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In Vitro Suppression of DNA Synthesis by a Remission Induction Agent and its Correlation with Response in Adult Acute Leukemia*

ANNE-MARIE MADDOX,†‡ DENNIS A. JOHNSTON,§ BARTHEL BARLOGIE,† MOHAMED HAQ,† MICHAEL J. KEATING† and EMIL J. FREIREICH†

*Departments of *Developmental Therapeutics and §Biomathematics, The University of Texas, M. D. Anderson Hospital and Tumor Institute at Houston, 6723 Bertner Avenue, Houston, TX 77030, U.S.A.*

Abstract—*Fifty-three adult patients who presented with previously untreated acute leukemia had leukemic cells incubated* in vitro *with five doses of cytosine arabinoside* (0.001–0.1 μg/ml) *and adriamycin* (0.1–10 μg/ml). *Thymidine incorporation was measured in treated and untreated cells. Greater than* 80% *suppression of pretreatment thymidine incorporation after treatment with* 0.004 μg/ml *of cytosine arabinoside correlated with complete remission (CR) of the patient. A Mahalanobis'* D^2 *score, which provides an index of the deviation of patient's* in vitro *drug response from the mean profile of responders, was calculated for each drug. Patients who had* D^2 *values greater than* 10 *for Ara-C were less likely to respond to therapy. Patients with low* D^2*s had rates of CR higher than predicted by the prognostic model employed at U.T. M. D. Anderson Hospital. However, unlike in our previous report, responders and non-responders were not clearly separable on the basis of the* D^2 *value.*

INTRODUCTION

DESPITE progress in treatment for adult leukemia, remission induction can still not be accomplished in 25–35% of adult patients with acute leukemia. Such primary induction failure may be the result of drug resistance of leukemic cells or limited host tolerance allowing fatal infection or hemorrhage, or a combination. In a recent study from U.T. M. D. Anderson Hospital, Estey *et al.* reported that chemotherapy resistance accounted for 22% of patients failing to respond to primary induction treatment [1].

While host tolerance may be improved through vigorous supportive care measures, chemotherapy resistance requires the use of more effective and more selective agents against leukemia. As primary induction failure is almost universally associated with early death, pretreatment knowledge of response likelihood may be important. Many investigators have developed remission induction models based on clinical and standard laboratory parameters [2]. However, there are differing results regarding the prognostic usefulness of pretreatment cytokinetic and *in vitro* growth parameters [3–8].

All these analyses, however, are based on population statistics. An ideal test for response prediction would be based on an individual patient's cellular response *in vitro* to potential induction agents. The development of stem cell assays for normal hematopoiesis, solid tissue neoplasms and leukemic cells has led several investigators to use such technology to test relative drug sensitivity by the reduction in survival of either primary or secondary leukemic colonies [9–12]. Unfortunately the usefulness of this type of assay is limited by low plating efficiencies, which allow it to be applied to only 50% of patients. In addition, it requires 7–14 days of incubation before results can be evaluated.

Other investigators have therefore undertaken studies to test whether inhibition of macromolecular synthesis, DNA, RNA and protein could be used as a substitute measurement for

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‡To whom reprint requests should be addressed at: 6431 Fannin, Room 5016, Division of Oncology/Hematology, Houston, TX 77030, U.S.A.

lethal cell injury [9–18]. We have previously reported on the usefulness of the observation of dose-dependent suppression of tritiated thymidine incorporation into the DNA of leukemic cells following exposure to adriamycin and cytosine arabinoside [19]. The present report extends this earlier study to 53 patients with acute leukemia. Although we still were able to recognize differences between responding and failing patients, the original complete separation of these subsets was unfortunately lost. However, when analyzed along with standard clinical and laboratory parameters predicting remission induction, this *in vitro* test ranked first.

MATERIALS AND METHODS

Patient population

Between July 1979 and November 1980 bone marrow cells from 53 adult patients with newly diagnosed acute leukemia undergoing remission induction treatment with anthracycline and cytosine arabinoside (Ara-C), vincristine and prednisone (OAP) were tested *in vitro*. During the first part of the study adriamycin was used for patients less than 50 yr of age (ADOAP) and rubidazone was administered for patients greater than 50 yr of age (ROAP). Since April 1980 patients with a predicted probability of response of less than 40% [2] received methanesulfon-*m*-anisidide-4′-(9-acridinylamino) (AMSA) and OAP (AMSAOAP), whereas patients who had a highly predicted probability of achieving complete remission (CR) received ADOAP [2]. All patients received 70–90 mg/m^2/day of cytosine arabinoside for 7 days and either AMSA 30 mg/m^2 daily for 7 days or rubidazone 200 mg/m^2 or adriamycin 55 mg/m^2 as a bolus on day 1. There were 42 patients with acute myelogenous leukemia (AML) and 11 patients had acute lymphocytic leukemia (ALL). The median age of all patients was 48 yr; 19 were men and 24 were women. The overall response rate was 62%. Table 1 shows the patient characteristics and response in each treatment group.

Complete remission was defined as the achievement of less than 5% blasts in the bone marrow with a normal peripheral hemogram consisting of a neutrophil count greater than 1000/μl, platelet count greater than 100,000/μl and a hemoglobin of 10 g/100 ml maintained for more than 30 days. Patients were considered as dying early if they died prior to being evaluable for the effects of two courses of chemotherapy. Patients were considered to have drug-resistant disease if they did not respond to two or more courses of chemotherapy for which they were evaluable. Cytoreduction was defined by the following formula:

$$\text{cytoreduction} = \frac{B_1C_1 - B_2C_2}{\Delta D(B_1C_1)} \times 100,$$

where B = percentage blasts, C = percentage cellularity, 1 = day 1 or pretreatment value, 2 = second sampling and ΔD = interval in days between samples. For example, a patient with 90% blasts and 100% cellularity prior to therapy for 50% blasts with 50% cellularity on day 10 would have:

$$\text{cytoreduction} = \frac{(90 \times 100) - (50 \times 50)}{10 \times (90 \times 100)} \times 100 = 7.9\% \text{ per day.}$$

In vitro *methods*

After informed consent was obtained prior to beginning chemotherapy, 1–2 ml of bone marrow was aspirated from the posterior iliac crest into 2 ml of RPMI 1640 media (Gibco, Grant Island, NY) that contained 250 units of preservative-free heparin. Mononuclear cell enrichment was accomplished by Ficoll-Hypaque gradient separation (density = 1.078 g/cm^3) after spinning at 1000 *g* for 20 min at 4°C [20]. Interphase cells were washed in phosphate-buffered saline (PBS) and resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 15% fetal calf serum and 25 mM Hepes buffer [21–23]. After incubation of 37°C in 5% CO_2 in air for 18 hr, 0.2 ml of a cell suspension of 10^6 cells/ml was transferred to wells of a microtiter plate. Triplicate wells were employed for each drug. Ara-C or adriamycin containing medium was then added in a volume of 0.2 ml to yield the final concentration; Ara-C ranged from 0.001 to 0.1 μg/ml and adriamycin from 0.1 to 10 μg/ml. After 3 hr of incubation with drug

Table 1. Patient characteristics, treatment and response

	No.	AML No.	ALL No.	Age Median (range)	Male/female	CR rate (%)
ADOAP	33	25	8	32 (16–58)	17/16	76
ROAP	12	11	1	58 (51–70)	4/8	50
AMSAOAP	8	6	2	64 (59–71)	8/0	28
Overall	53	42	11	48 (16–71)	17/24	62

0.02 ml tritiated thymidine (Schwartz-Mann, Orangeberg, NY, 55 μCi/ml; sp. act., 19 Ci/mM) was added to each well for a final concentration of 5 μCi/ml for an additional hour. After a total of 4 hr of drug incubation cultures were harvested onto filter paper disks using an automatic cell harvester (Titerek Flow Laboratories, Rockville, MD). Filter paper disks were allowed to dry and placed in 11 ml of Scintriverse universal cocktail solution (Fisher Scientific). Tritiated thymidine incorporation was measured using a liquid scintillation spectrophotometer (Packard 2420, Downer's Grove, IL). Relative DNA incorporation in 2×10^6 cells was calculated as a percentage of controls after background counts were subtracted. In addition, cell cycle distribution was always measured prior to the initiation of cultures using DNA flow cytometry of cells stained with ethidium bromide and mithramycin, employing a Phywe ICP II flow cytometer (Phywe, F.R.G.) [24, 25].

Cytosine arabinoside was obtained as Cytosar-U from Upjohn (Kalamazoo, MI). Adriamycin was purchased from Adria Laboratories (Wilmington, DE).

Statistical methods

Comparisons of normalized tritiated thymidine incorporations for each drug dosage point were performed in responding vs non-responding patients using Student's *t* test or the Mann-Whitney rank test [26]. Comparison of dose-response curves for responding vs non-responding patients was performed with the Hotellings T^2 analysis [27]. Maholanobis' D^2 test was used to compare individual dose curves with the average dose-response curve determined for responding patients. Scoring was performed as described in the previous report [19]. The D^2 index provides an index of the deviation of a patient's *in vitro* drug response curve from the mean profile of remission patients obtained for a given drug. Two patients with high degrees of sensitivity, that is, high percentages of suppression with small amounts of drug, also deviated by large amounts from the curve and was given a D^2 value of 1.

In vitro test results were analyzed along with standard clinical prognostic factors by employing Cox's regression analysis and logistic regression [28, 29].

RESULTS

Concentration-dependent suppression of thymidine incorporation was demonstrated for all patients with both adriamycin and Ara-C. After adriamycin, thymidine incorporation decreased in an exponential fashion, but without difference in the mean curves of responders and non-responders (Hotelling's *T* test; $P > 0.05$). In contrast, incubation with Ara-C induced inhibition of [^{3}H]-TdR incorporation that was exponential for the lower concentrations but reached a plateau at higher concentrations. There was a difference in the curves of the responders and non-responders for Ara-C, but it was not significant ($P > 0.05$).

In order to determine whether differences in suppression of DNA synthesis after drug treatment *in vitro* differentiated patients who responded to therapy and those who failed, individual counts and the entire curve were analyzed. First, we determined the percentage of suppression for each dose of drug for each patient. The best discrimination of patients who would respond to therapy and those who would fail was the *in vitro* suppression of greater than 80% of the pretreatment counts by Ara-C at 0.004 and 0.007 μg/ml, as can be seen in Table 2. The CR rate was 81% when greater than 80% of the counts were suppressed and 54% when less than 80% of the counts were suppressed by 0.004 μg/ml of Ara-C ($P = 0.056$). The discrimination provided by these measurements was unchanged when patients who were drug-resistant and who died early were analyzed separately. There was no association between achievement of remission and suppression of thymidine incorporation after incubation with any concentration of adriamycin.

Mahalanobis' D^2 test was employed to determine if deviation of a patient's curve from the profile of responding patients correlated with achievement of remission. The D^2 values of the 53 patients after

Table 2. CR rate at various levels of Ara-C-induced suppression of [^{3}H]-*TdR incorporation and suppression of control cpm of* [^{3}H]-*TdR remaining after treatment with Ara-C*

Ara-C (μg/ml)	>80% suppression	<80% suppression	*P*
0.001	67 (4/6)	78 (29/47)	N.S.
0.004	81 (13/16)	54 (20/37)	0.056
0.007	77 (14/18)	54 (19/35)	0.084
0.01	70 (19/27)	64 (14/22)	N.S.
0.1	63 (29/46)	44 (6/9)	N.S.

Ara-C exposure are illustrated in Fig. 1. The overlap of D^2 values between responding and non-responding patients persists when the mechanism of failure is included in the analysis [30]. As can be seen in Table 3, D^2 values greater than 10 identified 7 of 8 patients who failed to respond, whereas D^2 values of less than 10 were associated with remission in 31 of 45 patients. Although the highest D^2 values were found in patients with ALL, the discriminatory power was unchanged when AML and ALL were analyzed separately. Figure 2 shows the relationship of D^2 for *in vitro* treatment with adriamycin and response. As with Ara-C, the D^2s of responders tended to be lower than the D^2 of non-responders. The difference of the D^2 values of responders and non-responders was statistically significant using the Mann-Whitney test ($P = 0.02$) but not with Student's t test ($P = 0.229$).

Because suppression of DNA synthesis is affected by the number of cells capable of DNA synthesis, incorporation of the percentage of cells in S-phase potentially enhances the discriminatory power of an *in vitro* test. The S-phase compartment size was determined by measurement of DNA content. We corrected for the effect of DNA synthesis by dividing Mahalanobis' D^2 by the percentage of cells in S-phase. The value calculated for each patient did not, however, enhance the discrimination between responders and non-responders for either adriamycin or Ara-C.

Of crucial importance for a new test with clinical implications is its relation to already established factors. A model has been developed at U.T. M. D. Anderson Hospital for predicted probability of response (PPR) [2]. Using the parameters that are currently in the model—age, history of antecedent hematologic disorder, cytogenetics, blood urea nitrogen, hemoglobin and temperature—a predicted probability for CR

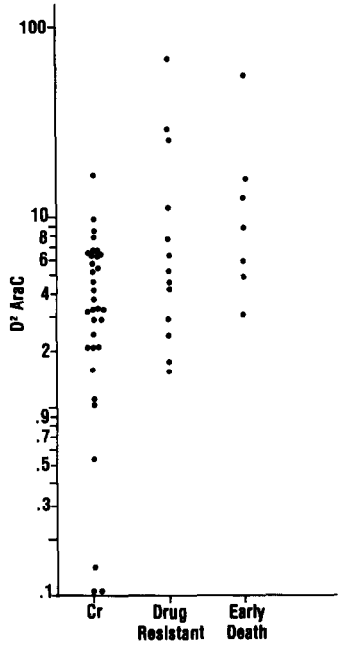

Fig. 1. D^2 Ara-C and response to therapy—D^2s *according to response status.* (a) *CR* (n = 33); (*b*) *drug-resistant* (n = 13); (*c*) *early deaths* (n = 7).

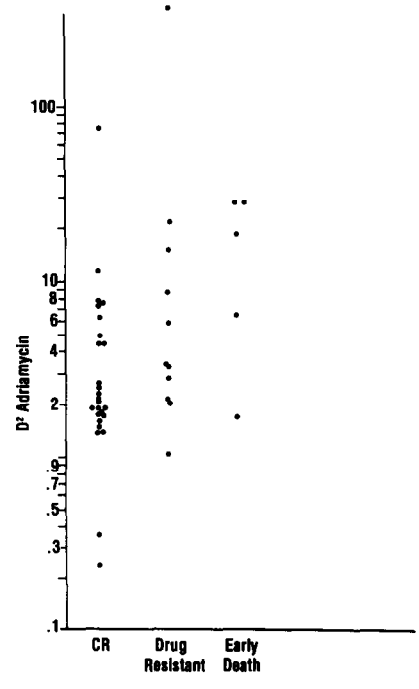

Fig. 2. D^2 *adriamycin and response*—D^2s *according to response status.* (*a*) *CR* (n = 26); (*b*) *drug-resistant* (n = 11); (*c*) *early deaths* (n = 5).

Table 3. D^2 *scores for Ara-c and adriamycin and CR rate*

	ARA-C		Adriamycin	
D^2 value	No. of patients	% CR	No. of patients	% CR
≥10	8	1/8 (13%)	8	2/8 (25%)
<10	45	31/45 (69%)	34	24/34 (71%)
Total	53	32/53 (60%)	42	26/34 (76%)
	$P = 0.0044$ Fisher's exact test		$P = 0.0247$ Fisher's exact test	

was derived for each patient. We examined the association of D^2 and the ratio of observed rate of CR to predicted probability of CR. For patients with D^2 values of 2–7 the ratio of the observed to predicted ratio of CR was greater than 1. With D^2 greater than 7 the observed:predicted ratio was less than 1 (Fig. 3).

When D^2 for Ara-C and adriamycin were included in this stepwise Cox regression analysis with the other pretreatment parameters, the D^2 value for Ara-C was selected as the single most important parameter ($P = 0.002$). Age was next most important ($P = 0.051$), followed by presence of metaphases, temperature, hemoglobin and antecedent hematologic disorder, none of which were statistically significant.

Because achievement of complete remission is influenced by host factors, we examined the relationship between cytoreduction, as a measure of *in vivo* drug sensitivity, and Mahalanobis' D^2 test. Cytoreduction was defined as the decrease in marrow blasts per day. Although the D^2 of patients with greater cytoreduction tended to be lower than those with less cytoreduction, there was not a statistically significant relationship (correlation coefficient $r = 0.03$; $P = 0.07$).

We also assessed the relationship between *in vitro* test results and length of CR. Although the initial counts of tritiated thymidine incorporation without drug were not associated with achievement of remission (the mean count of patients achieving CR being 14,875 and the mean for non-responders 13,501), the initial count was related to length of remission. Figure 4 shows the length of CR for patients with high initial counts (>10,000) ($P = 0.086$). There was no relationship between D^2 score and length of CR, but there was between D^2 score and survival duration. When only the patients who responded to therapy were examined, there was no association between D^2 score and survival duration. The association of low D^2 and survival duration was due to the low D^2s of patients achieving CR, who lived longer than patients failing to respond to therapy.

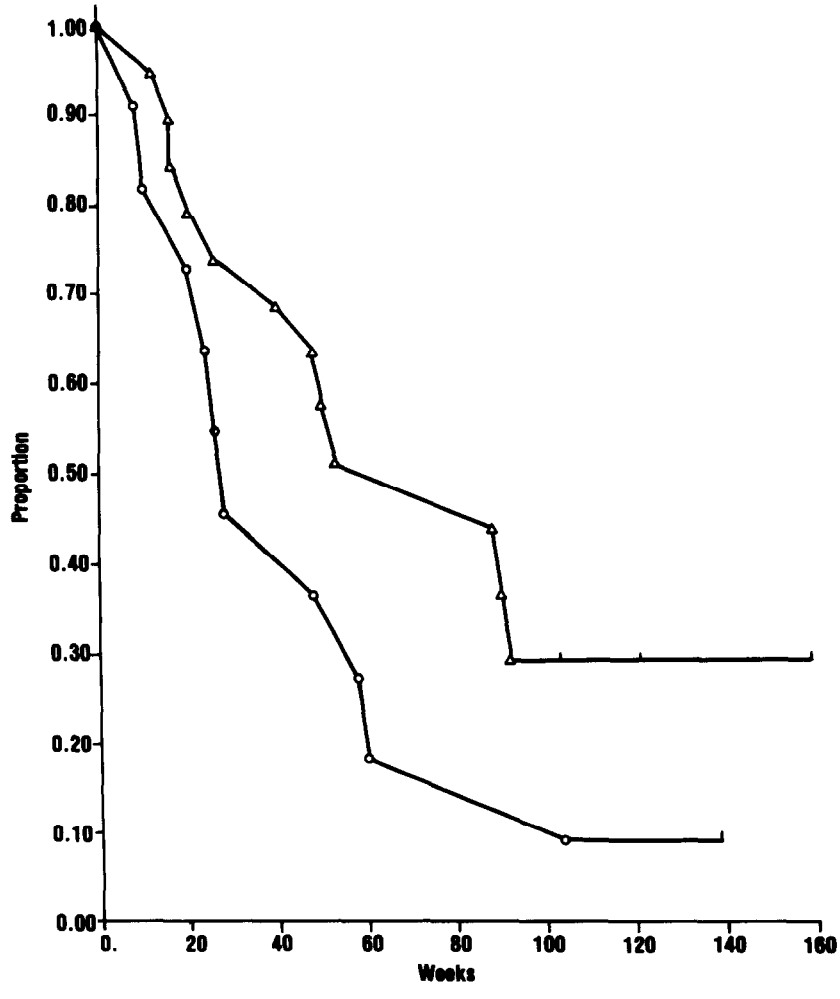

Fig. 3. Pretreatment thymidine uptake and remission duration. 0, Patients with thymidine uptake in bone marrow >10,000. Ten of 11 patients have relapsed; Δ, patients with thymidine uptake <10,000. Twelve of 19 patients have relapsed; ↑ indicates patient in CR at the time of analysis ($P = 0.086$).

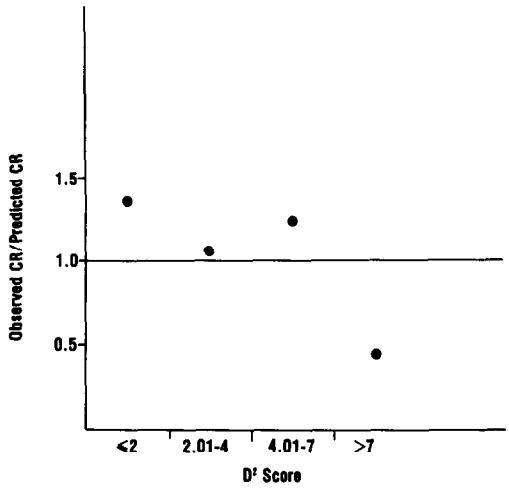

Fig. 4. D^2 *score and ratio of observed and predicted rate of complete remission. The* D^2 *scores are shown on the abscissa: (a)* ≤ 2.00 (n = 9); *(b)* 2.01–4.00 (n = 14); *(c)* 4.01–7.00 (n = 16); *(d)* ≥ 7.00 (n = 13). *The ratio of the observed rate of CR to the predicted rate of CR is shown on the ordinate.*

DISCUSSION

In this study we have investigated the dose-dependent suppression of DNA synthesis by Ara-C and adriamycin *in vitro* in 53 previously untreated patients with acute leukemia. These patients were subsequently treated with Ara-C and an anthracycline and the relationship of the *in vitro* results was compared to their response to chemotherapy. Although we found a relationship between the D^2 value and response, we did not observe the clear separation that was previously reported in our initial analysis of 14 patients [19].

Many different factors could account for the inability of the suppression of thymidine synthesis to correctly predict *in vivo* response. As with other studies that attempt to predict the effect of drugs on either macromolecular synthesis or colony formation [9–19] in short-term culture, we may be examining a small subpopulation of cells unable to adapt to culture or one that represents only a small proportion of a heterogeneous population. We also had not been able to control the role of proliferation. Most drugs are active against proliferating cells only, and the proportion of proliferating cells in culture may differ from the proportion *in vivo*. Thymidine suppression as a test for cytotoxicity by Ara-C is complicated by the possibility that it produces a static blocking effect, inhibiting DNA synthesis, rather than a cytotoxic effect. As a measure of drug-induced cytotoxicity, thymidine suppression results do not correlate with those of the tumor colony cell assay [16–19]. Maurer has recently described the technical problems that interfere with [^{3}H]-thymidine incorporation's accuracy as a measure of cell proliferation [31].

Another problem complicating the interpretation of results of *in vitro* drug sensitivity test is that patients are treated with combinations of drugs. The contribution of each individual drug in the combination is unknown. Our test, like other *in vitro* tests of drug sensitivity, is unable to control for host factors and complications that interfere with the attainment of complete remission. Also, this type of test does not predict for patients who achieve cytoreduction but who have repopulation of the bone marrow with leukemia. We were unable to enhance the discriminatory power of the test by differentiating type of failure, but this failure may be due to the small numbers.

Pretreatment thymidine uptake did not predict for CR. After treatment of the bone marrow cells *in vitro*, the best discriminator was a greater than 80% suppression of DNA synthesis after treatment with 0.004 μg/ml of Ara-C. In a recent publication [16] Epstein and Priesler, who used higher doses of Ara-C and adriamycin than we did, reported that there was no relationship between suppression of thymidine synthesis and response.

The D^2 value by itself did not discriminate between responders and non-responders. When we divided the patients on the basis of having a D^2 value above and below 10, we found that there was a significant correlation between response and low D^2. In logistic regression analysis, D^2 for *in vitro* testing of Ara-C was picked as the most important pretreatment factor. When we examined response to therapy in patients who were predicted to have high and low probabilities of CR by our model, we found that patients with low D^2s had higher than expected rates and those with high D^2s lower than predicted rates. Although this test by itself does not have sufficient sensitivity or specificity for a routine screening test of drug sensitivity in its present form, it does appear to add to the clinical predictive factors by discriminating on the basis of leukemic cell drug sensitivity.

Pretreatment thymidine uptake correlated with the length of CR. Crowther *et al.* had previously found that the labeling index correlated with the length of CR [4].

Although in multivariate analysis the suppression of DNA synthesis measured by D^2 was significant, and there is some indication that it measures some biologically important factor of leukemic cells important in determining drug sensitivity, the considerable overlap of values among patients did not allow it to be useful as a single parameter in helping to choose therapy.

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