Impact of in Vivo Administration of Interleukin 3 on Proliferation, Differentiation, and Chemosensitivity of Acute Myeloid Leukemia

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ABSTRACT

Early clinical trials of growth factor augmentation of induction chemotherapy for acute myeloid leukemia have yielded variable results. To test the hypothesis that this heterogeneity is a consequence of the pleiotropic effects of growth factors on leukemic cell biology, we measured the effects of in vivo interleukin 3 (IL-3) administration on leukemic cell proliferation and drug sensitivity. Thirty-four patients with acute myeloid leukemia with high-risk features or advanced myelodysplasia received IL-3 as a continuous infusion beginning 3 days prior to chemotherapy and continuing for the duration of intensive induction therapy. Bone marrow cells were studied prior to and after 3 days of IL-3 administration to assess changes in overall and leukemic progenitor cell [leukemia colony-forming unit (CFU-L)] proliferation, and progenitor cell sensitivity to 1-β-D-arabinofuranosylcytosine. The median fold increase in overall leukemic cell proliferation in response to IL-3, assessed as expression of the nuclear antigen Ki67 in 28 patients, was 1.2. The median fold increase in percentage of cells in S phase (assessed in 29 patients) was 1.3. Despite the increase in overall cell proliferation in 70% of cases, CFU-L number increased in only 4 of 20 patients successfully studied (median day 4:day 1 ratio of CFU-L number, 0.6). While this suggests possible terminal differentiation of leukemic progenitor cells, expression of CD34, HLA-DR, c-kit, CD15, and CD14 were not consistently affected by the cytokine. 1-β-D-Arabinofuranosylcytosine sensitivity of CFU-L increased significantly in 30% of cases, decreased in 30%, and was unchanged in 40%. Changes in overall cell proliferation (Ki67 expression) and CFU-L were independent predictors of change in 1-β-D-arabinofuranosylcytosine sensitivity; increase in percentage of cells in S phase in response to IL-3 was correlated with attainment of complete remission. While these findings support the concept of cell cycle recruitment, IL-3 has marked pleiotropic effects on proliferation, differentiation, and survival of leukemic progenitors which make the clinical impact of in vivo cytokine administration for individual patients difficult to predict.

INTRODUCTION

Because of the critical dependence of antileukemic chemotherapy on cycle-active cytotoxic drugs, recruitment of Go leukemic cells into active phases of the cell cycle may enhance the cure rate in AML. In vitro models have shown enhanced killing of leukemia cells by ara-C in the presence of myeloid growth factors (1, 2), increased ara-C uptake and 1-β-D-arabinofuranosylcytosine 5'-triphosphate retention following exposure to GM-CSF (3), and increased selectivity of ara-C uptake in leukemic cells when compared with normal hematopoietic cells (4, 5). Several Phase I and Phase II clinical trials of GM-CSF augmentation of AML chemotherapy have demonstrated the clinical feasibility of such an approach (6–9). Preliminary results of one randomized trial have suggested an improvement in disease-free survival with GM-CSF (10), and a randomized study of G-CSF augmentation of an unconventional induction regimen (fludarabine plus ara-C) showed improved survival in the G-CSF arm, although the effects of G-CSF were not confirmed in multivariate analysis (11). Conversely, data from the M. D. Anderson Cancer Center (using historical controls) have raised concerns about possible increased drug resistance in patients receiving GM-CSF to augment induction chemotherapy (8).

In 1992 we initiated a Phase I trial of IL-3 for cell cycle recruitment in high risk and relapsed cases of AML and advanced MDS undergoing intensive ara-C-based remission induction chemotherapy. Since IL-3 may affect growth and differentiation of somewhat more primitive normal hematopoietic cells than does GM-CSF, IL-3 may impact on the biology and chemosensitivity of a broader phenotypic spectrum of AML than does the latter cytokine. In fact, IL-3 stimulates the in vitro proliferation of more cases of AML than does GM-CSF (12), with a greater mean increase in proliferation index (13). In the current trial, IL-3 was begun 3 days prior to chemotherapy and continued for the duration of chemotherapy. The effects of IL-3 infusion on the proliferation, differentiation, and chemosensitivity of leukemic blasts and clonogenic precursors were studied.
**Table 1** Patient characteristics and impact of IL-3 on leukemic cell biology

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<th>Patient Age</th>
<th>Sex</th>
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<th>Entry criteria</th>
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<th>Karyotype</th>
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<th>S-phase fold increase</th>
<th>CFU-L fold increase</th>
<th>ara-C sensitivity</th>
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<tr>
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<td>No growth</td>
<td>CR</td>
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<tr>
<td>34 74 F M5</td>
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<td>7</td>
<td>ND</td>
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<td>1.1</td>
<td>No growth</td>
<td>No growth</td>
<td>NR</td>
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</table>

*a* Fold increase, day 4 value: day 1 value.
*b* Other complex cytogenetic abnormalities present.
*c* ND, not done; IL-3 D/C, IL-3 discontinued before study was completed; NE, not evaluable; NR, no response; Tox, treatment-related death; PRT, primary refractory; Rel, relapse.
*d* Difference between day 4 and day 1 value not statistically significant.

in bone marrow samples obtained prior to beginning the cytokine infusion and again prior to the initiation of chemotherapy. These studies were undertaken to test the hypothesis that the variability of results of clinical trials of growth factor augmentation are due to the pleiotropic effects of growth factors on leukemic cell biology and to the biological heterogeneity of AML.

**MATERIALS AND METHODS**

**Patients.** All adult patients with MDS and AML referred to The Johns Hopkins Oncology Center were assessed for eligibility in this trial beginning in August 1992. Eligible patients had AML in first relapse, AML which failed to go into remission after one course of infusional ara-C-based chemotherapy (primary refractory disease), refractory anemia with excess blasts, refractory anemia with excess blasts in transformation, or newly diagnosed AML with one of the following high-risk criteria: history of AHD, FAB subtype M6 or M7, secondary AML, or age ≥55 years. Patients were excluded for any of the following criteria: Karnofsky performance status <60%; total WBC >100,000/μL, serum creatinine ≥2.0 mg/dL; total bilirubin ≥2.5 mg/dL; aspartate aminotransferase, alanine aminotransferase, or alkaline phosphatase >5 times normal range; chemotherapy or growth factor administration within 4 weeks prior to study; ongoing disseminated intravascular coagulation; clinically evident leukostasis; and positive serum Β-human chorionic gonadotropin. All patients gave written informed consent as approved by the Institutional Review Board under Department of Health and Human Services guidelines.

Clinical characteristics of the 34 treated patients are listed in Table 1. The median age was 55 years, and 20 patients had karyotypic abnormalities associated with poor prognosis (14). While several patients had more than one high-risk feature making them eligible for the protocol, the primary (**i.e.,** most
potentially clinically significant) high-risk features were as follows: relapse (n = 7), primary refractory leukemia (n = 3), FAB M6 or M7 (n = 4), history of AHD (n = 8), advanced MDS (n = 5), and age (n = 7).

**Treatment.** Patients were treated with IL-3 at one of the following doses (μg/kg/day): 2.5, 5.0, 10.0, 20.0 (Table 1). IL-3 was produced by Sandoz (East Hanover, NJ) and supplied through the Cancer Therapy Evaluation Program of the National Cancer Institute. IL-3 was given as an i.v. continuous infusion from days 1 to 13. Chemotherapy was given during the IL-3 infusion as follows: ara-C, 0.667 g/m²/day on days 4–6 as an i.v. continuous infusion; daunorubicin, 45 mg/m²/day on days 4–6 as an i.v. injection; etoposide, 400 mg/m²/day on days 11–13 as a 4–6-h i.v. infusion. If the initial WBC count was >50,000/μl, chemotherapy was begun concurrently with the IL-3 infusion on day 1. If the WBC count rose to >75,000/μl before day 4, chemotherapy was begun early. Bone marrow aspirates were obtained before beginning the IL-3 infusion (day 1) and during the IL-3 infusion before beginning chemotherapy (day 4). If chemotherapy was initiated early, a second bone marrow was obtained at that time when possible. Supportive care was provided as previously described (15). Clinical details and outcome of the trial will be presented in a subsequent publication.

**Cells.** Mononuclear cells from heparinized samples of bone marrow aspirates were obtained by density centrifugation (specific gravity, <1.077 g/dl, Ficoll-Hypaque; Pharmacia, NJ) and used on the same day for all assays. Cytocentrifuge preparations of the mononuclear cells were stained with Wright’s stain and differential blood counts performed manually. The myeloid leukemia cell line KG1a was maintained in RPMI 1640 medium (Sigma, St. Louis, MO) and maintained at 37°C under 5% CO₂ as a control cell line for proliferation assays.

**Proliferation Assays.** The Ki67 antigen, a nuclear protein of unknown function, was originally described in phytohemagglutinin-stimulated lymphocytes, and was shown to be expressed as cells entered G₁ from G₀ with continued expression throughout the cell cycle (16, 17). Ki67 expression was measured in cells by a flow cytometric assay as described previously (18, 19). To exclude contaminating T cells from the analysis, cells were first stained with PE-labeled CD5 antibody (leul-PE; Becton Dickinson, Mountain View, CA) or PE-labeled isotype-matched control antibodies (Becton Dickinson) before fixation and permeabilization (18, 19). Anti-Ki67 and control mouse monoclonal IgG1 antibodies were purchased from Dakopatts. PE-labeled CD14 (TUK4), and isotype-matched fluorochrome-labeled irrelevant mAbs or control cells pulsed with medium instead of BrdUrd served as negative controls. Identically treated AML samples pulsed with medium instead of BrdUrd served as negative controls, and KG1a cells pulsed with BrdUrd were positive controls for the BrdUrd assay. Acquisition and analysis of the BrdUrd/PI-stained samples was performed using Cell-FIT software (Becton Dickinson). Analysis was performed using an electronic gate based on PI fluorescence pulse area and pulse width measurements to exclude cell doublets and aggregates according to the manufacturer’s instructions. Identical cells pulsed with medium instead of BrdUrd were used to determine the cutoff for BrdUrd expression, and all cells with significant BrdUrd signal were considered to be in S phase.

**Clonogenic Assay and ara-C Sensitivity.** CFU-L were quantified in methyl cellulose using a modification of McCulloch’s technique as described previously (21). Day 7 phytohemagglutinin-stimulated lymphocyte-conditioned medium was used as a source of growth factors, and CFU-L were quantified on day 5. CFU-L numbers were tested for significant impact of IL-3 infusion using two-tailed Student’s t test of the means of colony counts from triplicate or quadruplicate cultures plated on day 4 compared to CFU-L numbers on day 1 of cytokine infusion (P < 0.05). To determine the sensitivity of CFU-L to ara-C, the drug was incorporated into the methyl cellulose plates at a doses ranging from 0.5 to 5 μM. Each dose was studied in triplicate or quadruplicate. Semi-log dose-response curves of the percentage of surviving CFU-L versus ara-C dose were plotted and ara-C sensitivity was described as the slope of the regression line of the dose-response curve using least-squares analysis of the log-transformed data (21–23). Significant modulation of ara-C sensitivity by IL-3 was determined by comparing the 95% confidence intervals of the slopes on day 1 and day 4; samples were considered different if there was no overlap of the 95% confidence intervals.

**Immunophenotyping and Detection of Growth Factor Receptor Expression.** Surface antigens were detected following direct or indirect immunofluorescence staining and fixation in 1% formaldehyde as described previously (24). c-kit and IL-3 receptor expression were measured following binding of biotinylated (Steel Factor) or PE-labeled (IL-3) growth factor (R and D Systems, Minneapolis, MN) as described (13). Streptavidin-PE was purchased from R and D Systems. Control cells were incubated with streptavidin-PE only. Immunofluorescence was measured on a FACScan flow cytometer using FACScan Research Software; 10,000 events were acquired in list mode for each sample. Percentage of positive cells was determined using isotype-matched fluorochrome-labeled irrelevant mAbs or streptavidin-PE (for biotinylated samples) as controls. Cases were considered positive for a particular cell surface antigen if ≥20% of cells demonstrated significant specific fluorescence; growth factor receptors were considered positive if ≥10% of cells expressed significant fluorescence.

**Additional Antibodies.** FITC-labeled anti-HLA-DR (CR3/43), CD15 (C3D-1), PE-labeled CD14 (TUK4), and isotype-matched control antibodies were from Dakopatts. PE-la-
beled CD34 antibody (HPCA-2) was purchased from Becton Dickinson.

**Clinical Response Assessment.** Response was assessed by standard criteria (25). CR required normalization of blood counts with <5% blasts in the bone marrow and none in the peripheral blood. PR was scored if the bone marrow was cleared of blasts but evidence of myelodysplasia remained, either because of peripheral blood counts which stabilized in a nontransfusion-dependent range but did not meet the criteria for CR, or because of ongoing dysmorphic features in the bone marrow. In addition, PR was reported if previous bone marrow fibrosis persisted, despite the achievement of normal blood counts and differential WBC count.

**Statistical Methods.** The major statistical outcomes of this study were changes between day 1 and day 4 measurements of hematological variables, proliferative parameters, CFU-L number, and ara-C sensitivity. Changes in outcomes were calculated in two ways: as a ratio of day 4 value divided by day 1 value and as a logarithm of the ratio (i.e., difference on a log scale). Because of skewness in the distributions, we emphasize results from calculating the differences on a log scale. A two-tailed paired Student’s t test of the log-transformed values was used to test differences between means; means are reported ± SE. Differences between medians were tested using the Wilcoxon signed rank test (paired samples) or the Wilcoxon rank sum test (unpaired samples). Pairwise associations between outcome variables were summarized using correlation coefficients and multivariate associations were modeled using linear regression. Variables which were found to correlate well in pairwise analyses (r > 0.4) were entered into multiple regression models. Nonsignificant predictors were then removed from the model one at a time with reestimation of regression slopes and significance levels at each step (step-down procedure).

**RESULTS**

**Effect of IL-3 on Leukemic Cell Proliferation.** Bone marrow samples before and after IL-3 treatment were obtained from 29 of 34 patients. Day 4 marrows were not obtained from four patients for the following reasons: discontinuation of IL-3 (n = 2), urgent chemotherapy initiated early because of a rapidly rising WBC count (n = 1), and fibrotic bone marrow with insufficient cells obtained on day 1 for adequate study (n = 1). No patient had chemotherapy started simultaneously with IL-3. Ki67 expression was highly variable on both days 1 and 4, ranging from 1 to 84% on day 1 and 11 to 92% on day 4 (median, 40 and 45, respectively; P < 0.01, signed rank test). The ratios of day 4:day 1 for individual patients are shown in Fig. 1A. In this and subsequent Figs. 2–4, the patients are grouped according to whether the percentage of Ki67<sup>+</sup> cells significantly increased, decreased, or was not significantly changed on day 4 compared with day 1. The mean ratio of day 4:day 1 Ki67<sup>+</sup> expression was 1.4 ± 1.1 (P = 0.04; median ratio, 1.2). The change in Ki67<sup>+</sup> expression did not correlate with the dose of IL-3 administered. Ki67<sup>+</sup> expression was also not correlated with FAB classification (M0–M5 versus M6 or M7), history of AHD, or whether patients were relapsed.

IL-3 administration impacted on the S-phase fraction similarly to the Ki67<sup>+</sup> subset. Percentage of cells in S phase ranged from 1 to 23 on day 1 and 1.7 to 20 on day 4 (median, 5.8 and 8.7, respectively; P < 0.01, signed rank test). Day 4:day 1 ratios of the percentage of S-phase cells for individual patients are shown in Fig. 1B. The mean day 4:day 1 ratio of the S-phase percentage was 1.6 ± 1.1 (P < 0.008; median ratio, 1.3). The change in the S-phase percentage was not correlated with the dose of IL-3 administered or the clinical parameters noted above for Ki67.

**Changes in Clonogenic Leukemic Cell Proliferation.** Because the proliferative status of the bulk of bone marrow leukemia cells may not reflect changes in proliferation among clonogenic leukemia cells, changes in CFU-L number were measured before and after 3 days of IL-3 infusion. CFU-L were successfully cultured from the bone marrows of 21 patients (70%). The cloning efficiency of patient samples varied widely,

![Fig. 1 Impact of IL-3 administration on proliferation of leukemic cells. Proliferation was assessed as the percentage of cells in cycle (Ki67 assay, A) and percentage of cells in the S phase (B). Data represent the ratio of the day 4:day 1 value for each patient. Data are grouped according to the direction of change: increased, no change, or decreased. Change was considered significant if the value was increased or decreased by at least 10% (i.e., fold increase of 1.1 or 0.9). No attempt has been made to represent individual patients with the same symbol consistently between figures.](image-url)
The day 4:day 1 ratios of ara-C sensitivity are plotted in Fig. 3. Significance of change in ara-C sensitivity was determined by comparing the means of triplicate or quadruplicate cultures on days 1 and 4 using a two-sided Student’s t test (P < 0.05). Thus there is some apparent overlap in the magnitude of change between patients in the “no change” group (whose mean values on the 2 days were not significantly different) and in each of the other groups (whose mean values were significantly different).

![Fig. 2 Impact of IL-3 administration on CFU-L number. The mean CFU-L number from triplicate or quadruplicate cultures for each patient plated on day 4 was divided by the mean CFU-L number from cultures plated on day 1. Data are grouped according to the direction of change: increased, no change, or decreased. Change in CFU-L was considered significant if the mean CFU-L number on day 4 was significantly different from that on day 1 using two-tailed Student’s t test (P < 0.05). Thus there is some apparent overlap in the magnitude of change between patients in the “no change” group (whose mean values on the 2 days were not significantly different) and in each of the other groups (whose mean values were significantly different).](image1.png)

**Fig. 2** Impact of IL-3 administration on CFU-L number. The mean CFU-L number from triplicate or quadruplicate cultures for each patient plated on day 4 was divided by the mean CFU-L number from cultures plated on day 1. Data are grouped according to the direction of change: increased, no change, or decreased. Change in CFU-L was considered significant if the mean CFU-L number on day 4 was significantly different from that on day 1 using two-tailed Student’s t test (P < 0.05). Thus there is some apparent overlap in the magnitude of change between patients in the “no change” group (whose mean values on the 2 days were not significantly different) and in each of the other groups (whose mean values were significantly different).

Effect of IL-3 Infusion on Chemosensitivity of CFU-L.

The impact of the cytokine infusion on ara-C sensitivity was assessed in 18 patients (62%). Three patients in whom colony formation could be assessed formed too few colonies on either day 1 or day 4 to reliably quantify ara-C sensitivity on that day. The day 4:day 1 ratios of ara-C sensitivity are plotted in Fig. 3 according to the significance and direction of change of sensitivity. Significance of change in ara-C sensitivity was determined by comparing the 95% confidence intervals of the slopes of the ara-C dose-response curves on days 1 and 4 (see “Materials and Methods”). Using a slope of the ara-C dose-response curve of 1 as a cutoff for clinically relevant ara-C sensitivity (corresponding to a LD99% of 2 μM; Ref. 26), 9 of 18 samples were sensitive to ara-C on day 1; 9 were innately resistant. The median ara-C sensitivity on day 1 was 0.2 (range, 0.01–24). After 3 days of IL-3 infusion, the median ara-C sensitivity was 0.7 (range, 0.01–26). Five patients (patients 1, 14, 15, 20, and 28) had significant increases and five patients (patients 9, 18, 21, 22, 23) had significant decreases in ara-C sensitivity, with eight patients exhibiting no significant change. Two “resistant” cases were brought into the “sensitive” range (slope ≥ 1), while two sensitive cases demonstrated in vitro resistance to ara-C on day 4. Among the patients who demonstrated significant increases in ara-C sensitivity, the median ratio of day 4:day 1 ara-C sensitivity was 10 (range, 5–300); among those patients with significant decrements in ara-C sensitivity, the median ratio was 0.3 (range 0.04–0.7). No correlation was found between change in ara-C sensitivity and IL-3 dose.

Because of the marked heterogeneity of change in CFU-L ara-C sensitivity following IL-3 infusion, possible correlations between the log difference between day 4 and day 1 ara-C sensitivity and the log difference in Ki67, S phase, and CFU-L expression were tested. The change in ara-C sensitivity correlated with both the change in Ki67 (r = 0.55; P = 0.02) and change in CFU-L number (r = 0.4; P = 0.09) (Fig. 4). Multivariate analysis using these two variables to predict the change ranging from 0 to 1600 colonies/10⁵ cells on day 1 (median, 130) and 0 to 1150 on day 4 (median, 58). The ratios of day 4/day 1 CFU-L number are plotted in Fig. 2. Significance in change was determined by comparing the means of triplicate or quadruplicate cultures on days 1 and 4 using a two-sided Student’s t test (see “Materials and Methods”). CFU-L number increased significantly in 4 patients (19%), decreased significantly in 12 (57%), and was unchanged in 5 (23%). The median change in colony formation was a decrease of 40% on day 4.

Differential cell counts of Wright-stained cytocentrifuge preparations of BMMC from days 1 and 4 showed that change in percentage of blasts ranged from −60% to +49%, with a median change of +3%. The mean ratio of day 4/day 1 blast percentage was 0.6 ± 1.6 (P = not significant). Change in CFU-L number was not correlated with change in blast percentage when tested as continuous variables or when direction of change was compared through χ² analysis. The change in CFU-L number was also not correlated with change in Ki67 expression, IL-3 dose administered, FAB classification, history of AHD, or relapsed AML.

**Fig. 3 Impact of IL-3 administration on ara-C sensitivity of CFU-L.** ara-C sensitivity is reported as the slope of the log-linear dose response curve of ara-C. A slope of 1 or greater is considered clinically relevant ara-C sensitivity (corresponding to a LD99% of 2 μM; Ref. 26). Data represent the ratio of day 4/day 1 slopes calculated from the regression lines. Data are grouped according to the direction of change. Change in ara-C sensitivity was considered significant if the 95% confidence intervals of the slopes on day 4 and day 1 did not overlap. As in Fig. 2, there is some overlap in the magnitude of change between patients in the “no change” group (whose 95% confidence intervals of the dose-response slopes on day 1 and day 4 overlapped) and in the other two groups (whose 95% confidence intervals on the 2 days did not overlap).
Impact of IL-3 on Acute Myeloid Leukemia

Immunophenotypic Evidence of Differentiation. Because the number of clonogenic cells decreased on day 4 in many samples, immunophenotypic evidence of differentiation was sought in the more recently enrolled patients. In these patients, bone marrows from days 1 and 4 were studied for expression of the progenitor cell antigens c-kit, CD34, and HLA-DR, and the markers of monocytic and granulocytic differentiation CD14 and CD15 (Table 2). In normal myeloid differentiation, CD34, c-kit, and HLA-DR cease to be expressed, while levels of CD14 and/or CD15 increase, depending on the cell type. CD34 expression decreased significantly in only 1 of 12 patients studied. HLA-DR expression decreased in no patients; however, c-kit was down-regulated in 5 patients (median decrease, 40%). Four of five patients with significant decrements in c-kit expression had significant decreases in CFU-L growth on day 4 (the fifth patient demonstrated no CFU-L growth on either day). In contrast, two patients showed increased expression of CD34, HLA-DR, and c-kit, and three additional patients increased c-kit expression alone. No patient changed CD15 expression by more than 10% between days 1 and 4. Of the 2 of 12 patients who expressed CD14 on day 1, 1 increased expression and 1 decreased expression of this antigen on day 4.

Predictors of Biological and Clinical Response. Twenty-one patients (69%) had IL-3 receptor expression quantified using a flow cytometric assay utilizing binding of PE-labeled ligand. Twelve of these patients had detectable IL-3 receptors (range, 4–40% cells positive; median, 9%). Of these patients, 21 were evaluable for changes in Ki67 expression, 15 for changes in CFU-L number, and 13 for changes in ara-C sensitivity. Changes in these three parameters did not correlate with the percentage of cells which expressed measurable IL-3 receptors on day 1.

Twenty-five patients could be evaluated for clinical response to cytokine-supplemented chemotherapy (Table 1). Of the nine nonevaluable patients, three patients had IL-3 discontinued due to toxicity or deteriorating performance status. The other six died during remission induction. Of the 25 evaluable patients, 12 achieved CR, 5 were considered PR, and 8 were nonresponsive. Fourteen evaluable cases also had ara-C sensitivity successfully assessed on day 1. Three of five patients with day 1 ara-C sensitivity ≥1 (corresponding to a “drug-sensitive” LD99% of ≥2 μM; Ref. 26) achieved CR; three of nine patients with day 1 ara-C sensitivity <1 achieved CR. Twelve of the evaluable cases also had day 4 ara-C sensitivity successfully assessed. Only one of six patients whose ara-C sensitivity on day 4 was <1 achieved a CR. In contrast, four of six patients whose day 4 ara-C sensitivity was ≥1 achieved CR. Of these four patients, three had ara-C sensitivity in the sensitive range on day 1 as well. Of the two patients with day 4 ara-C sensitivity ≥1 who did not achieve CR, 1 had ara-C sensitivity of 1 on day 1; the other patient was resistant on day 1. The trend between remission achievement and day 4 ara-C sensitivity did not achieve statistical significance (P = 0.12, Fisher’s exact test).

Of the other biological parameters studied, only the change in percentage of cells in S phase predicted for remission achievement with patients whose day 4:day 1 ratio of S-phase cells was ≥1.16 more likely to achieve CR (P < 0.004, Fisher’s exact test).

Fig. 4 Correlation between proliferation and ara-C sensitivity. The differences between the day 4 and day 1 log-transformed data for Ki67 expression, CFU-L number, and ara-C sensitivity (corresponding to a ratio of day 4:day 1 parameters) were calculated and plotted. The lines represent results of linear regression. A, correlation between change in Ki67 expression and change in ara-C sensitivity (slope, 1.3; P = 0.02). B, relationship between changes in CFU-L number and ara-C sensitivity (slope, 0.5; P = 0.09). In multivariate analysis, the P values for these two relationships were 0.01 and 0.05, respectively.
DISCUSSION

The failure of current aggressive chemotherapy regimens to cure the majority of patients with AML, particularly those cases evolving from myelodysplasia and those with unfavorable FAB classifications or cytogenetic abnormalities (14) requires the development of new therapeutic strategies. In the absence of promising new chemotherapeutic agents, appropriate biomodulation of AML chemotherapy could positively impact on outcome in these diseases. The use of myeloid growth factors to recruit leukemic progenitor cells into cycle, sensitizing the cells to the cytotoxic effects of cycle-specific agents such as ara-C and, to a lesser extent, the topoisomerase II inhibitors, has grown out of a long history of preclinical and clinical studies suggesting the utility of this approach (1-4, 15, 27-29). However, the results of the early trials of growth factor augmentation of induction chemotherapy for AML have been variable.

The heterogeneity of these trial outcomes may reflect the pleiotropic biological effects of growth factors, as well as the heterogeneity of the diseases which are lumped together as AML. The effects of hematopoietic growth factors on the biology of normal and leukemic cells can be categorized into effects on proliferation, survival, and differentiation. Increased cycling of leukemia progenitor cells in response to growth factors would potentially increase their sensitivity to cytotoxic agents. However, recent evidence demonstrates that hematopoietic growth factors inhibit apoptosis (30), the primary mechanisms by which cells die in response to cytotoxic agents. Because inhibited apoptosis resulting from multiple different processes can produce drug resistance (26, 31-34), growth factor administration could actually decrease drug sensitivity. Differentiation of leukemic progenitor cells could be a beneficial effect of growth factor administration if this was associated with a loss in self-renewal capacity, but might have variable effects on drug sensitivity. In an in vitro model, myeloid growth factors shifted the growth of AML colony-forming cells from "self-renewing" to more differentiated colony growth, associated with a decrease in ara-C sensitivity (35). Thus, the clinical impact of growth factor administration in conjunction with chemotherapy for AML for an individual patient may be a complex function of the relative effects of the cytokine on proliferation, survival, and differentiation in that case. These effects in turn reflect the interactions of numerous intracellular pathways normally or aberrantly stimulated or inhibited by that particular cytokine in a particular case of AML.

In the current Phase I study of IL-3 in poor-risk AML, the pleiotropic effects of this cytokine were clearly demonstrated. While the dose of IL-3 administered to evaluable patients ranged from 2.5 to 10 µg/kg/day in this Phase I study, biological effects of the cytokine were seen at all doses, with no trend toward a dose-response effect. Hence it is likely that the cytokine has significant activity at all dose levels tested, and the variability in outcomes reflects the heterogeneity of the response of leukemic cells to the cytokine. The biological changes measured could not be predicted by percentage of cells with significant IL-3 receptor expression detected by the flow cytometric assay. The biological response to IL-3 and other myeloid growth factors has been demonstrated in vitro to correlate poorly with receptor expression measured by a variety of assays; significant biological responses have been seen in the absence of detectable receptors. It is likely that small numbers of IL-3 receptor are necessary to effect biological responses (13, 36, 37).

IL-3 moderately stimulated proliferation of leukemic cells in the majority of cases at all doses tested (median increase, 20-30%); however, the numbers of clonogenic leukemic cells decreased during the 3-day cytokine infusion in most cases in which colonies could be successfully grown. The decrease in CFU-L number despite increased proliferation in the majority leukemic cell population suggests that proliferation is associated with ongoing differentiation of the progenitor cells. Evidence of differentiation of the majority of the leukemia cells was modest: no net effect of the cytokine infusion was seen on the median blast percentage ( assessed morphologically); CD34 expression decreased in only 1 of 13 patients studied, with no patient demonstrating decreased CD15 expression. CD14 expression increased in one patient whose leukemia expressed this antigen; no significant impact was seen on CD15 expression. Down-regulation of c-kit expression in 5 of 15 patients studied may represent a differentiation event; however, the biological impact of expression of this receptor on AML cells has not been extensively studied.

The in vitro assay for ara-C sensitivity used in this study has previously been shown to correlate with remission attainment (26); in the small sample size in which clinical response could be compared with in vitro ara-C sensitivity in the present study, a trend was seen toward remission attainment in patients whose ara-C sensitivity was ≥1 on day 4 of therapy. The correlation with remission attainment must be viewed in the context of the fact that all patients were treated with two active agents besides ara-C, the topoisomerase II-active drugs daunorubicin and etoposide. Although topoisomerase II expression

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### Table 2

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</tr>
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*Bone marrow mononuclear cells from days 1 and 4 of cytokine administration were studied for antigen expression by two-color flow cytometry. Cases were considered positive if ≥20% of cells expressed significant fluorescence (≥10% in the case of c-kit). Significant increase or decrease in antigen expression was defined as a change in cellular expression of at least 10%.
varies during the cell cycle and increases after in vivo treatment with GM-CSF (38), we did not measure the impact of IL-3 on the CFU-L sensitivity to these drugs. Instead, we restricted our in vitro investigation to the effects of the cytokine on ara-C sensitivity, because ara-C forms the foundation of most first-line treatments for AML, and as the most cell cycle dependent of the three agents would be most likely to be influenced by changes in cell proliferation. IL-3 significantly altered clonogenic ara-C sensitivity in almost two-thirds of patients from whom colonies could be cultured; however, ara-C sensitivity was increased and decreased in equal numbers of cases.

The mechanism of increased ara-C resistance in a significant subset of patients is uncertain. The role of IL-3 as a survival (anti-apoptotic) factor may cause it to function as a chemoprotectant in these patients. Hematopoietic growth factors directly protected two mouse myeloid leukemia cell lines from chemotherapy-induced apoptosis (including ara-C; Ref. 39). Similar protective effects of IL-3 on killing of human factor-dependent AML cell lines and individual samples of primary AML by superpharmacological doses of ara-C (120 μM) have been demonstrated (40). Besides protection against apoptosis, recent data have suggested that driving cell cycling might paradoxically decrease ara-C sensitivity by increasing deoxynucleotide pools and thereby inhibiting deoxycytidine kinase, the rate-limiting enzyme for 1-β-d-arabinofuranosylcytosine 5′-triphosphate generation (41). However, the positive correlations demonstrated in the present study between changes in overall (Ki67) or clonogenic (CFU-L) proliferation and ara-C sensitivity make this latter explanation less likely.

The independent positive correlations between the changes in proliferation (Ki67 expression) and CFU-L number with change in ara-C sensitivity support the recruitment hypothesis. This is further supported by the apparent increased likelihood of achieving CR in patients in whom IL-3 increased the percentage of cells in the S phase by at least 16% (of the day 1 value). The small size of the data set precludes establishment of change in the S phase as an independent predictor. A major reason for the failure of IL-3 to impact positively on ara-C sensitivity in many of the cases may relate to its dual effects on proliferation and differentiation. While the majority of cases showed increased proliferation with IL-3, favoring increased ara-C sensitivity, the number of clonogenic cells decreased in many cases, potentially due to differentiation of clonogenic precursors, and favoring decreased ara-C sensitivity (Fig. 4B). The clinical impact of this putative differentiation effect apart from drug sensitivity is unknown. Enrollment in the present study was limited to patients with high-risk leukemia; 50% of the patients studied were innately ara-C resistant before beginning therapy (slope of dose-response curve <1; LD99% >2 μM; Ref. 26). Purely “kinetic resistance” may play a more important role in good-risk de novo leukemias. In the present study, patients with a history of AHD or who were treated with a diagnosis of MDS were less likely to have increased ara-C sensitivity following IL-3 than were patients with de novo leukemia. Nonetheless, the impact of cytokine administration on differentiation and survival of leukemic progenitor cells will need to be considered carefully in de novo leukemia as well.

The biological heterogeneity of AML makes uniform approaches to all patients unlikely to succeed. Progress in AML will more likely be made as therapy can be individualized based on the biology of the particular case of leukemia. Because of the heterogeneity of biological response to IL-3, future trials of cytokine augmentation of chemotherapy effect may be best planned following in vitro investigation of the cytokine effect on chemosensitivity. As the intracellular pathways which regulate proliferation, differentiation, and survival in hematopoietic cells become further elucidated, leukemic cells may eventually be screened at diagnosis to determine which patients are likely to benefit most from cytokines administered concurrent with or following chemotherapy, and those patients for whom cytokine administration may induce drug resistance and is best avoided.

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REFERENCES


Impact of in vivo administration of interleukin 3 on proliferation, differentiation, and chemosensitivity of acute myeloid leukemia.
