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Inhibition of DNA Synthesis by Cytosine Arabinoside: Relation to Response of Acute Non-lymphocytic Leukemia to Remission Induction Therapy and to Stage of the Disease*

HARVEY D. PREISLER,† JOSHUA EPSTEIN,† AZRA RAZA,* NOZAR AZARNIA,† GEORGE BROWMAN,‡ LYNDA BOOKER,‡ JACK GOLDBERG,§ ARLAN GOTTLIEB,§ JAMES BRENNAN,‖ HANS GRUNWALD,¶ KANTI RAI,¶ RALPH VOGLER,** LEE WINTON,** KENNETH MILLER†† and RICHARD LARSON‡‡

†*Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, U.S.A.,* ‡*Hamilton Clinic, Ontario Cancer Foundation, 711 Concession Street, Hamilton, Ontario L8V 1C3, Canada,* §*Upstate Medical Center, Department of Medicine, 750 East Adams Street, Syracuse, NY 13210, U.S.A.,* ‖*University of Rochester School of Medicine, Department of Medicine, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A.,* ¶*Long Island Jewish Hospital, Queens Hospital Center, Department of Hematology, 82–68 164th Street, Jamaica, NY 11432, U.S.A.,* *******Emory University School of Medicine, Leukemia Research Laboratory, 718 Woodruff Memorial Bldg., Atlanta, GA 30322, U.S.A.,* ††*New England Medical Center Tufts/Boston, Department of Hematology, 171 Harrison Avenue, Boston, MA 02167, U.S.A. and* ‡‡*University of Chicago, Hematology/Oncology, PO Box 420, 950 East 59th Street, Chicago, IL 60637, U.S.A.*

Abstract—*The sensitivity of leukemic marrow cell DNA synthesis to cytosine arabinoside (araC) exposure* in vitro *was studied in specimens obtained from patients with acute non-lymphocytic leukemia. Cells from patients who had been treated with araC in the past were more likely to be resistant to the effect of araC on DNA synthesis than cells obtained from patients who had not been so-treated previously. Resistance to the effects of araC on DNA synthesis was associated with the failure of high-dose araC therapy to induce remissions in relapsed patients, whereas remission induction failure in previously untreated patients was not associated with araC resistance.*

INTRODUCTION

CYTOSINE arabinoside (araC) is the primary drug for treatment of patients with acute non-lymphocytic leukemia (ANLL), and consequently many studies have attempted to define the factors which determine clinical response to chemotherapy. Unfortunately these studies have failed to demonstrate any relationship between either the uptake and phosphorylation of the drug by leukemic cells or the effects of the drug on leukemic cell DNA synthesis and the outcome of remission induction therapy [1–3]. The design and interpretation of these studies was confounded by the fact that the patients were not treated with single-agent araC therapy and no distinction was made between patients who did not enter remission because of resistant leukemia and treatment failure due to death during therapy. Thus data from patients who might have entered complete remission had they survived were pooled with data obtained from patients with truly araC-resistant leukemia, thereby preventing a direct comparison of the araC sensitivities of leukemic cells obtained from patients who entered remission with those of patients who failed therapy because of persistent leukemia.

The introduction of single-agent high-dose cytosine arabinoside therapy (HD araC) [4–6] and the classification of remission induction failures into documented drug-resistant failures and drug sensitivity 'inevaluable' or 'other' failures permits a more thorough examination of the factors which determine response to araC therapy [7–10]. In a prior publication we reported that pretherapy

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tumor cell mass, the percentage of cells in S phase and the metabolic sensitivity of ANLL cells to araC all play a role in determining response to HDaraC therapy [11]. The present communication will extend our initial report and will focus on the relationship between inhibition of DNA synthesis by araC and the outcome of remission induction therapy at different stages of ANLL.

MATERIALS AND METHODS

The leukemic marrow cells of 77 patients with acute non-lymphocytic leukemia (ANLL) as defined by the French–American–British working party [12] who were eligible to be treated with high-dose cytosine arabinoside (HDaraC) therapy [6, 11, 13] were studied for their sensitivity to araC. Thirty-four patients were treated and studied at the time of initial diagnosis and 43 patients at the time of first, second or third relapse. All relapsed patients had been treated in the past with regimens which included cytosine arabinoside. Patients $\leqslant$70 yr old were treated with a single course of 3 g/m^2 of cytosine arabinoside q 12 hr for 12 doses while patients >70 yr old received 2 g/m^2 [5, 13]. The cytosine arabinoside was administered as a 75-min infusion. Informed consent was obtained from each patient.

The outcome of treatment was characterized as complete remission as defined by Cancer and Leukemia Group B [14] or remission induction failure. Induction failure was divided into failure because of resistant disease (RD) (also referred to as persistent leukemia) or induction death ('other' failure) [7, 10]. Patients were classified as RD failure if the day 13 marrow biopsy cellularity was >5% on biopsy, the aspirate was $\geqslant$1 of 4+ cellular and contained leukemic cells or if leukemic cells repopulated the marrow after HDaraC therapy produced severe marrow hypoplasia. Patients were classified as being 'other' failures (i.e. failure not clinically ascribable to RD) if they expired early in therapy (<day 13) or if they died while their marrow was extremely hypoplastic. The distinction between RD failures and 'other' failures is considered of prime importance since the clinical sensitivity of the latter patient's leukemia to HDaraC was inevaluable, i.e. had the patients survived they might have entered CR (and hence had araC sensitive leukemia) or they might have proved to have RD. Thus to assess the reliability of a drug sensitivity assay it is important to include only those patients whose drug sensitivity was clinically evaluable [10].

Table 1 provides information regarding the outcome of remission induction therapy for the patients who were studied.

Assessment of in vitro *sensitivity to cytosine arabinoside*

The effects of araC on DNA synthesis are a reflection of a series of cellular events which ultimately play a role in determining the effect of the drug on the cell. These include uptake and activation of araC to the triphosphate form [15] and probably incorporation of araC into DNA [16]. These effects were studied from the perspective of both the immediate effects and persistent effects of araC on DNA synthesis. The former represents the effects of araC on DNA synthesis while the cells are being incubated in the presence of the drug while the latter assesses the effects of the drug on DNA synthesis after removal of araC from the medium. The persistence of effects may be a reflection of intracellular degradation of araCTP as well as cellular repair processes which may excise araC from DNA.

In vitro *studies of the properties of the leukemic cells*

Bone marrow was aspirated into a syringe containing 6% sodium citrate. The cell suspension was layered over Ficol-Hypaque (sp.gr. 1.077) and centrifuged at 1200 *g* for 30 min. The light density cells were recovered, washed twice and resuspended at 2×10^6 cells/ml in RPMI 1640 containing 10% fetal calf serum (v/v). The cells were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% room air and then used for the *in vitro* studies.

The 'immediate effects' of araC on DNA synthesis were measured by adding a sufficient amount of araC to the experimental cell suspension to bring the final araC concentration to the indicated level. After incubation for 1 hr, 50 μl of the cell suspension was placed in each of

Table 1. *Outcome of remission induction therapy*

	CR	RD	'Other' failures	No. of patients
All patients	19 (25%)	35 (45%)	23 (30%)	77
No prior therapy	11 (32%)	13 (38%)	10 (29%)	34
First relapse	4 (24%)	7 (41%)	6 (35%)	17
Second relapse	4 (21%)	9 (47%)	6 (32%)	19
Third relapse	0	6 (86%)	1 (14%)	7

five microwells containing 50 μl of [^{3}H]-TdR to make a final concentration of 10 μCi/ml and the incubation continued for an additional hour. The cells were then harvested using a multiple sample precipitator (Otto Hiller Co., Madison, WI) harvester. Control cells were incubated and processed under identical conditions except that they were not exposed to araC.

In an attempt to assess the ability of the cells to recover from the effects of exposure to araC, the persistence of effects of araC on DNA synthesis were assessed. To this end, after the initial incubation with araC for 1 hr as described above, a matched set of control cells and the araC exposed cells were washed and incubated for 3 hr more in araC-free complete medium at 37°C in a humidified incubator. [^{3}H]-TdR was then added for 1 hr and incorporation into DNA assessed as described above. The data are presented as percentage inhibition of DNA synthesis, calculated as follows:

$$\frac{\text{cpm for control cells} - \text{cpm for araC exposed cells}}{\text{CPM for control cells}} \times 100 = \% \text{ inhibition of DNA synthesis.}$$

Percentage of cells in S phase

Tritiated thymidine ([^{3}H]-TdR) labeling indices were assessed as previously described [17]. In brief, the leukemic cells were incubated for 60 min with 40 μCi/ml of [^{3}H]-TdR (sp. act. 60–70 Ci/mM). The incubation was terminated by the addition of 15 vols of cold medium containing 100 μg/ml of thymidine (TdR). The cell suspension was washed three times with cold medium containing TdR followed by two washes with cold medium without TdR and slides were made using a cytocentrifuge. The slides were then processed as decribed before [17]. Slides bearing the labeled cells were sent from each intergroup institution to Roswell Park Memorial Institute for autoradiographic study. Five hundred cell-labeling indices were performed by a single individual who did not know the outcome of treatment. Cells with more than five grains over the nucleus were considered to be labeled. DNA histogram analysis was performed using the ethidium bromide-mithramycin method [18] and an Ortho-diagnostics ICP20 with a 2150 computer.

Statistical methods

Since the distribution of the measured parameters were unknown, the distribution-free non-parametric Mann–Whitney test was employed to compare the percentage inhibition of DNA synthesis for patients with different responses to remission induction therapy.

The correlations between the effects of araC on DNA synthesis and the [^{3}H]-TdR labeling index and between the immediate and persistent effects of araC on DNA synthesis were analyzed using Pearson correlation coefficients. Additionally, both Kendall and Spearman rank correlations were used to double-check the Pearson statistics.

RESULTS

Direct effects of araC on DNA synthesis

Figure 1a presents the average effects of different concentrations of araC on [^{3}H]-TdR incorporation into DNA by the cells of ten patients who entered CR, ten patients who failed induction therapy because of clinically documented resistant disease and ten 'other' failures. At all araC concentrations save 300 μg/ml, the cells of CR patients were on average more sensitive to araC than were the cells of RD patients. The cells of 'other' failures exhibited sensitivity to araC which was intermediate between that of the CR and RD patients. Figures 1b,c,d provide the dose response data for the individual patients. The cells of CR patients were very sensitive to low levels of araC with little increase in the inhibition of [^{3}H]-TdR incorporation being evident at araC concentrations above 0.3 μg/ml (Fig. 1b). For eight of the ten patients DNA synthesis was inhibited by $>70\%$ by 0.3 μg/ml of araC. Additionally, the araC dose response curves for the cells of different CR patients were similar and are spaced close together in the graph. In contrast, the cells of RD patients exhibited significant resistance at araC levels up to and including 3 μg/ml. For example, DNA synthesis was inhibited by $\geqslant 70\%$ by 0.3 μg/ml of araC for only three marrow specimens and 3 μg/ml of araC inhibited DNA synthesis by $\geqslant 70\%$ in only six of the ten specimens. Additionally, the dose response curves for individual patients are widely spaced from each other on the graph at all araC levels up to 30 μg/ml (Fig. 1c). The dose response curves of 'other' failures were intermediate between those of CR and RD patients (Fig. 1d), with the dose response curves of some patients resembling those of CR patients and the dose response curves of other patients resembling those of RD patients.

Figure 2 relates inhibition of [^{3}H]-TdR incorporation by 0.3 or 3 μg/ml araC to the stage of the patients' leukemia. At both the 0.3- and 3-μg/ml concentrations the araC sensitivity of the cells of newly diagnosed patients to araC was relatively uniform, with only 22 and 10% of specimens being inhibited by $<80\%$ for the 0.3- and 3.0-μg/ml concentrations respectively. For the 0.3-μg/ml concentration DNA synthesis was

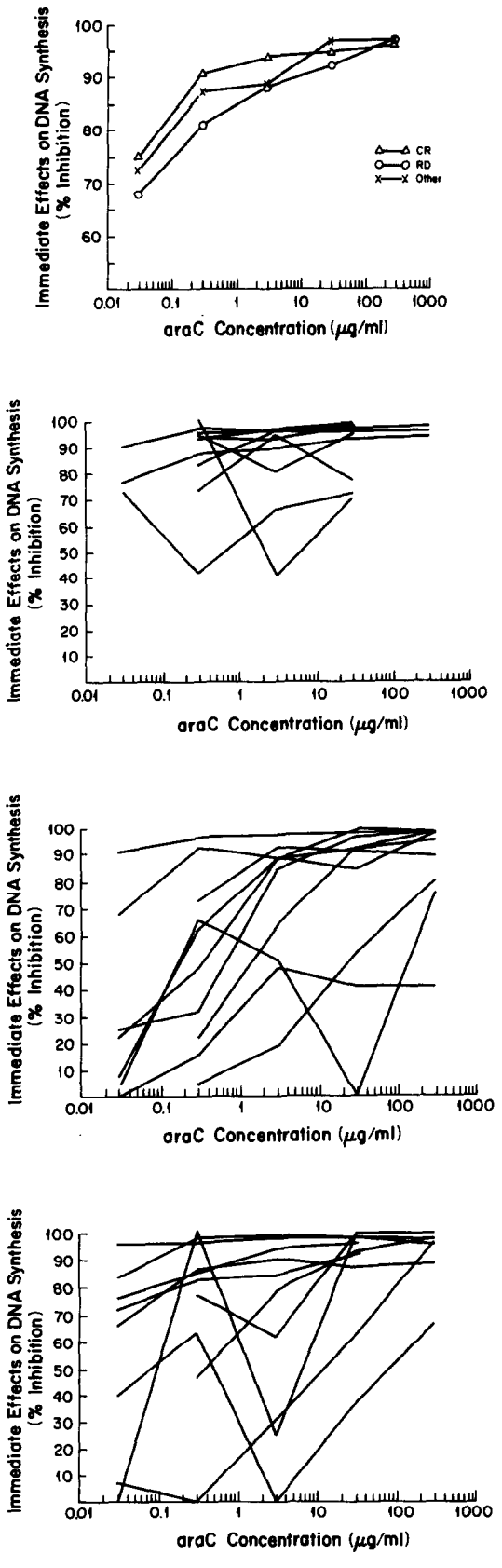

Fig. 1. Immediate effects of araC on DNA synthesis. (a) Average % inhibition of DNA synthesis vs concentration of araC for CR, RD and 'other' failure patients; (b) individual dose response curves for CR patients; (c) individual dose response curves for RD patients; and, (d) individual dose response curves for 'other' patients.

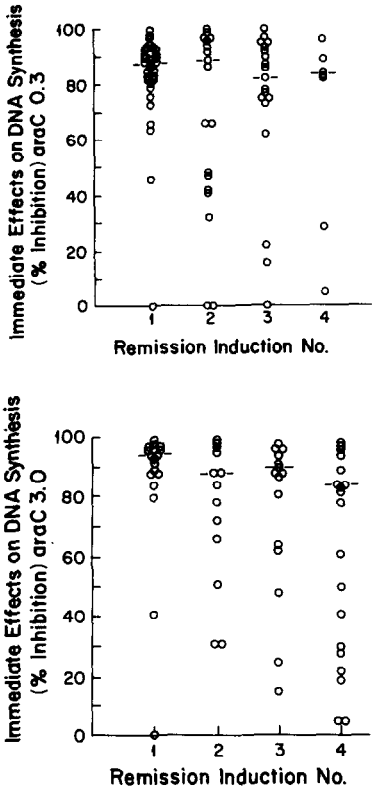

Fig. 2. Relationship between immediate effects of araC on DNA synthesis and stage of leukemia. (a) 0.3 μg/ml; and (b) 3.0 μg/ml. Bars indicate median values.

inhibited by <80% in 47, 54 and 43% of specimens obtained from patient in first, second and third relapse respectively. For the 3.0-μg/ml concentration the cells of 45, 31 and 40% of patients in first, second and third leukemic relapse were inhibitied by <80%. The median % inhibition of DNA synthesis for the cells of previously untreated patients when exposed to 0.3 and 3.0 μg/ml of araC was 87 and 94% respectively. Considering all relapsed patients together, the corresponding median values were 83 and 87%. The differences between the patients studied at the time of initial diagnosis or at the time of relapse for the 3.0-μm/ml concentration approach a level of statistical significance ($P = 0.08$).

The data presented in Fig. 3a demonstrate that the effects of araC at 0.3 μg/ml on [^{3}H]-TdR incorporation into DNA were significantly greater for the cells of patients who entered CR than for patients who failed therapy because of RD ($P = 0.007$), with mean ± S.E. for % inhibition and median values of 83 ± 6%, 90% and 71 ± 4% and 81% for CR and RD patients respectively. When these same data (araC 0.3 μg/ml) are presented separately for previously untreated patients and for relapsed patients (Fig. 3b,c) it beome apparent that RD treatment failure for previously untreated patients was not signifi-

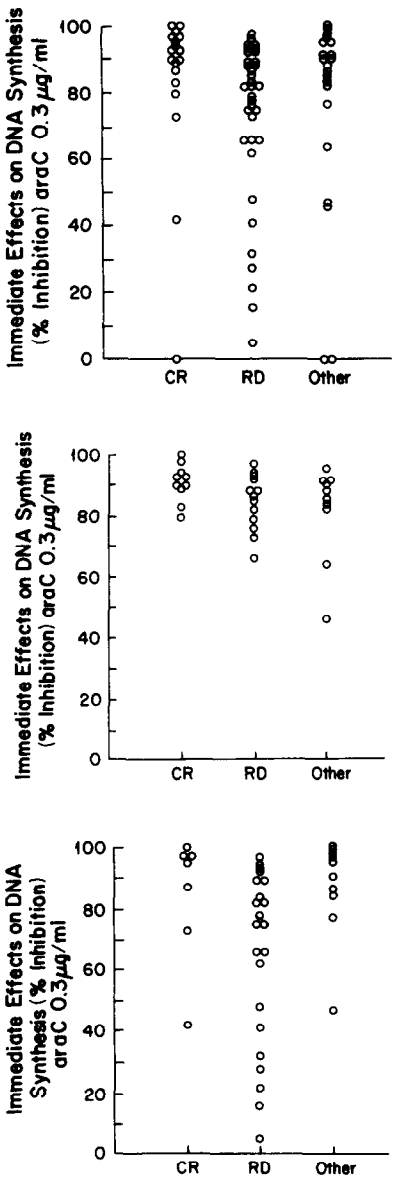

Fig. 3. Relationship between immediate effects of araC (0.3 μg/ml) on DNA synthesis and the outcome of remission induction therapy. (a) All patients; (b) previously untreated patients; and (c) relapsed patients.

cantly associated with cellular insensitivity to araC (median % inhibition values of 90 and 85% respectively for CR and RD patients). When the corresponding data for relapsed patients were considered together statistically significant differences were noted between CR and RD patients (median % inhibition values of 88 and 75% for CR and RD patients respectively, $P = 0.03$). A similar pattern was noted for the 3.0-μg/ml concentration of araC but statistically significant differences were not present. The use of araC at concentrations of 30 or 300 μg/ml dampened the differences between CR and RD patients. Exposure of cells to araC at 0.03 μg/ml provided data which were similar to those provided by the 0.3- and 3-μg/ml studies.

The median % inhibition of DNA synthesis for CR patients with no prior therapy whose cells were exposed to 0.3 or 3 μg/ml of araC was 90 and 93%, while the corresponding values for the cells of relapsed patients who entered CR were 88 and 94%. In contrast, the sensitivity to araC of marrow cells of RD patients who were treated at the time of relapse was significantly less than that of RD patients who had been treated at the time of initial diagnosis with araC at 0.3 and 3 μg/ml. The median % inhibition values were 75 and 87% for the former patients, as opposed to 85 and 90% for the latter patient group ($P = 0.05$ and 0.007 respectively; Fig. 3b,c).

Persistence of araC effects on [^{3}H]-TdR incorporation after removal of araC from the medium

Figure 4a presents information demonstrating that at all concentrations of araC the effects on [^{3}H]-TdR incorporation were more persistent for the cells of CR patients than for the cells of patients who would not enter CR. Figure 4b provides the detailed data for the relationship between the persistent effects of 0.3 μg/ml and the outcome of remission induction therapy for all patients. The median % persistent inhibition of DNA synthesis for CR patients was 73% while for RD patients it was 60% ($P = 0.06$). Figure 4c,d provide these data for newly diagnosed patients and for patients treated at the time of leukemic relapse. The trends are similar for both patient data sets but the differences between CR and RD patients were not statistically significant when analyses for each group were performed separately. Studies employing 0.03, 3.0, 30 and 300 μg/ml of araC provided similar data; only the data for 0.3 μg/ml were close to the level of statistical significance. Figure 4e demonstrates that the persistence of araC effects of 0.3 μg/ml on [^{3}H]-TdR incorporation after the araC was removed from the medium was comparable regardless of the stage of the patients leukemia. The same observations were made at the other concentrations of araC. It should be noted, however, that the persistence of araC effects were >0 for all previously untreated patients while zero values were obtained for 4/14 and 4/16 patients in first or second relapse respectively.

Collateral measurements in patients who were RD treatment failures but whose cells were sensitive (>80% inhibition) to the immediate effects of araC

Table 2 provides data regarding pretherapy tumor cell mass (as estimated by marrow cellularity), the percentage of cells in S phase and the persistence of araC effects for 15 RD treatment

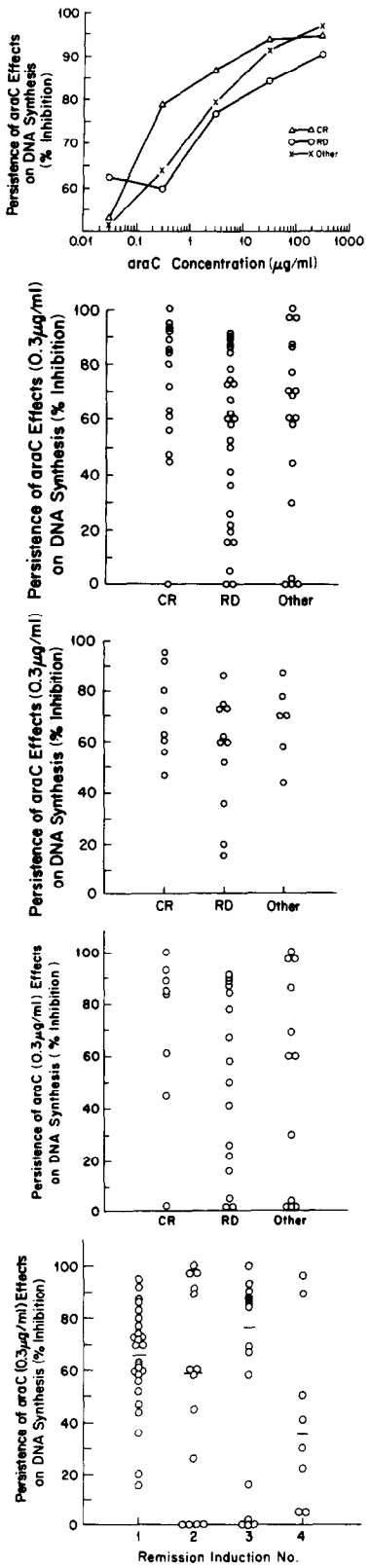

Fig. 4. Persistence of araC effects on DNA synthesis. (a) Average dose response curves for CR, RD and 'other' failure patients; (b) relationship between persistence of araC effects (0.3 μg/ml) and outcome of therapy—all patients; (c) relationship to outcome of therapy for previously untreated patients; (d) relationship to outcome of therapy for relasped patients; and (e) relationship between persistence of araC effects in DNA synthesis and stage of leukemia.

failures whose leukemic cells were sensitive to the immediate effects of araC. Nine of the 12 patients for whom pretherapy biopsy cellularity data were available had high tumor cell masses (marrow cellularity $\geqslant 70\%$), three of ten patients with available data gave evidence of having few cells in S phase ($<6\%$), while the persistence of araC effects on DNA synthesis was low ($<70\%$) for four of the 15 patients for whom information was available. Taken together, all but two of these 15 RD treatment failures whose leukemic cell sensitivity to the immediate effects of araC on DNA synthesis was high were associated with a high pretherapy tumor cell mass (nine patients), with few cells in S phase (four patients) or with lack of persistence of araC effects (four patients). Some patients had more than one adverse prognostic sign.

[^{3}H]-TdR labeling index (LI) studies were performed on cells obtained from 31 patients whose cells were also studied for sensitivity to 0.3 μg/ml of araC and for 22 patient studies at 3 μg/ml. The Pearson correlation coefficient between the LI and the immediate and persistent effects of araC at 0.3 μg/ml on DNA synthesis were 0.1846 and 0.1122 respectively, while the comparable correlation coefficients between the LI and the immediate effects of 3.0 μg/ml araC were 0.3109 and 0.0889 respectively.

DISCUSSION

The data presented here demonstrate that the inability of cytosine arabinoside to inhibit leukemic cell DNA synthesis *in vitro* was associated with a reduced likelihood of a complete remission being produced by HDaraC therapy. Dose response curves demonstrated that only at araC concentrations $\geqslant 30$ μg/ml were the inhibitory effects of araC comparable on the cells of CR and RD patients. These concentrations are not sustainable *in vivo* with current HDaraC therapy [6, 19, 20]. Even at these extremely high concentrations, however, the cells of some clinically resistant patients were insensitive to araC. Of the araC concentrations tested, the highest correlation between *in vitro* sensitivity and treatment outcome was noted when 0.3 μg/ml was used for the *in vitro* test.

Analysis of the effects of araC on leukemic cells obtained at the time of initial diagnosis or at the time of leukemic relapse demonstrated that leukemic cell insensitivity to araC was uncommon for previously untreated patients but was common for patients treated with araC in the past. These data suggest that selection of rare drug-resistant cells present at the time of initial

Table 2. Collateral characteristics of RD Patients whose leukemic marrow cells were sensitive to the immediate effects of araC on DNA synthesis (>80% inhibition)

Remission induction (RI) attempt: patient No.	Pretherapy marrow biopsy cellularity	Persistant effects of araC (3.0 μg/ml) on DNA synthesis (% inhibition)	% marrow cells in S phase LI	% marrow cells in S phase Flow cytometry
RI No. 1				
1	90%	85%	ND*	13%
2	70%	78%	10%	10%
3	90%	55%	25%	26%
4	ND	52%	ND	3%
5	90%	84%	ND	ND
6	ND	90%	5%	10%
7	ND	68%	6%	19%
RI No. 2				
1	90%	90%	19%	ND
2	90%	65%	ND	ND
RI No. 3				
1	90%	78%	7%	13%
2	80%	74%	ND	9%
3	65%	92%	8%	4%
4	50%	100%	ND	ND
RI No. 4				
1	50%	96%	12%	15%
2	85%	84%	ND	ND

*ND = not done.

diagnosis or the appearance of new araC-resistant mutant cells which appeared during initial therapy had occurred. Consistent with these observations was the fact that cellular metabolic resistance to araC [21] was a significant cause of remission induction failure only for relapsed patients. The data presented in Table 2 demonstrate that factors in addition to cellular metabolic sensitivity to araC play a role in determining response to HDaraC therapy. As might be expected, the data regarding the araC sensitivity of the cells of patients who were 'other' failures were intermediate between those of the cells of CR and RD patients, demonstrating that potential CR and RD patients were present within this treatment failure category.

The immediate effects of araC on DNA synthesis and the persistence of these effects after removal or araC from the medium were each correlated with outcome and were also correlated with each other, with correlation coefficients of 0.4872 ($P<0.001$) and 0.5797 ($P=0.001$) for the immediate and persistent effects of 0.3 and 3.0 μg/ml of araC respectively. Though statistically significant, these correlations were weak, and in some individual patients profound immediate araC effects on DNA synthesis were rapidly reversible upon transfer of the leukemic cells to araC-free medium. While prior exposure to araC was associated with an increased likelihood of the presence of cells resistant to the immediate effects of araC on DNA synthesis, this was not the case for the persistence of araC effects after removal of araC from the medium. These observations suggest that clinical resistance to araC on the basis of a decreased intracellular half-life of araCTP or on the basis of the 'repair' of DNA into which araC has been incorporated were not common mechanisms of resistance to araC but rather that the development of araC resistance was probably associated with decreased uptake or phosphorylation of the drug, increased levels of competing metabolites or a decrease in the sensitivity of DNA polymerase to araC. The observations that araC effects may not be 'persistent' in the cells of some patients suggests that araC administered by continuous infusion might be more active therapeutically in these patients than the intermittent bolus therapy utilized in the studies reported here.

In conclusion, the studies described here have demonstrated that cellular metabolic resistance to araC was not commonly associated with HDaraC remission induction failure for patients treated at the time of initial diagnosis. On the other hand, metabolic resistance was a significant problem for patients with relapsed disease. In assessing cellular sensitivity to araC, both the direct and persistant effects of araC on DNA synthesis should be measured.

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