Cell Kinetic Responses in Childhood Acute Nonlymphocytic Leukemia During High-Dose Therapy With Cytosine Arabinoside

By L. A. Smets, J. Taminiau, K. Hählen, F. de Waal, and H. Behrendt

Sequential bone marrow aspirates obtained from 10 children with relapsed acute nonlymphocytic leukemia (ANLL) after a high dose of cytosine arabinoside (Ara-C; 1000 mg/sq m) were analyzed by flow cytophotometry. The drug caused elimination of proliferating cells followed by a synchronous wave of cell recruitment. Among individual patients, considerable variation was observed in the degree of recruitment as well as in the time of appearance of the recruitment maximum (range 17–36 hr). However, both parameters appeared inversely correlated with the proliferative status in the bone marrow before treatment. In 6 other patients, cell kinetic responses were studied during treatment with repeated Ara-C injections individually according to the expected optima of recruitment. Waves of recruitment could be observed during 4–5 consecutive injections. The results suggest that in childhood ANLL, characteristic and individual cytokinetic responses to treatment with high-dose Ara-C can be monitored during therapy. These observations may allow the development of individual treatment schedules.

THE PROGNOSIS of children with acute nonlymphocytic leukemia is very poor. Long-term remissions are achieved only in a small minority of the patients and there is an obvious need for better treatment strategies. Ara-C, either single or in combination with other drugs, is recently used in very high doses to obtain complete remissions in (childhood) ANLL refractory to other schemes. Since this drug is highly cell cycle phase specific, nonproliferating cells will be refractory to treatment and can possibly serve as a focus of relapse. Strategies aiming at improved therapy by the eradication of nonproliferating tumor cells are based on the recruitment of resting cells and on synchronization of proliferating cells in drug-sensitive phases of the cell cycle. Unfortunately, such efforts have met with limited success in human patients. Positive as well as negative conclusions have been reached regarding the relevance of cell kinetic parameters for the treatment of human ANLL. On the other hand, optimal effects of chemotherapy have been recorded in animal models using schemes derived from cell kinetic observations. Aggietta and Colly demonstrated an important cell kinetic factor in the therapeutic response of a realistic rat AML model treated with high-dose Ara-C. They observed a strong synergistic effect of repeated injections provided that these coincided with the maximum of recruitment induced by the previous dose. In human patients, Lampkin et al. observed a therapeutic advantage in the use of Ara-C by manipulation of the mitotic cycle and individualized treatment schedules.

While the many studies on cell kinetic alterations induced by Ara-C in low doses or by continuous infusion have not led to unequivocal results, equally little is known regarding the cell kinetic responses after high-dose administrations. Based on the data obtained in a model system, a pilot study on the treatment of childhood ANLL with high-dose Ara-C was started by the Dutch Study Group for Childhood Leukemia. In order to most rationally administer the drug, cell kinetic perturbations have been measured by flow cytophotometry in bone marrow specimens obtained at various time intervals after administration of a bolus injection of Ara-C (1,000 mg/sq m) and during treatment with repeated injections.

MATERIALS AND METHODS

Patients

Cell kinetic studies after the first injection were performed in 10 children with relapsed ANLL (Table 1). A bone marrow aspirate was obtained from the patients just prior to the intravenous bolus administration of Ara-C (500–1000 mg/sq m) and 4 additional samples at different time intervals thereafter up to 48 hr. Subsequently, 6 children with ANLL (3 relapsed, 3 with primary disease) were studied during further treatment with individualized injection intervals.

Samples

Samples were defibrinated and diluted with buffered saline. Erythrocytes, mature myeloid, and dead or moribund cells were removed by centrifugation on Ficoll-Isopaque gradients. Cells from the interface were collected, washed, and fixed in acI.0% Contamination of marrow aspirates by nucleated cells from the peripheral blood was controlled by differential counting of erythrocytes and...
operative Group Classification.

coefficients of 2%-4% were analyzed by planimetry

peripheral blood admixture was necessary in only 1 of

and blood according to Holdninet et al.'5 Connection for

Sample Analysis

cells from the peripheral blood.

The histographs in Figs. 1 and 3 were

after tenfold amplification of the S and G2/M phase

graphs of approximately

clumps.

previously'4 was less laborious and produced no cell

added. This modification of methods published pre-

nucleated cells in corresponding samples of marrow

and blood according to Holdninet et al.'5 Correction for

for peripheral blood admixture was necessary in only 1 of

55 bone marrow aspirates containing >25% nucleated

cells from the peripheral blood.

Sample Analysis

For analysis by flow cytophotometry, approximately

2×10^6 fixed cells were incubated at 37°C in 1 ml

RNase for 30 min. Next, 1 ml of pepsin solution (2

mg/ml 0.4% HCl) was added without centrifugation,

and after 20 min, 2 ml of a staining solution containing

ethidium bromide (20 mg/liter), stain Hoechst 33258

(4.0 mg/ml), and bovine serum albumin (5.0 g/liter)

in double-strength buffer at pH 7.3 was similarly added. This modification of methods published previous-

ly'4 was less laborious and produced no cell

Samples were analyzed with the ICP I 1 pulse

cytophotometer equipped with a sheet flow cell. Histo-

graphs of approximately 5×10^6 cells with variation

coefficients of 2%-4% were analyzed by planimetry

after tenfold amplification of the S and G2/M phase

compartments.14 The histographs in Figs. 1 and 3 were

redrawn from the original recordings for three-dimen-
sional representation.

RESULTS

Bone Marrow Kinetics After the First

Ara-C Injection

Serial bone marrow aspirates were obtained from

children with ANLL: 1 pretreatment sample and 4

samples after a bolus injection with Ara-C (1000 mg/sq m) with a median time interval of 12 hr in the first 3 patients entering this study. In subsequent patients, the postinjection aspirates were obtained at 4–6-hr intervals before and after the expected maximum of recruitment, allowing a more detailed analysis. Recruitment responses were only determined in series of measurements containing evaluable histo-

graphs before and after the recruitment maximum (compare Table 2). This procedure allowed the deter-

mination of the recruitment maxima within limits of

4–6 hr and independent of the variations in sampling
time.

A characteristic response on Ara-C was observed in

8 of 10 patients as illustrated in Fig. 1. The drug

caus ed an initial reduction of cells in S (channels 45–75) and G2/M phase (channel 80) of the cell cycle

followed by a wave of cell proliferation with a distinct

maximum. In patient R.K. (Fig. 1), the prethenapy

value of S-phase cells was 7.0% and a maximum of

20.0% was registered after 30 hr, declining to 13.0% at

t40. Synchronization was apparent from the small

number of early S-phase cells at t24 and the accumula-

tion of cells in early and mid-S-phase at t30. Note that in the histograms of samples at or after the maximum

(i.e., at t30 and t40), cells with a DNA content

equivalent to G2 phase and mitosis (channel 80) were

observed in relative amounts similar to the pretreat-

ment sample at t0. These observations are representa-

tive of all evaluable patients, summarized in Table 2. By comparing all histograms of these patients, a

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>FAB Classification</th>
<th>Percent Nucleated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. A.W.</td>
<td>14</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M4</td>
<td>76</td>
</tr>
<tr>
<td>2. G.O.</td>
<td>12</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M2</td>
<td>72</td>
</tr>
<tr>
<td>3. R.K.</td>
<td>8</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M2</td>
<td>55</td>
</tr>
<tr>
<td>4. M.G.</td>
<td>14</td>
<td>F</td>
<td>ANLL; relapse</td>
<td>M2</td>
<td>75</td>
</tr>
<tr>
<td>5. I.v.L.</td>
<td>2</td>
<td>F</td>
<td>ANLL; relapse</td>
<td>M5</td>
<td>25</td>
</tr>
<tr>
<td>6. W.v.H.</td>
<td>9</td>
<td>M</td>
<td>ANLL; primary</td>
<td>M2</td>
<td>49</td>
</tr>
<tr>
<td>7. E.V.</td>
<td>9</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M5</td>
<td>82</td>
</tr>
<tr>
<td>8. J.M.</td>
<td>3</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M5</td>
<td>25</td>
</tr>
<tr>
<td>9. E.H.</td>
<td>15</td>
<td>F</td>
<td>ANLL; relapse</td>
<td>M4</td>
<td>70</td>
</tr>
<tr>
<td>10. R.R.</td>
<td>10</td>
<td>M</td>
<td>ANLL; relapse</td>
<td>M2</td>
<td>48</td>
</tr>
</tbody>
</table>

*Classification according to the French-American-British (FAB) Cooperative Group Classification.

Table 2. Summary of Cytokinetic Responses in Bone Marrow of ANLL Patients Following a Single Bolus Injection of Ara-C

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose (mg/sq m)</th>
<th>Percent S Before Treatment (A)</th>
<th>Percent S at Recruitment Maximum (B)</th>
<th>Ratio B/A</th>
<th>Time of Recruitment Maximum (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. A.W.</td>
<td>1,000</td>
<td>3.5</td>
<td>15.5</td>
<td>4.4</td>
<td>36</td>
</tr>
<tr>
<td>2. G.O.</td>
<td>1,000</td>
<td>5.5</td>
<td>16.0</td>
<td>2.9</td>
<td>30</td>
</tr>
<tr>
<td>3. R.K.</td>
<td>1,000</td>
<td>7.0</td>
<td>20.0</td>
<td>2.9</td>
<td>30</td>
</tr>
<tr>
<td>4. M.G.</td>
<td>1,000</td>
<td>8.5</td>
<td>18.5</td>
<td>2.2</td>
<td>24</td>
</tr>
<tr>
<td>5. I.v.L.</td>
<td>1,000</td>
<td>8.5</td>
<td>15.0</td>
<td>1.8</td>
<td>22</td>
</tr>
<tr>
<td>6. W.v.H.</td>
<td>1,000</td>
<td>11.0</td>
<td>19.0</td>
<td>1.7</td>
<td>22</td>
</tr>
<tr>
<td>7. E.V.</td>
<td>500</td>
<td>12.5</td>
<td>28.0</td>
<td>2.2</td>
<td>18</td>
</tr>
<tr>
<td>8. J.M.</td>
<td>1,000</td>
<td>14.0</td>
<td>24.5</td>
<td>1.7</td>
<td>17</td>
</tr>
<tr>
<td>9. E.H.</td>
<td>1,000</td>
<td>10.5</td>
<td>Not evaluable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. R.R.</td>
<td>500</td>
<td>6.0</td>
<td>Not evaluable</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
transit time through S-phase during recruitment of maximally 12 hr could be estimated. Accordingly, the observed maxima resulted from a synchronous wave of actively cycling cells rather than from a transient arrest in S-phase.

Among the various patients, considerable variation was observed in the time of appearance of the recruitment maximum, i.e., between 17 and 36 hr. However, this time interval was inversely proportional to the S-phase percentages before treatment. Thus, slowly proliferating tumors (low S-phase percentage) responded by a late appearance of the synchronous wave of compensatory growth. The correlation between pretherapy S-phase percentages and the appearance of the subsequent maximum is given in Fig. 2. In addition, the number of S-phase cells measured at the recruitment maximum was always higher than in the pretreatment samples. The ratio, indicating the potentiation of the tumor cell population for a second dose of Ara-C, was again inversely proportional to the proliferative activity before treatment (Table 2).

In two patients (Table 2, E.H. and R.R.), no evaluable responses could be derived from the histograms, which showed only minor variations. This could not be ascribed to apparent failures in the measurements nor to an unusual clinical response, since both patients went into complete remission. In case of patient R.R., the bone marrow aspirates obtained at t28 and t38 contained 5.5% and 1.5% S-phase cells, respectively. In retrospect, a recruitment maximum after about 34 hr (cf. Fig. 2) might have been missed by these measurements except for the decrease after 38 hr. This cannot explain the failing results in patient E.H., from whom samples were obtained at t20 and t24 with an expected maximum of recruitment at t22. However, in this patient the relapse was detected when she was referred to the hospital for bone marrow transplantation after previous induction therapy. In this early stage of the disease, the developing leukemia may have contained only a small proportion of noncycling cells, and accordingly a weak recruitment response that went undetected.

It appears from these results that childhood ANLL responds to high-dose application of Ara-C similarly to that observed in a rat AML model. However, the timely aspects of this response are highly individual and related to the proliferative status of the tumor cell population before treatment.

**Bone Marrow Kinetics During Therapy**

In a pilot clinical study, 6 young ANLL patients were treated with 12 injections of high-dose Ara-C. The injection intervals for each patient were individually determined from the pretreatment S-phase percentage. For this purpose, the curve in Fig. 2 was used to estimate the time of the recruitment maximum. The injection interval was then fixed as the expected recruitment time minus 4 hr. Since the appearance of a recruitment maximum can be inferred from Fig. 2 within accuracy limits of 4–6 hr only, an earlier injection interval may reduce the risk of recruited cells having entered the insensitive G2 phase of the cell cycle before administration of the next dose. Moreover, if the timing is appropriate, the recruited cells will be exposed to subsequent Ara-C injections while accumulated in the most sensitive early S-phase. As shown in Fig. 3, this prediction could be confirmed in aspirates.
Fig. 3. DNA per cell distributions in bone marrow aspirates of an ANLL patient obtained before Ara-C injections 1–3. The injection intervals were individually determined as outlined in the text. Note progressive accumulation in early S phase. Magnification as in Fig. 1.

Fig. 4. The percentages of S-phase cells recorded from DNA per cell distributions in bone marrow aspirates obtained just prior to consecutive injections with Ara-C. The samples were obtained from 6 patients treated for ANLL using individual injection intervals as described in the text.

To further study the cell kinetic reactions during therapy, S-phase percentages were determined in 5 more patients, each contributing 1–3 aspirates randomly obtained before the various injections. In view of the large variation among individual patients related to the pretreatment S-phase values, this investigation was limited to new patients with initial values ranging from 4% to 6% and corresponding injection intervals of 27–31 hr. As shown in Fig. 4, the recruitment response was detectable for about 4–5 drug applications and was maximal as a consequence of the second dose. The characteristic DNA/cell distribution with increased numbers of early S-phase cells (cf. Fig. 3) could be observed in most samples obtained before injections 2–5. Samples obtained afterwards did not demonstrate this characteristic distribution anymore. No S-phase cells could be recorded in the 2 samples obtained after the last injection (no. 12), corresponding with clinical aplasia of the patients. In additional samples obtained from 2 patients between injections 3 and 4 and injections 4 and 5, low values of 7.0% and 3.0%, respectively were recorded, indicating the persistence of cyclic responses similar to those illustrated in Fig. 1 after the first injection.

All 6 patients (3 relapsed, 3 in primary disease) went into complete remission after 12 injections with Ara-C followed by 1 or 2 doses of adriamycin (40 mg/sq m). Aplasia occurred in all patients and lasted for 2–3 wk. No unusual or limiting toxicity was experienced during this period. Of the patients with previous relapse, one received bone marrow transplantation after remission induction. Another patient (J.V.) developed a second relapse within 2 m. At that time the leukemia was characterized by chromosomal hyperdiploidy and a very high proliferative activity with 19.0% S-phase cells. These findings indicated the emergence of a clonal variant from the original diploid tumor with 6.0% S-phase cells at diagnosis. In view of the particular cell kinetic properties, reinduction was successfully attempted by a 1-wk infusion of Ara-C and one dose of adriamycin. The other four patients received consolidation therapy at 5-wk intervals consisting of 6 doses of Ara-C and 1 dose of adriamycin and were still in remission after 6 mo.

From these data it is concluded that the recruitment response to high-dose Ara-C observed after the first injection is maintained during at least three more injections administered with individualized time intervals. The large increments in the proportions of sensitive S-phase cells during this period probably reflect considerable potentiation of the tumor cell population for successive Ara-C injections.

DISCUSSION

The objective of this study was to investigate whether cell kinetic responses observed in a rat AML model after high-dose Ara-C could be monitored in children with (relapsed) ANLL. The results with 8/10
patients have indeed shown that high-dose Ara-C induced cell kinetic responses qualitatively similar to those seen in the rat model. As illustrated in Fig. 1, an initial decrease in S and G2/M phase cells was followed by a synchronous wave of cells traversing these cycle phases. As shown in Fig. 2 and Table 2, the data also revealed considerable variation among individual patients with regard to the degree of recruitment and the time of appearance of the recruitment maxima. Both parameters appeared inversely related to the pretreatment S-phase percentage. Using individualized injection intervals, the characteristic response persisted during 4–5 successive injections (Figs. 3 and 4). During this period, the number of sensitive S-phase cells increased 2–6-fold, indicating considerable potentiation relative to the untreated tumor cell population. These data may suggest that efficient and individual treatment schedules can be designed based on cell kinetic measurements.

However, in the literature, conflicting results have been reported as to the relevance of cell kinetic studies during chemotherapy of acute leukemia. Using autoradiography, increased labeling indices have been found by Lampkin et al., whereas others did not obtain evidence for significant recruitment or synchronization. Drenthe-Schonk et al. found no recruitment of leukemic cells in 11 adult ANLL patients treated with Ara-C (100–1000 mg/sq m). In contrast, our findings are confirmatory of those reported by Lampkin et al.

In their study, partial synchronization was induced by an intravenous bolus injection of Ara-C followed by a 12-hr infusion during the subsequent maximum of DNA synthesis. The interval, determined by mitotic counts and 3H-TdR incorporation rates, ranged from 18 to 24 hr. This time interval corresponds with the recruitment times determined in many patients of the present study. However, the variation among individual patients was considerably larger in our study and varied between 17 and 36 hr. The following factors may have contributed to this difference: (1) the composition of the patient sample, (2) the cytolytic effect of high-dose as compared to low-dose Ara-C as discussed below, and (3) the direct measurement of cell cycle distribution by flow cytometry.

Apart from the possibility that the responses to Ara-C in childhood ANLL are different from those in the adult leukemia, a number of other possibilities may explain the contradictory results mentioned before. First of all, we have observed considerable variation in the degree and the time of appearance of the recruitment response among individual patients. Therefore, a minimum of 5 samples with variable sampling times is required to determine a recruitment response that may go undetected with less samples at fixed sampling times. Moreover, in most studies, Ara-C was administered in low doses (80–120 mg/sq m) or by continuous infusion. Under these conditions, the drug is primarily cytostatic in patients as well as in model systems. The rapid cytolytic action of high-dose Ara-C, on the other hand, will give rise to a defined signal for recruitment of resting cells. Finally, the use of Ficoll-Isoopaque density separation removed most mature normal cells and dead or moribund tumor cells from our preparations. Control studies (unpublished) revealed a five-fold enrichment of cycling blasts from the peripheral blood leukocytes of patients with chronic myelocytic leukemia. In a patient with highly differentiated ANLL, the peripheral S-phase cells (amounting to 2.5%) were only found in the cells harvested from the gradient interface but not in the bulk of more mature pathologic cells sedimenting into the high-density solution. As a result, the methods employed in this study focus on the proliferating fraction of the tumor cells. The combined effects of sampling time, drug concentration, and sample preparation have probably permitted the detection of characteristic and individual responses in most of the patients in this study.

It is not directly obvious from the results whether the Ara-C-induced perturbations reflect a wave of resting cells induced to proliferate (recruitment) or a temporary arrest in S phase (synchronization). We favor the idea that the response is largely determined by recruitment for the following reasons. In many patients (Table 2), the actual "movement" of the cells from early S to late S and G2/M phase could be inferred from the pre- and postmaximum samples within 8–12 hr. Synchronization, however, would inhibit transition to G2/M phase as actually shown in patients treated with low dosages. Finally, one may expect maximal recruitment responses in tumors with the highest proportion of noncycling cells, whereas synchronization will be maximally demonstrated in actively growing populations. As shown in Table 2, tumors with low S-phase percentages (i.e., small growth fractions) displayed the highest degree of potentiation, a response compatible with a recruitment type of reaction.

In conclusion, the results demonstrate the basic possibility of measuring the characteristic cell kinetic perturbations during Ara-C treatment of childhood ANLL. Most importantly, the data accounted for individual differences among patients with regard to the relevant reactions, confirming and extending other observations. Since flow cytometric data can be obtained within 2 hr after sampling, a direct interaction between laboratory findings and clinical practice could be accomplished.
However, the relevance of cell kinetic parameters at diagnosis for the subsequent clinical course has been doubted by some authors. Notably, in acute lymphocytic leukemia of childhood, recent improvements in prognosis are mainly due to systemic consolidation therapy and prophylactic therapy after remission induction. In the acute nonlymphocytic leukemia, however, relapses are often observed within 2–3 mo, meaning that initial tumor cell reduction had been achieved over a few decades only. Accordingly, more efficient remission strategies are a minimum requirement to improving the prognosis of this mostly fatal disease. Using individualized schedules, remission rates as high as 81% have been achieved, and in our study, complete remission occurred in 14 of 16 children. Moreover, the emergence of a cell kinetic variant during the relapse of patient J.V. (see Results) also indicated a possible role of individual cell kinetic properties in the clinical outcome. The possible existence of cell kinetic variants apart from drug-resistant ones should be taken into account for optimal consolidation and maintenance schedules. Whether individualized therapeutic schemes with high-dose Ara-C guided by cell kinetic parameters can improve prognosis beyond current achievements remains to be established by an ongoing clinical study.

REFERENCES

Cell kinetic responses in childhood acute nonlymphocytic leukemia during high-dose therapy with cytosine arabinoside

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