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Effects of Cytosine Arabinoside on DNA Synthesis as Predictor for Acute Myelocytic Leukemia (AML) Patients' Response to Chemotherapy*

JOSHUA EPSTEIN and HARVEY PREISLER

Department of Medical Oncology, Sections of Cell Biology and Myeloproliferative Diseases, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, U.S.A.

Abstract—*Bone marrow cells from 25 AML patients were exposed to cytosine arabinoside and the effects of the drug on the incorporation of ^{3}H-TdR by the cells were measured and compared to the response of the patients to antileukemia chemotherapy with ara C and an anthracycline. The majority of the leukemic cells of the patients in whom bone marrow hypoplasia was not achieved as a result of treatment were classified as resistant to the drugs administered. Incubation with 0.3 μg/ml ara C inhibited DNA synthesis in 11 of 12 resistant patients by 20–95%. Exposure to 3.0 μg/ml drug inhibited the incorporation of ^{3}H-TdR in 10 of 11 resistant patients by 38–98%. The in vivo resistance of the leukemic cells to ara C was not reflected by the in vitro response of the synthesis of DNA by these cells to ara C. The effects of ara C on DNA synthesis were also not correlated to the drug's effects on the clonogenic cells in 21 bone marrow specimens. We conclude that the effects of ara C on DNA synthesis are not predictive to a patient's response to therapy, nor do they reflect the cytocidal effects of the drug on the clonogenic cells.*

INTRODUCTION

THE DEVELOPMENT of reliable methods for predicting the *in vivo* sensitivity of tumor cells to chemotherapeutic drugs will facilitate the administration of effective anti-cancer therapy and at the same time will prevent the administration of ineffective drugs which produce only toxic effects. Several attempts to predict the response of tumor to drugs by measuring the inhibition of DNA synthesis produced by these agents have been reported in the literature [1–5]. These studies indicated that although some correlation exists between the effects of ara C on DNA synthesis and response, the results obtained from sensitive and resistant cells overlapped more than should be desired from a predictive assay.

We have recently reported a good correlation between the lethal effects of cytosine arabinoside (ara C) on leukemic cells which clone *in vitro* and the response of patients with acute myelogenous leukemia (AML) to chemotherapy using an ara C-anthracycline regimen [6]. The clonogenicity assay, however, is impractical for routine screening purposes since it requires many cells, takes a long time to complete, and because approximately one-third of marrow specimens obtained from patients with AML do not clone *in vitro*. Measuring the effects of cytosine arabinoside on the incorporation of ^{3}H-thymidine (^{3}H-TdR) does not require many cells and can be completed in a matter of hours.

To determine whether measuring the effects of ara C on DNA synthesis is a suitable assay for predicting the *in vivo* sensitivity of leukemic cells to the drug, we have compared the effects of ara C on DNA synthesis by leukemic bone marrow cells and the response of the patients to anti-leukemia chemotherapy. To determine the ability of this assay to reflect the cytocidal effect of the drug, we have also looked for a correlation between the effects of ara C on the synthesis of DNA by bone marrow cells obtained from leukemic and non-leukemic patients and the drug's effects on the *in vitro* clonogenic potential of these specimens.

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MATERIALS AND METHODS

Chemicals

Tissue culture media and fetal calf serum

were purchased from GIBCO. ^{3}H-Thymidine (49 C/mmole) was obtained from Amersham. Cytosine arabinoside was the product of the UpJohn Company. For the clonogenicity assays, we used Difco's bacto agar.

Specimens

Heparinized specimens of citrated bone marrow aspirates were obtained as indicated from previously untreated patients with acute myelocytic leukemia (AML), and from relapsed AML patients prior to therapy. For studying the effects of ara C on the clonogenic cells, bone marrow specimens were also obtained from AML patients in complete remission, one patient with acute lymphocytic leukemia in complete remission, and from a patient with a solid tumor without bone marrow involement. All patients in complete remission, as well as the patient with the solid tumor, had not received chemotherapy for at least 6 weeks prior to the time when the bone marrows were obtained. Patients with AML were treated with RPMI protocol No. 970701 (ara C + adriamycin or daunorubicin) [7] and those who failed to enter complete remission were classified on the basis of the type of failure [8] (Table 1).

Table 1. Classification of remission induction in AML

Failure type	Definition
I	Failure to produce marrow hypocellularity at any time during therapy
II	Attainment of marrow hypocellularity (< 1 + cellular) but regrowth of leukemic cells within four weeks
III	Patient survives more than four weeks with hypocellular marrow; peripheral blood cytopenia without detectable leukemia
IV	Patient expires with a hypocellular marrow without evidence of residual leukemia
V	Inadequate trial. Patient expires within 7 days after cessation of induction therapy

Incubation with Ara C

Bone marrow cells free of red blood cells were pre-incubated for 60 min at 37°C at a cell density of 5×10^6 cells/ml in complete medium (RPMI 1640 containing 10% heat inactivated fetal calf serum). Cytosine arabinoside was added at the appropriate concentration and incubation continued for an additional 60 min. After incubation with the drug, the cells were washed twice with 15 ml of complete medium each wash, resuspended in ara C-free media and then studied. Control cells were treated in an identical way but were incubated without ara C.

Incorporation of ^{3}H-TdR into DNA

Samples of the washed cells as well as the appropriate control cells were diluted to 2×10^6 cells/ml. Aliquots of 50 µl cell suspensions were pipetted into microtiter plate wells containing 50 µl warm ^{3}H-TdR (49 C/mmole, 20 µCi/ml in complete medium), agitated and incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then collected on fiberglass filters using a mash harvester, washed, and the radioactivity was counted in a scintillation counter.

Clonogenicity

In order to evaluate the relationship between the effects of ara C on DNA synthesis and the drug's effects on the ability of cells to proliferate, we compared the effects of ara C on the incorporation of ^{3}H-TdR and its effects on the colony formation *in vitro* by the same marrow samples. Bone marrow specimens were obtained from eight AML patients in complete remission and four patients with active AML. Chemotherapy was administered to the patients with active AML, and on the basis of response, the majority of leukemic cells of three patients were judged to be drug-sensitive (one complete remission and two Type IV failures). Bone marrow specimens were also obtained from one patient with a solid tumor and one with lymphoma, both without evidence of bone marrow involvement. The cells were treated with ara C as described above. After removing cells for measuring the incorporation of ^{3}H-TdR, samples of the drug-treated cells as well as the matching control cells were cultured in soft agar as described earlier [9].

One hundred thousand cells in 0.1 ml of complete medium were mixed with 0.75 ml of 0.3% agar and plated over a 2.5 ml underlayer of 0.5% agar made 20% with colony stimulating factor. The cultures were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% room air. After 7 days of culture, three plates were fixed with 3% glutaraldehyde and the number of cluster–colonies counted by a single observer. This individual did not know the identity of the plates being counted since each plate had been coded by a different individual according to a computer generated random number sequence. The latter individual also decoded the experiments once counting was complete.

Ten cluster–colonies were picked from each

experimental group, smeared on a slide, and stained with Wright–Giemsa and the morphological type of cells was determined.

Evaluation of results

Comparing the results of the *in vitro* inhibition of DNA synthesis by ara C to the *in vivo* sensitivity of the leukemic cells to the drug presented two problems: the *in vivo* sensitivity of the leukemic cells to treatment had to be determined from the patients' responses to treatment, and the *in vivo* sensitivity of the cells to ara C had to be separated from their sensitivity to the anthracycline. Since failure to enter complete remission does not necessarily indicate leukemic cell resistance to the drugs administered, the patients in whom complete remission was not achieved were classified on the basis of their marrow responses, as has been described in detail by Preisler [8] (Table 1). Thus, the majority of the leukemic cells of patients who were Type I failures were considered to be resistant to therapy since marrow hypoplasia did not occur as a result of treatment. By contrast, the majority of the leukemic cells of patients who were Type II or IV failures were regarded as drug sensitive as evidenced by the significant marrow hypoplasia produced by chemotherapy. It would be impossible to discern the effects of ara C on cells of patients who responded to therapy. However, cells which were determined as being resistant to therapy by the above-mentioned classification clearly are resistant to both drugs. Thus, resistance to ara C can be accurately estimated. Results are presented for sensitive cells so that the distribution of the *in vitro* effects of ara C on these cells could be compared with that of the resistant cells.

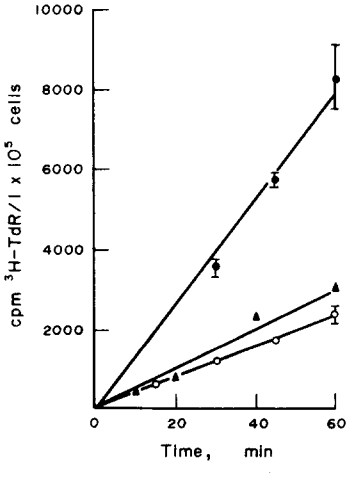

Fig. 1. Time course of ^{3}H-TdR incorporation by bone marrow cells. Each data point is the mean of three replicate measurements. Standard errors are represented by the bars. Results are presented for three marrow samples. 1×10^5 cells were incubated as indicated with ^{3}H-TdR (40 Ci/mmole, 10 µl/ml) harvested and radioactivity incorporated into macromolecules determined using a scintillation counter.

Analytical procedures

Linear regression and *t*-test analysis of the results were done using the Institute's computer.

RESULTS

Effects of ara C on DNA synthesis

Figure 1 shows the time course of the incorporation of ^{3}H-TdR by the cells obtained from three different patients. In all cases, the incorporation was linear for at least 60 min. Since some cell populations were expected to have low incorporation, we chose to use 60 min incubations. The relation between the cell number in each well and the amounts of ^{3}H-TdR incorporated is demonstrated in Fig. 2. For three different cell populations the

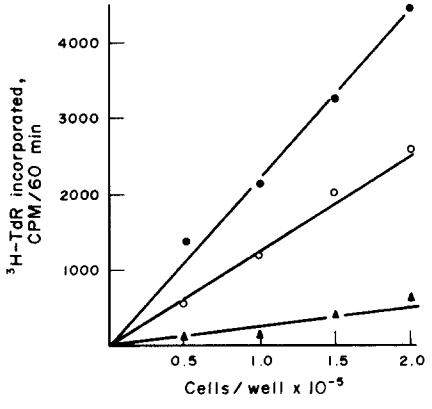

Fig. 2. Incorporation of ^{3}H-TdR by bone marrow cells as function of cell numbers. Cells were incubated with ^{3}H-TdR for 60 min and the radioactivity incorporated counted. Each data point is the mean of three replicate measurements. The standard error of the means is covered by the area of each data point.

incorporation of ^{3}H-TdR was a linear function of the cell number between 5×10^4 and 2×10^5 cells/well. Each data point in Figs. 1–5 represents the mean value of three replicate measurements. When the standard error of the mean was greater than the size of the illustrated data point, it is represented by a bar. The mean value of the zero time incorporation for all experiments was 56 cpm/10^5 cells with a standard error of 8.

The effects of four concentrations of ara C on the incorporation of ^{3}H-TdR by bone marrow cells from four resistant and four treatment-sensitive AML patients are demonstrated in Fig. 3. The cpm incorporated by

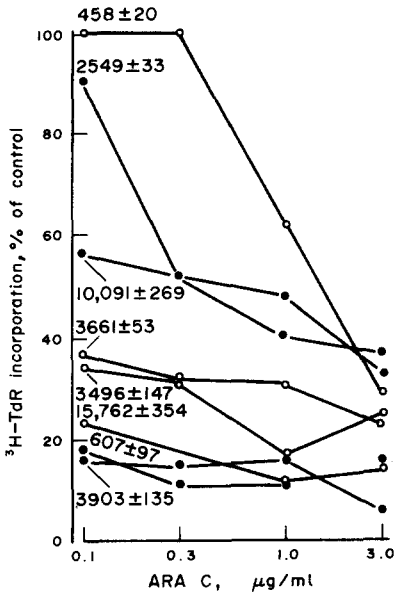

Fig. 3. Effects of 60 min *incubation with ara C on the incorporation of* ^{3}H-TdR. *Bone marrow cells of AML patients were incubated with ara C, washed, and then incubated with* ^{3}H-TdR *for* 60 min. *The radioactivity incorporated was determined as described in the Methods section.* ○ = *sensitive cells.* ● = *resistant cells. Each point is the average of three replicate measurements. The numbers represent the cpm incorporated by control cells* ± S.E.

the control cells in each experiment are given along with the standard errors. The dose–response curves for both resistant and sensitive patients show great variability reflecting individual differences between patients. No distinction can be made between the effects of ara C on the incorporation of ^{3}H-TdR by the marrow cells of resistant and sensitive patients. We have extended this study to 17 additional AML patients. Since the availability of cells was limited, we measured the effects of only two drug concentrations. Ara C concentrations of 0.3 and 3.0 μg/ml were chosen to match the drug concentrations used to determine the drug's effects on CFUc [6]. The results of these measurements together with the data from Fig. 3 are presented in Fig. 4. The means ± standard errors of the effects of 0.3 ara C on the incorporation of ^{3}H-TdR were 39 ± 8 and 40 ± 9 for the cells obtained from sensitive and resistant patients, respectively. Similarly, the mean values for 3.0 μg/ml drug were 23 ± 4 and 31 ± 9 for sensitive and resistant patients, respectively. For both drug concentrations the differences between the means were not significant by the Student *t*-test.

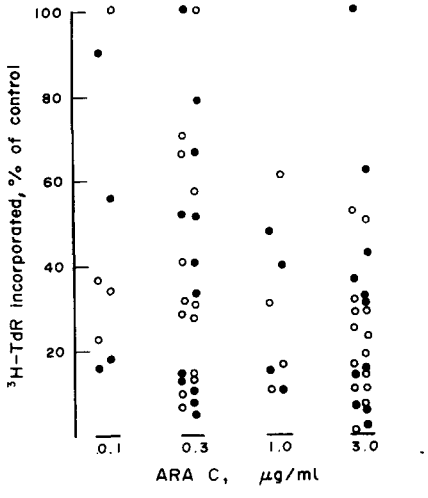

Fig. 4. Effects of ara C on DNA synthesis. Bone marrow cells of AML patients were incubated with ara C and the incorporation of ^{3}H-TdR *measured as described.* ● = *resistant cells.* ○ = *sensitive cells (see text for definition). Each point is the mean value of three replicate measurements.*

Effects of ara C on clonogenicity and DNA synthesis

Aliquots of cells from 22 bone marrow samples were cloned in soft agar subsequent to incubation with 0.3 and 3.0 μg/ml ara C, and their growth relative to the growth of control cells (not exposed to ara C) was plotted

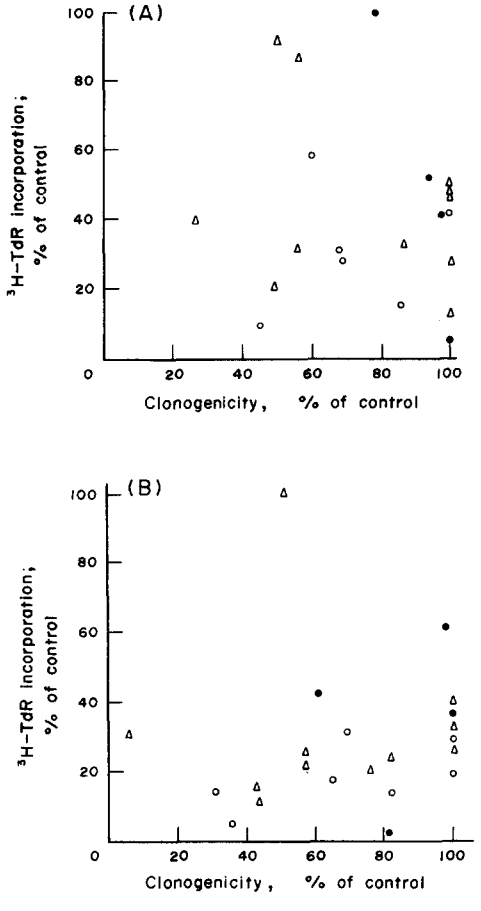

Fig. 5. Relationship between the effects of ara C on DNA synthesis and clonogenicity. Bone marrow cells were incubated with 0.3 (A) *and* 3.0 (B) μg/ml *for* 60 min. *Part of the cells were used to measure DNA synthesis and part were clones in agar.* △ = *bone marrow of individuals without active leukemia.* ○ = *cells of clinically sensitive patients.* ● = *cells of clinically resistant patients (see definitions in text).*

against their residual DNA synthesis (Fig. 5). The number of colonies–clusters in the control plates varied from 62 ± 3 to 1260 ± 122 for the leukemic marrows and from 51 ± 4 to 696 ± 33 for the non-leukemic marrows. The colonies–clusters were granulocytic or macrophagic. The colony/cluster ratio was not affected by treatment with ara C. For both ara C concentrations, there was no correlation between the effects of drug treatment on the incorporation of ^{3}H-TdR and its effects on clonogenicity ($r = 0.154$ and 0.126 for 0.3 and 3.0 μg/ml ara C, respectively). The correlation coefficient calculated for the effects of 3.0 μg/ml ara C, Not counting the single data point showing no inhibition of DNA synthesis, was 0.386.

DISCUSSION

The AML patients in this study were treated with a combination of ara C and an anthracycline [7]. It would, therefore, be impossible to distinguish between the effects of ara C and those of the anthracyclines in patients in whom severe marrow hypoplasia was achieved by chemotherapy. For this reason, classifying the leukemic cells of patients in whom marrow hypoplasia was achieved or who entered complete remission as sensitive to ara C could be an over-estimate. On the other hand, the leukemic cells of patients in whom marrow hypoplasia was not achieved were indeed resistant to both drugs so that resistance is an accurate estimate.

The effects of 60 min incubation with ara C on DNA synthesis by the bone marrow cells of patients whose leukemic cells were resistant to chemotherapy varied widely from 0 to 98% inhibition. The results of these studies, as demonstrated in Fig. 4, clearly indicate that the effects of a wide range of concentrations of ara C (0.1–3.0 μl/ml) on DNA synthesis cannot be used to accurately predict the *in vivo* response of a patient's leukemic cells to anti-AML chemotherapy.

The inability of the *in vitro* inhibition of DNA synthesis by ara C to predict *in vivo* leukemic cell kill by the drug is also expressed in the lack of correlation between this effect of ara C and the drug's effects on the clonogenic cells. This indicated that the inhibition of DNA synthesis produced by 60 min incubation with ara C does not reflect the cytocidal effects of the drug.

Cytosine arabinoside exerts a maximal lethal effect on cells during the S phase of the cell cycle by interfering with the synthesis of DNA. Ara C also has a cytostatic effect arresting cells at the G_1/S transition [10]. Both effects will contribute to the inhibition of DNA synthesis in our studies, but only the inhibition of DNA synthesis by the cytocidal mechanism would correlate with cell kill. The inhibition of DNA synthesis resulting from the synchronizing effect would most likely be reversible and, therefore, would not correlate with the effects of ara C on the CFUc. It is possible that the inhibition of ^{3}H-TdR incorporation which results from 60 min incubation with ara C is primarily the reversible, synchronizing effect and, hence, is not correlated to the lethal effects of the drug. A more direct measurement of the reversibility of the inhibition of DNA synthesis by ara C is required to assess this possibility.

An alternative and perhaps more likely explanation for the lack of correlation between ara C effects on DNA synthesis by the marrow cell population as a whole and the drug's ability to kill clonogenic cells *in vitro* or leukemic stem cells *in vivo* is that these cell types are present as a small subpopulation of the bone marrow cells which may differ in their properties from the majority of the cells in the specimens. Hence, perturbation in the bone marrow cell population as a whole do not necessarily indicate that similar perturbations occurred in the subpopulation of CFUc or in the leukemic stem cell [11].

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