Europ. J. Cancer Vol. 17, No. 5, pp. 549–555, 1981
Printed in Great Britain

0014-2964/81/050549-07$02.00/0

Dose-Dependent Suppression of DNA Synthesis *In Vitro* as a Predictor of Clinical Response in Adult Acute Myeloblastic Leukemia*

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Abstract—*We determined for* 14 *patients with acute myeloblastic leukemia, prior to therapy with an anthracycline-ara-C combination, the relationship of clinical response to dose-dependent DNA synthesis inhibition produced by each agent on each patient's cultured leukemic cells. Using a microculture system ara-C and adriamycin sensitivity* (D^2) *was determined for each patient based upon each individual's dose response curve. The* 9 *patients achieving complete remission and one patient who died during induction had* D^2 *values to both agents less than* 7, *while* 4 *non-responding patients had* D^2 *values in excess of* 9. *Correlation of* D^2 *levels with* in vivo *chemotherapy-induced bone marrow cytoreduction was noted for adriamycin*($P<0.005$) *and for ara-C* ($P=0.1$). *A relationship between* in vitro *ara-C and adriamycin sensitivity* ($P<0.05$) *suggests that they act upon similar leukemic cell populations. Inhibition of thymidine synthesis over a range of concentrations deserves further study as a rapid* in vitro *test for drug sensitivity in acute myeloblastic leukemia.*

INTRODUCTION

SUCCESSFUL treatment of bacterial infections has been greatly facilitated by the availability of *in vitro* screening assays of a battery of antibiotics against bacterial isolates. Such tests rely upon qualitative and quantitative measurements of the inhibition of bacterial growth by medium containing defined antibiotic concentrations. Availability of a similar predictive test for human tumor cells would greatly enhance treatment results in those tumors for which there is a variety of active chemotherapeutic agents available, such as in adult acute leukemia [1–8].

Several investigators have examined the feasibility of *in vitro* prediction of clinical antitumor effect. To this end clinical tests of drug uptake and activation have been examined for ara-C with conflicting results [9–12]. Other studies have evaluated the effect of drugs on macromolecular synthesis and survival of target cells in short-term culture [13–19]. Unfortunately, none of these approaches has found wide clinical application, notably because of considerable progress in empirically designed induction regimens.

In the absence of recent significant changes in the management of acute leukemia; and with the problem of both primary induction failures, particularly in older patients, and relapse of disease previously in remission, re-examination of the feasibility of *in vitro* predictive tests seems warranted. Thus, a variety of available antitumor agents could be screened and, in the case of differing sensitivities, the most effective agent could be chosen, avoiding various potential toxicities.

The purpose of the present analysis is to determine, in retrospect, whether dose-

Accepted 6 August 1980.
*Supported in part by Grants CA28153 and CA11520 from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.
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dependent effects of currently employed anti-leukemic drugs on DNA synthesis of bone marrow cells from patients with acute leukemia reflect *in vivo* drug responsiveness. We have therefore measured the dose-dependent suppression of thymidine incorporation of leukemic cells in liquid culture following *in vitro* exposure to adriamycin and ara-C, the major components of our present induction regimen for acute myeloid leukemia. The *in vitro* culture results in the first 14 patients tested were predictive of *in vivo* cytoreduction during the first 2 weeks of therapy and of subsequent remission status.

MATERIALS AND METHODS

Patient population

All adult patients with acute myeloid leukemia scheduled to receive induction treatment with ara-C–anthracycline combinations between July and December of 1978 were studied. Patients 50 years of age or younger were treated with a combination of adriamycin, vincristine, ara-C and prednisone (ADOAP) [7]; while those exceeding 50 years were treated with a similar combination in which the anthracycline rubidazone was substituted for adriamycin (ROAP) [8]. To appreciate the direct *in vivo* cytotoxic effects of induction treatment on leukemic infiltrate, without regard to host tissue factors, the speed of bone marrow cytoreduction was determined. Cytoreduction was defined as;

$$\frac{B_1C_1 - B_2C_2}{I \times B_1C_1} \times 100,$$

where B = % blasts, C = % cellularity [20], 1 = day 1 or pre-treatment value, 2 = day of second sampling, and I = interval in days between samples. For example, a patient with 90% blasts and 100% cellularity prior to therapy and 90% blasts and 90% cellularity on day 10 would have cytoreduction;

$$= \frac{(90 \times 100) - (90 \times 90)}{10 \times (90 \times 100)} \times 100 = 1\% \text{ per day.}$$

Complete remission was defined as the achievement for ≥ 30 days of a normal cellular M_1 bone marrow with a peripheral blood absolute granulocyte count exceeding 1000 mm^3, a platelet count $> 100{,}000\ mm^3$, and a hemoglobin concentration > 10 g‰.

Sample processing and culture methods

After obtaining informed consent 2 ml of bone marrow was aspirated from the posterior iliac crest. The specimen was immediately placed into a tube containing 2 cm^3 of RPMI 1640 medium (Gibco, Grand Island, New York) with 250 units/ml of preservative-free heparin. Mature granulocytes and red cells were removed by Ficoll Hypaque gradient separation (density = 1.078 g/cm^3, 1000 ***g*** for 15 min at 4°C). Interphase cells were collected, washed, and resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium with 15% fetal calf serum and 25 mM Hepes and were incubated at 37°C, 5% CO_2 in 95% room air atmosphere. After incubation for 24 hr, aliquots of ara-C (Cytosar-U, Upjohn, Kalamazoo, Michigan) or adriamycin (Adria Laboratories, Willmington, Delaware) were added to cell suspension aliquots to yield drug concentrations specified in Results. Immediately after the addition of drugs, 2×10^5 cells were placed into microtiter wells (0.2 ml/well) with triplicate wells per drug concentration point [21–23]. Cell suspensions without drugs served as controls and were run in batches of six. After 3 hr incubation, 0.02 ml of tritiated thymidine (Schwartz-Mann, Orangeberg, New York) (55 μCi/ml, S.A. = 19 Ci/mM) was added to each well for a final concentration of 5 μCi/ml for another 1 hr. After a total of 4 hr of drug incubation, cultures were harvested onto filter paper discs with an automatic cell harvester (Titerek, Flow Laboratories, Rockville, Maryland). Filter discs were allowed to dry and were placed in 11 ml of Scintiverse Universal Cocktail Solution (Fisher Scientific, Houston, Texas). Tritiated thymidine incorporation was measured utilizing a liquid scintillation spectrophotometer (Packard 2420, Downer's Grove, Illinois). Relative DNA incorporation per 200,000 cells was calculated as percentage of control after background counts were subtracted.

Statistical methods

Three statistical techniques were utilized in analysing dose response data. First, comparison of single drug dosage points in responding versus non-responding patients was made using Student's t-test. Second, comparison of entire dose response curves in all responding patients versus all non-responding patients was made using Hoteling's T^2 analysis [24]. Finally, Mahalanobis' D^2 test [24], a var-

iation of Hotelling's T^2 test, was used to compare individual dose response curves with the mean dose response determined for responding patients. This type of analysis was used to score the deviation of dose response points in each patient from that of all patients with complete response. Scoring was obtained in the following manner: If $\bar{X}=(\bar{X}_1, \bar{X}_2, \ldots, \bar{X}_p)^T$ represents a p-dimensional calculated mean profile of individuals who achieve complete remission; S is calculated $p \times p$ sample variance–covariance matrix of the N individuals achieving remission; and $Y=(Y_1, Y_2, \ldots)^T$ is a p-dimensional dose response profile from a given leukemic patient; then the distance (squared) from Y to $\bar{X}$ is defined by the quadratic form

$$D^2(Y)=N(Y-\bar{X})^T S^{-1} (Y-\bar{X})/(N+1),$$

where T denotes vector transpose (to column vectors) and -1 denotes matrix inversion. The computed distance of the individual patient profile from the mean profile of patients in response constitutes the D^2 or 'sensitivity index', with low numerical values indicating greater sensitivity or being more closely associated with response. It may be shown statistically that $[D^2(Y)/N-1]\ [(N-p)/p]$ is distributed as a central F-distribution with p and $N-p$ degrees of freedom, respectively, under the null hypothesis that Y represents a normal profile. The D^2 value provides an index of the magnitude of deviation for each leukemic patient's mean dose response data from the mean profile in responders obtained for a given drug.

RESULTS

Clinical analysis

Fourteen patients, 10 females and 4 males, ages 16–66 years (median 44 years), were analysed. Five patients, all over the age of 50, received the ROAP regimen, while the remainder received ADOAP therapy. Complete remission was achieved in 9 patients, while 5 failed the induction regimen. All patients had pretreatment bone marrows with $\geq 75\%$ leukemic cells and total infiltrate (% blasts $\times$ % cellularity $\geq 50\%$).

In vitro *culture results*

Concentration dependent suppression of thymidine incorporation by adriamycin and ara-C was determined for each patient, and the results normalized to pretreatment measurement. Thus, a family of similarly shaped curves was generated for each drug. In one patient with an insufficient number of cells available for the entire study, only the adriamycin dose response function was determined. The mean dose response curve for responding patients was compared to that for non-responding patients for adriamycin and for ara-C (Fig. 1). Adriamycin suppressed thymidine incorporation in an exponential fashion. In contrast, the ara-C concentration inhibition of [^{3}H] TdR incorporation was exponential for the lower concentrations merging into a plateau at higher concentrations.

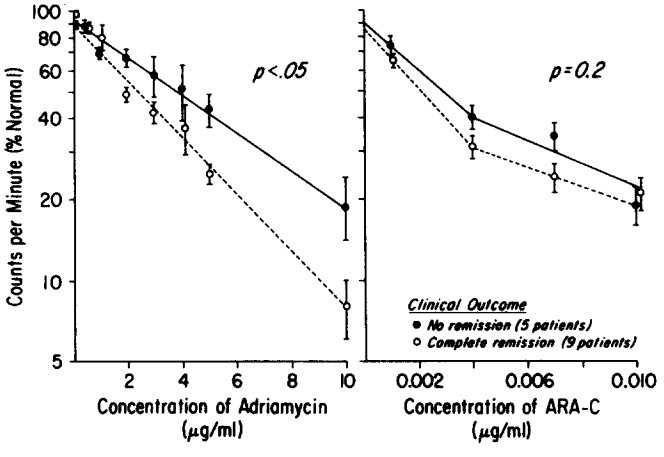

Fig. 1. Dose-dependent effects of adriamycin and ara-C on thymidine incorporation in cultured leukemic cells from 14 patients with acute myeloblastic leukemia.

Considering adriamycin, *t*-test comparison between responding and non-responding patients was made for each drug concentration (0.1–10 μg/ml). At low concentrations (0.1, 0.5, 1.0 μg/ml) no significant difference in the adriamycin effect on thymidine incorporation was noted. At 2, 5 and 10 μg/ml, however, a significant difference in DNA synthesis inhibition was noted, with responders incorporating less thymidine than non-responders ($P<0.05$). A similar trend was seen at 3 and 4 μg/ml. Considering the entire range of concentrations, a significantly lower rate of thymidine incorporation was seen in patients achieving complete remission than in non-responders ($P<0.05$ by Hoteling's T^2 analysis).

For ara-C the range of dosage concentration utilized was 0.001–5 μg/ml. For the lowest concentration range responders showed slightly less thymidine incorporation, while a significant separation of the two patient groups in a similar direction at 0.004 and 0.007 μg/ml of ara-C was noted ($P<0.05$). At concentrations above 0.01 μg/ml no significant difference in groups was noted, with a gradual decrease in thymidine incorporation rate merging into a plateau beyond the 0.001 μg/ml of ara-C concentration. The difference in [^{3}H] TdR incorporation for responders and non-responders for the entire concentration range using Hoteling T^2 test shows a trend for less thymidine incorporation in responders ($P=0.2$).

Drug sensitivity indices were then determined for each patient (Mahalanobis' D^2 test) for adriamycin and ara-C (Fig. 2). All patients achieving complete remission to the anthracycline-ara-C combination *in vivo* had D^2 values <7 for both drugs *in vitro*. With the exception of one patient, four of the five non-responding individuals had D^2 values above 9. The one non-responding patient with a D^2 value of 2.7 for ara-C and 6.5 for adriamycin died on day 15 of induction therapy from candida septicemia with hypocellular bone marrow and no evidence of leukemia on post mortem examination. A matched comparison of D^2 values for adriamycin and ara-C in all 14 patients indicate a significant correlation ($P<0.05$ with $R=0.61$).

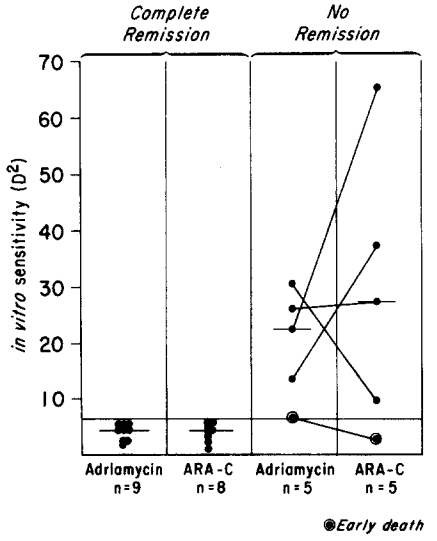

Fig. 2. D^2 sensitivity index determinations for adriamysin and ara-C in responding and non-responding patients with acute myeloblastic leukemia.

Patients who achieved complete remission had a mean cytoreductive rate of $0.8\pm0.01\%$/day while patients not achieving remission had a mean cytoreductive rate of $0.05\pm0.02\%$/day ($P=0.2$). For all 14 patients a negative correlation was noted between the rate of leukemic cytoreduction and D^2 values for adriamycin ($R=0.74$, $P<0.005$), while there was less correlation between these two parameters for ara-C ($R=-0.329$, $P=0.1$).

Since the four of the nine responding patients remain in complete remission at the time of this analysis, a comparison of remission duration and survival with *in vitro* sensitivity could not be performed.

DISCUSSION

The results of our study indicate that observation of adriamycin and ara-C-induced dose-dependent inhibition of DNA synthesis can be utilized as a measure of *in vivo* drug sensitivity. The sensitivity (D^2) determinations in 14 patients receiving adriamycin and ara-C therapeutically showed excellent correlation with bone marrow cytoreduction and with complete remission. *In vitro* studies have shown that tests of metabolic activity such as thymidine incorporation cannot be equated with survival measured by clonogenic assays [25]. Interestingly, in our system, we have noted dose response functions for adriamycin and for ara-C which are qualitatively similar to survival curves obtained from exposure of established cell lines and cultured leukemic cells to these agents [13, 26–28]. The close quantitative correlation between *in vitro* DNA synthesis inhibition and *in vivo* effects suggest that our method is a reflection of *in vivo* drug cytotoxicity. Unlike survival assays, requiring prolonged culture periods [13], results of thymidine incorporation are available within 48 hr and can therefore influence prospectively the treatment of a rapidly progressive malignant disease such as acute leukemia. In

addition the suspension culture assay, with shorter growth requirements, bypasses the necessity for specific growth factors necessary for reproducible and efficient growth of leukemic stem cells in semi-solid systems [13, 29–31].

For ara-C, which predominantly kills S-phase cells [28], inhibition of thymidine incorporation has been reported to be predictive of clinical response in leukemia [15, 17]. Considering the concentration levels necessary for suppression of DNA synthesis, [^{3}H] TdR incorporation was reduced at concentrations consistent with *in vivo* plasma levels in patients undergoing treatment [32]. In contrast, the levels of adriamycin effective *in vitro* exceed ten-fold the therapeutic levels normally achieved in man [33]. Adriamycin, as a DNA intercalating agent [34], interferes primarily with G_2 phase traverse [26, 27]. Higher concentrations were required to directly inhibit ongoing DNA synthesis. The predictive value of our 4-hr assay, limited to high adriamycin concentrations, suggests that for this agent, as for ara-C, DNA synthesis inhibition can reflect ensuing *in vivo* cytotoxicity. These considerations point out that clinically useful information can be gained by testing concentrations outside the therapeutic dose range.

Previous investigations of the usefulness of thymidine incorporation assays for *in vivo* prediction of remission in acute leukemia were not stringent and include partial remissions [17] and less rigorous definitions of cytoreduction [15]. Considering the results of Raich [17], who determined DNA synthesis suppression at one ara-C concentration for patients receiving a variety of regimens, complete remission was predicted in 5 of 6 patients and failure in 5 of 7. Complete remissions were achieved in 46% of patients with AML. Cline and Rosenbaum [15], studying the effect of a range of ara-C concentrations on thymidine incorporation in 8 patients with leukemia, found correlation with *in vivo* cytotoxicity but not with remission. In the present study the *in vitro* evaluation using a range of drug concentrations enabled a successful determination of patients with resistant leukemia. This indicates particular sensitivity of our method in view of the more effective chemotherapy utilized (65% complete remissions). In addition our definition of cytoreduction applied only to bone marrow rather than the less rigorous one of Cline and Rosenbaum [15] who considered a decrease in peripheral blood or bone marrow leukemic cells as cytoreduction.

Our results suggest that multiple drug *in vitro* sensitivity screening procedures should be performed for all patients with acute leukemia. If the current conclusions are confirmed in a prospective fashion, newly diagnosed and relapsing patients with high *in vitro* resistance to a planned induction regimen could be treated with other more effective agents, avoiding needless toxicity. Since D^2 sensitivity values for adriamycin and for ara-C showed significant correlation, the question of how adriamycin contributes to ara-C containing regimen arises. While two effective agents may intensify cytotoxicity toward the same subpopulation of cells, it may neglect resistant clone(s) responsible for relapse. Thus, pretreatment identification of other effective agents may be utilized to intensify induction regimens, increasing the cure rate in acute myeloblastic leukemia.

Acknowledgements—The authors gratefully acknowledge the assistance of Dr. Avrum Z. Bluming for his critical review of the manuscript and Ms. Marilyn Schindler for her excellent secretarial help.

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