Metabolism of Ara-C by Blast Cells From Patients With ANLL

By Douglas D. Ross, Bruce W. Thompson, Christopher C. Joneckis, Steven A. Akman, and Charles A. Schiffer

The dose–response relationship between extracellular concentration of cytosine arabinoside (ara-C) and intracellular formation of the putative active metabolites of ara-C [ara-C incorporation into DNA and intracellular pools of ara-C in triphosphate form (ara-CTP)] was investigated in blast cells obtained from patients with acute nonlymphocytic leukemia (ANLL) by exposing these cells in vitro to 10, 100, or 1,000 nM/L of ara-C. We studied 23 untreated patients who subsequently achieved complete remission (CR) with a regimen using daunorubicin and conventional doses of ara-C (ara-C-sensitive group), and 30 patients judged to be ara-C-resistant either by failing initial induction therapy (16 patients) or by having relapsed on an ara-C–containing maintenance regimen (14 patients). In both patient groups, ara-C incorporation into DNA and intracellular ara-CTP both displayed statistically significant increases in response to increasing extracellular concentrations of ara-C (P < .0001 in both cases), with the rate of increase of ara-CTP greater than that of ara-C incorporation.

DNA polymerase and that small amounts are incorporated into the DNA molecule. In tissue culture systems, incorporation of ara-C into DNA correlates directly with the degree of cytotoxicity. Thus, the efficiency of incorporation of ara-C into DNA (defined as the ratio of ara-C incorporation to ara-CTP pools) decreased by 58% with each tenfold increment in the extracellular concentration of ara-C (P < .0001), presumably as a result of the inhibitory effect of ara-CTP on DNA polymerase. Using an analysis of covariance, modest differences were found in the levels of ara-C and ara-CTP pools between the ara-C-resistant group and the ara-C-sensitive group.

THE ANTIMETABOLITE ara-C is one of the most effective agents currently available for the treatment of ANLL in adults. When combined with the anthracycline antibiotic daunorubicin, high remission induction rates have been reported for previously untreated patients. Unfortunately, most patients eventually relapse and die of leukemia despite maintenance or continued therapy with ara-C-containing regimens, suggesting the emergence or expansion of ara-C–resistant leukemic cell populations.

Considerable evidence has accumulated concerning the mechanism of ara-C cytotoxicity to established leukemic cell lines. After entering the cell, ara-C must be converted to the triphosphate form (ara-CTP) by a series of kinase enzymes. Ara-CTP is considered to be the active metabolic form of ara-C and is a powerful inhibitor of DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7), competitive with the natural substrate dCTP. Although inhibition of DNA polymerase would surely retard cell growth, inhibition of DNA synthesis per se is not necessarily a cytotoxic event. It has been recognized that ara-C is a weak substrate for DNA polymerase and that small amounts are incorporated into the DNA molecule. In tissue culture systems, incorporation of ara-C into DNA correlates directly with the degree of cytotoxicity. Indeed, recent studies in HL-60 cells of the relationship among ara-CTP pools, formation of ara-C (DNA), and clonogenic survival demonstrated that incorporation of ara-C into DNA was the single most powerful predictor of cell killing by ara-C.

Various biochemical mechanisms of cellular resistance to ara-C have been recognized in cultured cells or in experimental animal tumor models. Cells have been identified with high levels of cytidine deaminase (EC 3.5.4.5) or dCMP deaminase (EC 3.5.4.3) which convert ara-C or ara-CMP to dUMP. Cells deficient in the nucleoside kinase enzyme (deoxycytidine kinase, EC 2.7.1.74) have been identified, which are resistant because of inability to phosphorylate ara-C. Other cells have been found to be resistant to ara-C by their high intracellular levels of dCTP, which inhibit ara-C phosphorylation and which competes with ara-C for DNA polymerase.

A DNA polymerase insensitive to inhibition by ara-CTP or one that fails to recognize ara-CTP as a substrate is another means by which cells can become resistant to ara-C. A DNA repair enzyme activity has been described recently that removes ara-C residues from DNA. Thus, enhanced intracellular activity of such an enzyme may be a mechanism of cellular resistance to ara-C. Finally, increased dephosphorylation or intracellular catabolism of ara-CTP offers a potential mechanism of cellular ara-C resistance.

Most of the cellular mechanisms of ara-C resistance mentioned above have been identified in ara-C–resistant cells in culture. However, at present, little data exist as to the mechanism(s) operative in blast cells from leukemia patients clinically resistant to ara-C. Intracellular kinase and deaminase studies have failed to predict clinical outcome, as have studies of cellular ability to form ara-CTP, although the ability of blast cells to retain intracellular ara-CTP
following in vitro exposure to ara-C has been reported to correlate with remission duration in those patients who subsequently achieved a complete remission. More recently, the intracellular pharmacokinetic studies of Plunkett and co-workers suggest that retention of ara-CTP may be a determinant of response to therapy with high-dose ara-C.

Of the mechanisms of cellular resistance to ara-C outlined above, all should be manifested either by diminution in the steady-state intracellular concentration of ara-C, diminution of ara-C incorporated into DNA, or both. It should be noted that those mechanisms which involve enhanced cellular catabolism of ara-CTP and/or enhanced excision and repair of ara-C-induced DNA damage may only become apparent by observing the intracellular half-life (1/2) of ara-CTP (ara-CTP retention) or of retention of ara-C incorporated into DNA following exposure of cells to ara-C.

In the present studies, we measured intracellular steady-state concentrations of ara-CTP and ara-C incorporated into DNA in blast cells from patients with ANLL exposed to varying concentrations of ara-C in vitro, in an effort to understand the dose–response relationship between ara-C and formation of its metabolites in ANLL blast cells, and to determine if differences in these ara-C-metabolic parameters and dose–response can be detected between groups of patients clinically sensitive and resistant to conventional doses of ara-C. Extracellular concentrations of 10, 100, and 1,000 nmol/L of ara-C were used, since 100 to 1,000 nmol/L approximates the steady-state plasma level of ara-C attained during conventional-dose continuous infusion of ara-C.25

MATERIALS AND METHODS

Patient selection and characteristics. In all patients, the diagnosis of ANLL was established on the basis of microscopic examination of Wright's-stained specimens of bone marrow, assisted by examination of specially stained specimens [a-naphthyl esterase, Sudan black, periodic acid-Schiff]. These studies were performed between August 1982 and January 1985. The median age of the patients studied was 52 years, with 43% females and 57% males. This approximates the overall demographics of ANLL patients treated at our center. Patients were subdivided into three groups based on their clinical response to ara-C-containing regimens: complete remission (CR) group (ara-C-sensitive patients), no response (NR) group (ara-C-resistant patients); and relapsed (REL) group (ara-C-resistant patients).

Complete remission group. The complete remission (CR) group comprised 23 patients who were untreated at the time of study who subsequently achieved CR with induction therapy consisting of daunorubicin (45 mg/m²/d; days 1, 2, and 3) and ara-C (100 or 200 mg/m²/d, days 1 through 7 by continuous intravenous infusion). All but four of the 23 patients in this group have had remission durations >6 months (overall range 2 to 19+ months), and 12 patients (52%) remain in CR at the present time. All patients received ara-C–based maintenance or consolidation regimens. The projected median duration of CR (Kaplan-Meier) for this group is 11 months. All marrows studied were obtained before any treatment was given.

No response group. The no response (NR) group (ara-C-resistant patients) comprised 16 patients who were either untreated at time of study (13 patients) or previously treated (three patients), but who failed to attain remission with an ara-C–containing regimen at any time during the course of their disease. For the previously treated patients, marrow was collected for study after a suitable period elapsed from the last treatment attempt to allow marrow recovery from that treatment (2 to 3 weeks). All patients in the NR group received an initial induction regimen identical to that described for the CR group above. All patients in this group survived their induction attempt and were fully evaluable for response to the ara-C–containing regimen.26

Relapsed patient group (ara-C-resistant patients). Fourteen patients were studied at time of relapse. Seven patients in this group relapsed while on an intensive ara-C–containing maintenance regimen, and marrow was obtained for these studies prior to further treatment with investigational agents. The other seven patients had a previous CR but had relapsed and failed to respond to an ara-C–containing reinduction regimen. Of the REL group, 7 were in first relapse, 4 were in second relapse, 2 were in third relapse, and 1 was in fourth relapse at the time of study.

No patient was studied during therapy or within 2 weeks of the completion of a chemotherapeutic regimen. The data presented are from 53 different patients. Thus the patients within a response category are unique to that category. In no case was a patient included in different response categories nor was any patient included twice in the same category.

Bone marrow collection and preparation. Bone marrow (4 mL) was aspirated from the posterior iliac crest of patients using 0.1 mL of preservative-free heparin (1,000 U/mL) as an anticoagulant. Informed consent for marrow collection for this study was obtained as reviewed and approved by the University of Maryland at Baltimore Human Volunteers Institutional Review Board. Marrow was placed in a 15-mL sterile conical centrifuge tube (Costar Plastics, Cambridge, Mass) and then diluted with an equal volume of RPMI 1640 medium (without serum). Marrow cells were dispersed by gentle pipetting and 5 mL of Ficoll-Hypaque ("LSM": Litton Bionetics, Inc) were layered under the marrow suspension. Mononuclear cells were collected at the Ficoll-Hypaque and medium interface after centrifugation (400 g for 40 min, 4°C). RBCs contaminating the mononuclear cell fraction were lysed by exposure to 0.74% NH₄Cl, following which the partially purified marrow mononuclear cells were washed once with ice-cold RPMI 1640 medium (without serum). Cells were resuspended in a volume of growth medium [RPMI 1640 medium containing 10% vol/vol heat-inactivated (56°C for 30 min, fetal calf serum (FCS))] sufficient to achieve the desired final cell concentration (~0.8 to 1.2 x 10⁶ cells per milliliter). This procedure yields mononuclear cells of which 80% to 100% have the morphologic appearance of myeloid blast cells when viewed by light microscopy. Viability (trypan-blue dye exclusion) was routinely 80% to 100%. Average blast cell yield from the marrows studied was ~2 x 10⁶ cells.

Blast cell culture and exposure to ara-C or thymidine (dThd). Cells in growth medium (cell concentration, constant within each experiment, ranged from 1 to 2 x 10⁶ cells per milliliter among experiments) were aliquoted into sterile 13- by 100-mm borosilicate glass culture tubes (1.8 mL per tube), and the appropriate amount of ³H-ara-C or ³H-dThd was then added in a volume of 0.2 mL of culture medium to achieve the desired final concentration. Stock 5-³H-ara-C (15 Ci/mmol) was obtained from Amersham/Searle, Inc (Arlington Heights, Ill). The final concentrations in culture/specific activities used were 10 nmol/L (10 Ci/mmol), 100 nmol/L (5 Ci/mmol) and 1,000 nmol/L (1 Ci/mmol). 5-Methyl ³H-dThd (New England Nuclear, Inc, Boston, specific activity 6.7 Ci/mmol) was added to the appropriate culture tubes to achieve a final concentration of 300 nmol/L. Cultures were incubated at 37°C, 7.5% CO₂, for 22 hours, after which ara-C incorporation into
DNA, dTthd incorporation into DNA, and intracellular ara-CTP was determined by methods described below. Zero-time "background" tubes were prepared by incubating cells in the absence of isotope for the 22-hour period and then adding the appropriate amount of $^3$H-ara-C or $^3$H-dTthd after cooling the cells on ice for 15 minutes. Degradation of ara-C in culture medium alone after 22-hour incubation was minimal. Degradation of ara-C in the blast cell culture ranged from 23% to 37% for 10 nmol/L of ara-C, and 1% to 22% for 1,000 nmol/L of ara-C, using high-performance liquid chromatography (HPLC), as described previously. The major breakdown product was ara-U.

Overall ability of patient-derived blast cells to metabolize ara-C, including the effects of intracellular degradation of ara-C. Incorporation of $^3$H-dTthd into DNA was linear throughout the 22-hour period of incubation.

**Determination of intracellular ara-CTP pool size.** Following incubation, cultures were chilled on ice for 15 minutes, and cells were collected by sedimentation (400 g for 10 minutes, 4°C), washed twice with ice-cold phosphate-buffered saline (PBS), and then extracted twice with 200 μL of 60% methanol in H2O at 30°C × 10 minutes. The two methanol extracts of each culture were combined; debris was removed by sedimentation (13,000 g for 10 minutes, 4°C). Methanol extraction was used since we found that ara-CTP is stable for at least 2 months in 60% methanol at –20°C. Ara-CTP content of the methanol extracts was determined as reported previously by injecting 80 μL of the extract, along with appropriate internal standards, into a model 3500B HPLC (Spectra-Physics, Santa Clara, Calif.) equipped with a 4.6 mm × 25 cm Partisil SAX/10 anion exchange column (Whatman Inc, Clifton, NJ) and a 2.1 mm × 7 cm guard column filled with anion exchange packing. Samples were eluted with a mobile phase of KH2PO4 (500 mmol/L, pH 4.6) under isocratic conditions with a flow rate of 1.52 mL/min. Fractions (0.5 mL) of the eluate were collected and absolute radioactivity (DPM) was determined with a Searle Mark III liquid scintillation spectrometer, using quenched internal standards and a $^{133}$Ba external standard. Positive identification of the ara-CTP peak was made with the use of authentic nonradiolabeled ara-CTP (Sigma Chemical Co, St Louis) as an internal standard. This procedure permits effective separation of ara-CTP from dCTP.

**Determination of incorporation of $^3$H-ara-C or $^3$H-dTthd into DNA.** Following incubation, $^3$H-ara-C incorporation into DNA of the blast cells was determined exactly as described previously for HL-60 cells. $^3$H-dTthd incorporation into DNA also was determined by the same procedure except that cells were incubated with $^3$H-dTthd instead of $^3$H-ara-C. Triplicate determinations were made for each experimental point except in those few patients in whom blast cell yield from the marrow was low, in which case determinations were made in duplicate. Radioactivity in zero-time background tubes was subtracted from the radioactivity of the appropriate experimental tubes. Radioactivity was converted to molar quantities using the known specific activity of the radiolabel.

**Statistical methods.** The data were analyzed by several statistical methods. When comparisons were made by t test, Student's t test was used if a comparison was made between two groups of individuals, and the paired t test was used when the observations being compared were from the same subject.

The statistical models were fitted using the GLM procedure in SAS (SAS Institute, Cary, NC) using an IBM 4341 computer. Data were missing for one of the three ara-C concentrations for 5.9% of all observations. SAS treated missing values in such a way that any observation that had a missing value for any dependent or independent variable was excluded from the analysis.

**RESULTS**

Our research objectives were threefold: to study the relationship of dose of ara-C (10, 100 or 1,000 nmol/L) and the formation of potentially active metabolites of ara-C in blast cells from ANLL patients, to determine if the formation of such active metabolites of ara-C could be used to predict clinical response in individual patients, and to determine collectively if differences in ara-C metabolism exist among the three patient groups (CR, NR, and REL). To accomplish these objectives, using an in vitro culture system, we related the extracellular concentration of ara-C (dose) and the clinical response of a patient to three dependent variables which represent important events in ara-C metabolism: the amount of ara-C incorporated into DNA, the intracellular concentration of ara-CTP, and the ratio of these two measures, as a measure of efficiency of ara-C incorporation into DNA. The data and geometric means are shown in Figs 1 through 3. These plots are semilogarithmic. The means ± SE of the three dependent variables for each response group at each extracellular concentration of ara-C used are given in Table 1.

The data presented show a high degree of variability among patients in the amount of ara-C metabolite formed at any dose of ara-C (interpatient variability), contrasting with
the variability associated with making the measurements of ara-C metabolite in a single patient (experimental variability), which was small. For example, each data point in Fig 1 represents the mean of duplicate or triplicate experimental determinations for a single patient made at the corresponding dose of ara-C, with a SD for each point that was typically < 15%. In addition, what is not apparent from Figs 1 through 3 is the fact that the rank placement of a particular patient's ara-C metabolite measurement at a given dose of ara-C tended to be constant at all other doses of ara-C. Thus, patients with relatively low incorporation at 10 nmol/L of ara-C also tended to have relatively low incorporation at 100 and 1,000 nmol/L as well. Thus, the variability seen in Figs 1 through 3 is really variability associated with differences among patients (interpatient variability) rather than experimental variability associated with the measurement of the ara-C metabolites of a given patient.

The data in Table 1 and Figs 1 through 3 reveal a clear positive relationship between ara-C dose and formation of ara-CTP or incorporation of ara-C into DNA in each clinical response group. Statistically significant increases were present at each increment of extracellular ara-C concentration by paired t test (P < .0001 in each case). Similarly, the efficiency of ara-C incorporation (ratio of incorporation/ara-CTP) decreased significantly with each increment in ara-C dose (P < .0001).

When the various clinical response groups (CR, NR, REL) are compared, Figs 1 through 3 reveal considerable overlap in ara-C metabolite formation among all clinical response groups. Indeed, no significant differences were found in ara-C incorporation, formation of intracellular ara-CTP, or efficiency of ara-C incorporation among the various clinical response groups by Student's t test (P > .05 in all cases). By inspection of these data, one can see readily that it would be impossible to predict, based on the ara-C metabolic variables of a given patient, the clinical response group in which that patient lies. Thus, at present, we are unable to fulfill our second goal, namely, the development of a test that will predict clinical response in an individual patient.

To ascertain collective trends among the patients studied, the data were evaluated by an analysis of covariance, a statistical method that uses the concepts of both analysis of variance and linear regression. In computing the analysis of covariance, a natural logarithmic transformation was performed to stabilize the variance. For this reason, geometric means are provided in Figs 1 through 3. The results derived from this analysis agree substantially with the results obtained by t tests, as described above. Specifically, in relating ara-C dose with metabolite formed, we found that a 3.6-fold increase in ara-C incorporation into DNA and an 8.5-fold increase in intracellular ara-CTP can be expected.
Consistent ara-C. metabolic variable per tenfold increase in extracellular concentration of ara-C. This case, the barge amount of interpatient variability encountered prevented discernment of statistically significant differences. In contrast, the second analysis (which assumed that the patients were an entire population) found that the differences seen in the three dependent variables (Table 2) were large enough that they could not be due to experimental (or assay) variation, and thus they were statistically significant (P values, Table 2). However, conclusions drawn from this latter analysis must be limited to the group of patients studied and cannot be extended to the general population of ANLL patients.

Six patients in the CR group were studied again at the time of relapse. These patients were not included in the REL group as described above. No statistically significant differences in the metabolism of ara-C were found among these patients at time of relapse as compared with their values before treatment.

Because ara-C is an S phase-specific agent, a possible confounding factor was the rate of DNA synthesis in each patient marrow sample. For each patient, we measured the uninhibited rate of DNA synthesis in the bone marrow cultures during the 22-hour period by measuring the rate of 3H-dThd incorporation into DNA (means for each response group are shown in Table 4). No statistically significant differences in the rate of 3H-dThd incorporation were found among the means of response groups by t test (Table 4). When the rate of DNA synthesis in each patient marrow sample was used as a covariable in the analysis of covariance, there was still a highly significant relationship between the ara-C metabolic variable (ara-CTP, incorporation or ratio) and the dose of ara-C.

### DISCUSSION

These studies demonstrate a dose–response relationship for ara-C incorporation into DNA and formation of intracellular ara-CTP in blast cells from patients with ANLL. In response to doses of ara-C from 10 to 1,000 nmol/L, the increase in the rate of formation of ara-CTP is greater than that of ara-C incorporation into DNA. Thus, the efficiency of ara-C incorporation into DNA decreased significantly as the extracellular concentration of ara-C was increased. We attribute this effect in part to inhibition of DNA polymerase by the increased intracellular concentration of ara-CTP and in part to the fact that the extracellular concentrations of ara-C used were well below the K_m of deoxycytidine kinase for phosphorylation of ara-C (~20 μmol/L), as evidenced by the linear and nearly direct proportional relationship we observed between extracellular ara-C concentration and intracellular pools of ara-CTP (Table 2). The studies of Riva and colleagues, using high extracellular concentrations of ara-C (in excess of 20 μmol/L), found that the efficiency of ara-C incorporation increased with increasing ara-C dose, presumably due to saturation of the ara-C phosphorylating mechanism. We emphasize that, although we found that efficiency of incorporation decreased with increasing ara-C concentration, in no case did we observe an actual decrease in the absolute amount of ara-C incorporated into DNA by raising the extracellular concentration of ara-C.

The decrease in efficiency of ara-C incorporation with increasing dose of ara-C means that, relative to intracellular ara-CTP, a proportionately larger amount of ara-C is incor-

### Table 1. Arithmetic Means ± SE for ara-C Incorporation Into DNA, Formation of ara-CTP and Efficiency of ara-C Incorporation

<table>
<thead>
<tr>
<th>Clinical Group</th>
<th>Extracellular Concentration of ara-C (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>54 ± 7 (23)* 165 ± 21 (23) 834 ± 202 (23)</td>
</tr>
<tr>
<td>NR</td>
<td>55 ± 12 (16) 131 ± 21 (16) 609 ± 105 (16)</td>
</tr>
<tr>
<td>REL</td>
<td>50 ± 8 (14) 126 ± 23 (14) 524 ± 77 (13)</td>
</tr>
</tbody>
</table>

intracellular ara-CTP (μmol/10⁶ cells)

| CR       | 116 ± 23 (20) 1,006 ± 207 (23) 7,931 ± 1,425 (23) |
| NR       | 105 ± 21 (13) 994 ± 188 (13) 10,139 ± 1,850 (13) |
| REL      | 179 ± 71 (11) 942 ± 236 (13) 7,781 ± 1,478 (12) |

Efficiency of ara-C incorporation into DNA (ratio of incorporation to intracellular ara-CTP for each patient, expressed as %)

| CR       | 137 ± 59 (20) 57 ± 17 (23) 28 ± 11 (23) |
| NR       | 42 ± 10 (12) 25 ± 14 (13) 8 ± 2 (13) |
| REL      | 56 ± 13 (11) 23 ± 6 (12) 12 ± 4 (12) |

Abbreviations as in Table 1.

*Numbers in parentheses refers to the number of patient marrows studied at that particular ara-C dose level.

†One patient was excluded whose ratio was 3,500%, which is >3 SD from the mean which includes this value.

for each 10-fold increment in the extracellular concentration of ara-C (P = .0001 in both cases) (Table 2). Because the rate of increase of ara-CTP was greater than that of ara-C incorporation, the efficiency of ara-C incorporation (ratio of incorporation to ara-CTP) decreased by a multiple of 0.42 (ie, a 58% decrease) with each tenfold increase in ara-C dose (Table 1).

In comparing the various clinical response groups, two separate analyses of covariance were done. The first analysis made the assumption that the patients studied were representative of the general population of patients with ANLL. The second analysis considered a special case in which the patients we studied constituted a unique population, not necessarily representative of the general population of ANLL patients. Small differences in the levels of the three dependent variables were noted (Table 3, adjusted mean values). The CR group, overall, incorporated more ara-C than did the other groups (NR and REL). However, in the first analysis, these differences were not statistically significant, consistent with the results of the t tests described above. In this case, the large amount of interpatient variability encountered prevented discernment of statistically significant differences. The decrease in efficiency of ara-C incorporation with increasing ara-C dose means that, relative to intracellular ara-CTP, a proportionately larger amount of ara-C is incor-

### Table 2. Analysis of Covariance: Relation of ara-C Metabolic Variable to ara-C Dose

<table>
<thead>
<tr>
<th>Metabolic Variable</th>
<th>Slope*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C incorporation into DNA</td>
<td>3.6</td>
<td>.0001</td>
</tr>
<tr>
<td>ara-CTP</td>
<td>8.5</td>
<td>.0001</td>
</tr>
<tr>
<td>Ratio (incorporation/ara-C)</td>
<td>0.42</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

* Slope equals multiple of change in cytosine arabinoside (ara-C) metabolic variable per tenfold increase in extracellular concentration of ara-C.
The GLM procedure of SAS was used to calculate that an increase in extracellular ara-C concentration from 10 to 1,000 nmol/L causes only a 12-fold increase in ara-C incorporation into DNA, whereas intracellular ara-CTP increases 70-fold. Ten to 100 nmol/L of ara-C approximates plasma concentrations obtained with "low-dose" ara-C therapy (eg, 10 to 20 mg/m² q 12 hours). Thus, in comparing the various clinical response groups, covariance analysis provided single "adjusted mean" values for each response group. Adjusted mean values were obtained using the least squares option of the GLM procedure of SAS. Covariance analysis adjusts the means of the dependent variables (ara-C incorporation, ara-CTP or ratio) by regression to estimates of what they would have been had they had a common independent variable (ara-C dose). Therefore, in comparing the various clinical response groups, covariance analysis provided single "adjusted mean" values for each response group. Adjusted mean values were obtained using the least squares option of the GLM procedure of SAS.

### Table 3. Analysis of Covariance: Adjusted Mean Values

<table>
<thead>
<tr>
<th>Ara-C Metabolic Variable</th>
<th>Response Group</th>
<th>Adjusted Mean</th>
<th>Units</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-C incorporated into DNA</td>
<td>CR</td>
<td>146.9</td>
<td>f mol/10⁶ cells</td>
<td>.0079</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>124.6</td>
<td></td>
<td>.0002</td>
</tr>
<tr>
<td></td>
<td>REL</td>
<td>109.7</td>
<td></td>
<td>.005</td>
</tr>
<tr>
<td>Intracellular ara-CTP</td>
<td>CR</td>
<td>569.1</td>
<td>f mol/10⁶ cells</td>
<td>.51</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>823.0</td>
<td></td>
<td>.0001</td>
</tr>
<tr>
<td></td>
<td>REL</td>
<td>746.2</td>
<td></td>
<td>.0002</td>
</tr>
<tr>
<td>Efficiency of ara-C incorporated into DNA</td>
<td>CR</td>
<td>27.4</td>
<td>Ratio, %, ie:</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>15.6</td>
<td>(incorporation ara-CTP)</td>
<td>.0001</td>
</tr>
<tr>
<td></td>
<td>REL</td>
<td>18.4</td>
<td>x 100</td>
<td>.0001</td>
</tr>
</tbody>
</table>

Covariance analysis adjusts the means of the dependent variables (ara-C incorporation, ara-CTP or ratio) by regression to estimates of what they would have been had they had a common independent variable (ara-C dose). Thus, in comparing the various clinical response groups, covariance analysis provided single "adjusted mean" values for each response group. Adjusted mean values were obtained using the least squares option of the GLM procedure of SAS.

*Test of the hypothesis that the three means are equal if the patients studied are considered to be a unique population.

ara-C, cytosine arabinoside; CR, complete remission; NA, no response; REL, relapse.

When our patients were considered as a unique population, those who entered CR (estimated clinically to be cytotoxic) to ara-C incorporated more ara-C into DNA with an equal or lower intracellular pool of ara-CTP than did those who were estimated clinically to be resistant to ara-C. Our finding of higher intracellular ara-CTP pools in clinically resistant patients agrees with the findings of Haanen and co-workers, who observed increased ara-CTP formation in response to 1,100 nmol/L of ara-C in seven of eight ANLL patients at time of relapse as compared with their pretreatment (ie, at time of presentation) values. However, the significance of our findings cannot be extended to the general ANLL population at present since the interpatient variation encountered makes any comparison among response groups statistically nonsignificant when the patients are considered as a sample of the general ANLL population. Similarly, the interpatient variability encountered makes prediction of clinical outcome by such studies impossible in individual patients.

In cell lines selected for resistance to ara-C, common mechanisms of resistance are impaired phosphorylation of ara-C or increased intracellular pools of dCTP. Both mechanisms should result in a markedly lower intracellular pool of ara-C as compared with nonresistant cells. Not only were we unable to demonstrate impaired production of ara-CTP in the resistant patients, but neither did we encounter any patient in this study whose blast cells were totally unable to generate intracellular ara-CTP. This includes patients whom one might consider to be "super-resistant" to ara-C: those in the NR group or those who relapsed while receiving the drug. This finding, combined with the trend for greater efficiency of ara-C incorporation into DNA in our clinically sensitive patients, implies that within our population of ANLL patients with varying clinical sensitivity to ara-C, differences may have existed at the level of DNA polymerase. We cannot, however, rule out enhanced intracellular dephosphorylation of ara-CTP or excision and repair of ara-C/DNA, since these mechanisms may only become apparent when the intracellular retention of ara-CTP or incorporated ara-C is determined, which was not done in our study.

The differences we observed among the clinical response groups were relatively small. For example, the adjusted mean for ara-C incorporation in the CR group was only ~25% greater than those of the NR or REL groups (Table 3). This small difference is not surprising when one considers that in ANLL, the frequency of drug-resistant cells necessary to cause the state of clinical resistance to therapy may be low (eg, <10% of the malignant cell population), since most patients in whom conventional ANLL induction therapy fails to produce CR achieve considerable marrow hypoplasia within 10 to 14 days following therapy. This implies that treatment with an ara-C regimen causes at least 1 log tumor kill (90% reduction of tumor), even in clinically resistant patients. If such is the case, the ara-C metabolites

### Table 4. In-Vitro Rate of ³H-dThd Incorporation Into DNA (pmols/10⁶ Cells ± SE)

<table>
<thead>
<tr>
<th>Clinical Group</th>
<th>Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR (N = 21)</td>
<td>10.9 ± 1.6</td>
</tr>
<tr>
<td>NR (N = 14)</td>
<td>17.2 ± 4.0</td>
</tr>
<tr>
<td>REL (N = 13)</td>
<td>9.2 ± 2.6</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

Marrow blast cells were incubated in vitro with ³H-dThd in the absence of ara-C. There were no statistically significant differences in the means by Student's t test (CR v NR, P > .5; NR v REL, 0.1 > P > .05; CR v REL, p > .5).
formed by the more populous nonresistant cells may mask the contribution of the resistant cells to the mean ara-C metabolite value obtained for the marrow blast cell sample. Indeed, the variations in ara-C metabolism of the nonresistant cells may have contributed markedly to the interpatient variability we observed in these studies. Thus, in detecting ara-C-resistant cells among a heterogeneous population of blast cells obtained from an ANLL patient, it may be necessary to use a sensitive method capable of analyzing multiple events in single cells. We are currently pursuing such an approach, using flow cytometry.

REFERENCES

17. Leclerc JM, Cheng YC: Demonstration of activities in leukemic cells capable of removing 1-β-D arabinofuranosylcytosine (ara-C) from ara-C incorporated DNA. Proc Am Assoc Cancer Res 25:19, 1984
Metabolism of ara-C by blast cells from patients with ANLL

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