lymphocyte analysis was performed by counting 10,000 cells from each sample with an Ortho 30L cytofluorograph. The proportion of lymphocytes stained by each monoclonal antibody was converted to absolute number per µL by multiplying by the absolute number of lymphocytes per µL derived from the complete blood count.

T-lymphocyte purification. Purified T lymphocytes were used to assay proliferative responsiveness to IL-2 and were isolated by using PBMC through scrubbed nylon columns (type 200 nylon fiber; Fenwall Laboratories, Deerfield, IL). Recovery of lymphocytes eluted from the columns ranged from 60% to 75% and contained >90% T lymphocytes as determined by direct immunofluorescence staining with FITC-conjugated OKT3 monoclonal antibody. Cell recovery was comparable in samples from control subjects and patients on or off chemotherapy.

Mitogen-induced proliferation. PBMC (1 x 10^6 cells/culture) were cultured in triplicate with PHA (4 µg/mL; HA16; Burroughs Wellcome, Oxnard, CA) and Con A (20 µg/mL; Calbiochem, La Jolla, CA), and PWM (40 µg/mL; GIBCO Laboratories, Grand Island, NY) for 88 hours using standard microculture technique.

Proliferative responsiveness to exogenous IL-2. The proliferative response of purified T lymphocytes to exogenous IL-2 was measured using the method of Larsson as modified by Borzy.

In brief, purified T lymphocytes were suspended in RPMI-1640 (GIBCO) supplemented with 2 mmol/L-glutamine (GIBCO), 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO), hereafter termed as culture medium (CM), with 10 µg/mL Con A added and incubated for four hours at 37°C to initiate activation. After washing with CM containing methyl-a-D-mannopyranoside (20 mg/mL), the Con A-activated cells (2 x 10^6 cells/culture) were suspended in CM supplemented with 10% heat-inactivated pooled, human AB serum (Medical Specialities Laboratory, Boston) and cultured in the absence and in the presence of a saturating amount of exogenous human recombinant IL-2 (rIL-2) (400 U/mL; 210 mmol/L; Genzyme, Boston) for 88 hours. The concentration of rIL-2 used was determined in preliminary experiments to produce proliferation on the plateau of the dose-response curve. Cellular proliferation was estimated by the incorporation of tritiated thymidine (³H-TdR).

Results are expressed as net counts per minute (cpm) defined as mean cpm of triplicate cultures with IL-2 minus mean cpm of cultures without IL-2. Cultures of Con A-activated cells without IL-2 always produced cpm that were <10% of cpm of IL-2-supplemented cultures. IL-2 added to cells not previously exposed to Con A resulted in minimal proliferation (<15% of the cpm observed in Con A-activated, IL-2-supplemented cultures).

IL-2 production. PBMC (4 x 10^6) were suspended in CM supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO) and PHA (4 µg/mL) and were cultured in 1-mL aliquots in 17 x 100 mm round-bottom snap-top plastic culture tubes (Becton Dickinson, Oxnard, CA) for 48 hours at 37°C in a humid environment of 5% CO2 in air. After incubation, cells were centrifuged and supernatants were collected and stored at -70°C until assayed for IL-2 activity. Cells cultured without PHA produced no detectable IL-2.

IL-2 assay. IL-2 activity was assayed in culture supernatants by their ability to support proliferation of IL-2 responsive, six-day-old, Con A-stimulated, normal human T lymphoblasts as previously described.

In brief, 1 x 10^6 T lymphoblasts were suspended in CM with 10% heat-inactivated FBS and cultured with serial twofold dilutions of test supernatants for 18 hours, pulsed with ³H-TdR for an additional six hours, and then harvested. These T lymphoblasts were IL-2 dependent since in the absence of IL-2 they did not proliferate and die. In addition, their proliferative responsiveness to standard IL-2-containing supernatants or rIL-2 could be inhibited by the addition of a polyvalent rabbit anti-human IL-2 antibody (Genzyme). Carryover of PHA activity in IL-2-containing supernatants was insignificant, since absorption of supernatants with chicken erythrocytes, which bind PHA, did not diminish the proliferative capacity of the supernatants. The ability of chicken erythrocytes to remove PHA was demonstrated by the fact that CM supplemented with PHA and then absorbed with chicken erythrocytes did not support the proliferation of Con A-stimulated lymphoblasts. Results are expressed as units (U)/mL of IL-2 activity calculated by probit analysis of mean net cpm from cultures containing test supernatants at the 50% of maximal ³H-TdR incorporation endpoint of the laboratory standard IL-2-containing supernatant.

The laboratory standard supernatant was standardized against a human IL-2 reference reagent containing 500 reference U/mL of activity produced by the leukemia T-lymphocyte line Jurkat (Biological Response Modifiers Program, National Cancer Institute, National Institutes of Health [NIH]), and IL-2 activity in test supernatants is expressed in NIH reference units. The laboratory standard was determined to contain 20 NIH U/mL of IL-2 activity. For purposes of comparison, under these assay conditions, 50% of maximal ³H-TdR incorporation by six-day-old, Con A-stimulated T lymphoblasts represents 0.22 NIH U/mL.

IL-2R assay. PBMC (4 x 10^6) were cultured with PHA (4 µg/mL) for 48 hours at 37°C in a humid environment of 5% CO2 in air. Cell-surface IL-2R expression on cultured cells was determined by direct immunofluorescence staining with FITC-conjugated anti-human IL-2R (anti-Tac) monoclonal antibody (Becton-Dickinson). Staining with FITC-conjugated murine control monoclonal antibody of similar isotype was also performed on cells from each culture. Between 100 and 200 cells from each assay were examined by incident-light fluorescence microscopy, and results are expressed as net percent positive cells.

Non-specific staining with the control antibody was always <3%. Less than 1% of cells not cultured with PHA had detectable IL-2R.

Statistical analysis. Comparison between on therapy, off therapy, and control groups were performed using the nonparametric Mann-Whitney U test with two-tailed P values of <0.05 considered significant. Correlations within the on therapy and off therapy groups were performed using the Spearman rank correlation test with two-tailed P values of <0.05 considered significant.
RESULTS

Lymphocyte phenotypes. The mean absolute number of peripheral blood lymphocytes as well as the mean absolute number of T cells and T-cell subsets (CD3+, CD4+, and CD8+ T lymphocytes) from children on maintenance chemotherapy were all significantly lower than control subjects (Table 1); however, the percentages of CD3+, CD4+, and CD8+ lymphocytes in peripheral blood and the ratio of CD4+/CD8+ were all similar in the on therapy and control groups showing that chemotherapy decreased the T lymphocyte subset number in a uniform manner thus preserving their relative relationships. Interestingly, although the group of children off all chemotherapy had significantly greater (P < .001) mean absolute numbers of lymphocytes, T cells, and T-cell subsets as compared with the on therapy group, these absolute values for the off therapy group were still significantly below those of control subjects (Table 1). Similar to the on therapy group, the proportions of T cells and T-cell subsets in the off therapy group were not different from control subjects.

Analysis of lymphocyte phenotypes in PBMC and T-cell enriched populations was also performed. The proportion of CD3+, CD4+, and CD8+ cells and the ratio of CD4+/CD8+ cells in separated PBMC populations from patients on and off therapy and control subjects were similar. However, the proportion of CD3+ and CD4+ cells in the T-cell enriched populations from the on therapy group were significantly (P < .01) lower than both the off therapy group and control subjects, but there was no difference in the proportions of T cells or T-cell subsets between the off therapy group and control subjects (data not shown).

Proliferative responses to mitogens. As standard indicators of lymphocyte proliferative capacity, the responses to optimum concentrations of PHA, Con A, and PWM from patients on and off therapy and control subjects were determined as shown in Table 2. The geometric mean response to PHA by the on therapy group was somewhat lower than the control value while the mean responses to Con A and PWM were slightly higher; none of these differences, however, was statistically significant. For the off therapy group, mean responses were also slightly lower for all three mitogens as compared with control subjects, but again these differences were not statistically significant. There was no correlation between the absolute number of lymphocytes, T cells, or T-cell subsets, and mitogen responses in either the on or off therapy groups.

Proliferative responsiveness to rIL-2. The proliferative responsiveness of Con A-activated, purified T lymphocytes from patients on therapy to a saturating amount (400 U/mL; 210 nmol/L) of rIL-2 was decreased compared with control subjects (P < .005) as shown in Fig 1. The IL-2–induced proliferative responsiveness of purified T cells from patients off therapy was similar to that of patients on therapy and was also decreased as compared with control subjects with the difference being statistically significant (P < .05) (Fig 1). There was no correlation between the absolute number of lymphocytes, T cells, or T-cell subsets, and IL-2 responsiveness in either the on or off therapy groups.

IL-2 production. PHA-induced production of IL-2 by PBMC from patients on therapy was decreased as compared with control subjects (P < .05) (Fig 2). Similarly, IL-2 production by PBMC from patients off therapy was also decreased and was significantly less than control subjects (P < .002) as shown in Fig 2. Seven patients in the on therapy group and ten patients in the off therapy group produced <2 U/mL of IL-2 activity. Three patients, two in the on therapy group and one in the off therapy group, had high IL-2 production values (>20 U/mL). Interestingly, two of these three patients, including the patient with the highest IL-2 production value of 63.8 U/mL, subsequently had a relapse of their ALL. No spontaneous IL-2 activity was detected in non-PHA-stimulated cultures of patient or control PBMC. There was no correlation between the level of IL-2 activity produced by patients on or off therapy and the absolute number of T cells or T-cell subsets, mitogen responses, or responsiveness to IL-2.

IL-2R expression. As shown in Fig 3 the PHA-induced expression of cell surface IL-2R on PBMC from patients on, as well as off, therapy was significantly decreased as compared with control subjects (P < .002 for on therapy group v control group; P < .01 for off therapy group v control group). The patients off therapy had values slightly, but not significantly, greater than the patients on therapy. Less than 3% of fresh PBMC expressed IL-2R in all three groups. After 48 hours of culture, non-PHA stimulated IL-2R expression for all three groups was <6% positive cells. IL-2R expression did not correlate with the absolute number of T cells or T-cell subsets, mitogen responses, responsiveness to IL-2, or IL-2 production within either the on therapy or off therapy groups.

Effect of time on or off chemotherapy. The length of time in months that patients in the on therapy group had received chemotherapy was not statistically correlated with the absolute number of T cells or T-cell subsets, mitogen responses, IL-2 responsiveness, IL-2 production, or IL-2R expression. There was also no correlation in the off therapy groups.

Table 1. Lymphocyte Phenotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean ± SD Range</th>
<th>Mean ± SD Range</th>
<th>Mean ± SD Range</th>
<th>Mean ± SD Range</th>
<th>Mean ± SD Range</th>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>Cells/μL</td>
<td>Cells/μL</td>
<td>Cells/μL</td>
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</tr>
<tr>
<td>CD3+ Cells</td>
<td>800* ± 280-2,668</td>
<td>78 ± 12</td>
<td>626* ± 152-1,734</td>
<td>44 ± 12</td>
<td>325* ± 94-406</td>
</tr>
<tr>
<td>CD4+ Cells</td>
<td>2,366 ± 1,710-3,560</td>
<td>71 ± 11</td>
<td>1,673* ± 957-2,722</td>
<td>40 ± 10</td>
<td>938* ± 479-1,680</td>
</tr>
<tr>
<td>CD8+ Cells</td>
<td>3,044 ± 2,108-4,218</td>
<td>77 ± 12</td>
<td>2,335 ± 1,491-3,422</td>
<td>48 ± 10</td>
<td>1,449 ± 877-2,320</td>
</tr>
</tbody>
</table>

*p < .001 as compared with control groups.
†p < .05 as compared with control groups.
Table 2. Lymphocyte Proliferative Responses to Mitogens

<table>
<thead>
<tr>
<th>Group</th>
<th>PHA</th>
<th>Con A</th>
<th>PWM</th>
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<tbody>
<tr>
<td>On therapy (N = 14)</td>
<td>154,300</td>
<td>42,600</td>
<td>10,400</td>
</tr>
<tr>
<td>Off therapy (N = 19)</td>
<td>100,500-236,600</td>
<td>25,200-71,800</td>
<td>5,300-20,700</td>
</tr>
<tr>
<td>Control subjects (N = 15)</td>
<td>144,000</td>
<td>26,200</td>
<td>5,100</td>
</tr>
<tr>
<td></td>
<td>119,800-173,100</td>
<td>17,700-36,800</td>
<td>3,400-7,500</td>
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<tr>
<td></td>
<td>163,400</td>
<td>33,900</td>
<td>9,000</td>
</tr>
<tr>
<td></td>
<td>126,000-212,100</td>
<td>20,300-56,500</td>
<td>3,800-21,300</td>
</tr>
</tbody>
</table>

NOTE: Data are cpm (geometric mean cpm; 95% confidence interval).

...group between the number of months off all chemotherapy, which ranged from 2 to 138 months, and any of these studied parameters.

Effect of intensity of chemotherapy. The possibility that the intensity of chemotherapy might affect the degree of disruption of the immunologic system was analyzed. For the on therapy and off therapy groups the values for each immunologic parameter were compared for patients treated with standard therapy vs. patients treated with more intensive therapy (the BFM protocol or cyclic intensified maintenance therapy). No significant differences were observed.

DISCUSSION

This study demonstrates that patients with ALL in complete remission receiving standard maintenance chemotherapy for between 6 and 35 months have significant defects in the production of, proliferative responsiveness to, and expression of cell-surface receptors for IL-2. Similar defects were also observed in patients with ALL in complete remission and off all maintenance chemotherapy from 2 to 138 months, demonstrating that these defects of IL-2 physiology were very long lasting. These prolonged abnormalities in the IL-2 system are most likely lingering effects of systemic chemotherapy that impacts both IL-2 producer cells as well as IL-2 responder cells, although it is possible that these abnormalities are a primary defect, associated with the predisposition...
to leukemia, or to the leukemia process itself. Against a primary role for the predisposition to leukemia or the leukemic process itself in explaining these IL-2 defects is the observation that in more than half of patients with ALL at the time of diagnosis, and before any therapy, IL-2 production is markedly elevated. In addition, decreased IL-2 production has been reported in adult patients with nonlymphoid cancers receiving chemotherapy. Similar abnormalities of IL-2 production, response, and receptor expression have also been observed in children with nonlymphoid tumors receiving chemotherapy studied in our laboratory (data not shown). These observations suggest that these IL-2 defects are not due to leukemia, but more likely are generalizable defects caused by chemotherapy. Defects of IL-2 physiology present before the leukemic process cannot be excluded.

Two of the maintenance chemotherapeutic agents received by the patients in this study (prednisone and 6-mercaptopurine) have been shown to disrupt the IL-2 system. Substantial inhibitory effects on IL-2 production in vitro as well as inhibition of IL-2 mRNA synthesis and cell surface IL-2R expression. In addition to having detrimental functional effects on lymphocyte IL-2 production and IL-2R expression, these chemotherapeutic agents decreased the absolute number of lymphocytes, T cells, and T cell subsets thereby also decreasing the frequency of IL-2 producer and responder cells. The decreased IL-2 proliferative responsiveness observed in these patients is likely due to a diminished number of responder cells as suggested by the decreased number of cells expressing IL-2R after mitogen stimulation. Likewise, the decreased IL-2 production is most likely due to a decreased frequency of IL-2 producer cells present in these patients, although a qualitative defect in IL-2 producer cell function is also possible. Although not looked for directly, it is unlikely that suppressor cells are responsible for the observed IL-2 defects in these patients since the proportion of regulatory cells was unchanged by chemotherapy. In addition there was no evidence for cells that could suppress IL-2 response in other IL-2 deficient states.

Surprisingly, there was no significant improvement in these abnormalities of the IL-2 system even when measured in patients off all chemotherapy for up to 138 months, suggesting that these IL-2 defects are indeed very longlasting, or perhaps permanent. Further study of these off therapy patients is needed to determine when, or if, recovery of these IL-2 functions occurs. Increases in the absolute number of lymphocytes and T cells were observed within a few months following the cessation of chemotherapy; however, these values remained significantly less than normal. Moreover, there was no significant difference in the magnitude of the IL-2 defects observed in patients receiving different chemotherapy protocols, despite the fact that these protocols differed in the number and types of chemotherapeutic agents used, suggesting that, as far as IL-2 producer and responder lymphocyte function is concerned, standard treatment regimens were as damaging as the more intensive protocols.

Half of the patients in the on therapy group and more than half of the patients in the off therapy group produced very low (<2U/mL) levels of IL-2 activity, demonstrating a profound and persistent defect in the ability of these patients' lymphocytes to produce IL-2. Only three patients produced high (>20 U/mL) levels of IL-2 activity and, of interest, two of these three patients, including the one individual with the highest IL-2 production value of 63.8 U/mL, subsequently had a relapse of their ALL, compared to two relapses among the remaining 30 patients with normal or low IL-2 production levels. It is possible that the increased IL-2 production observed in these two relapsed patients may represent either an immunologic response to the acute leukemia or a leukemia-induced disruption of IL-2 regulation, as suggested by recent reports of elevated serum IL-2R levels and elevated IL-2 production by lymphocytes from children with newly diagnosed ALL.

The proliferative response to mitogens (PHA, Con A, and PWM) by PBMC from patients on or off therapy were similar to control subjects, a result in agreement with previous studies showing that the response to PHA was normal in patients with ALL receiving maintenance chemotherapy, and suggesting that mitogen-induced lymphocyte proliferation is an incomplete index of immunocompetence in treated cancer patients. In contrast, the assays of IL-2 production and responsiveness appear to be much more sensitive in detecting the adverse effects of chemotherapy on lymphocyte function, not only during treatment, but for a prolonged period after the cessation of therapy.

The biologic significance of these findings relates to the potential adverse consequences of the profound IL-2 defects
on T-lymphocyte function. Prolonged abnormalities of the IL-2 system result in a functional T-cell immunodeficiency that may play a role in the increased susceptibility to viral and parasitic infectious diseases as well as in the increased risk of second malignancies in this immunosuppressed patient population. The possibility that those patients with the lowest levels of IL-2 production and response are at the greatest risk for these late complications needs to be investigated by active surveillance and close follow-up of such patients.

Finally, that a profound and long-lasting defect in IL-2 production is present in the majority of long-term survivors of ALL suggests a potential immunoenhancing role for rIL-2 in these patients since most of the patients in both groups could respond, albeit subnormally, to rIL-2. The judicious use of exogenous IL-2 in patients with known defects in IL-2 production who develop serious infections, such as disseminated disease with varicella-zoster virus, or second cancers may be warranted.

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Prolonged defects of interleukin-2 production, responsiveness, and receptor expression in patients with acute lymphoblastic leukemia

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