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Detection of 1-β-D-Arabinofuranosylcytosine Incorporation into DNA *in Vivo*[1]

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ABSTRACT

The incorporation of (1-β-d-arabinofuranosylcytosine (ara-C) into the DNA of leukemic cells is highly correlated with cytotoxicity *in vitro*. However, the measurement of ara-C incorporation into leukemic cell DNA *in vivo* during ara-C therapy has been limited by the lack of a suitably sensitive method. A quantitative assay procedure has therefore been developed to determine incorporation of unlabeled ara-C into DNA. This method involves DNA isolation from patient myeloblasts, enzymatic digestion of the DNA, high pressure liquid chromatography separation of the nucleosides, and determination of ara-C in the eluate fractions by radioimmunoassay. Using this approach, incorporation of unlabeled ara-C into DNA of HL-60 cells is log linear over concentrations of 1 to 100 μM ara-C. Furthermore, the extent of ara-C incorporation into DNA as determined by this method correlates significantly with measurements of [^{3}H]ara-C (DNA) formation under similar conditions. This approach has also been applied to clinical samples. Myeloblasts from 6 patients receiving high-dose continuous-infusion ara-C therapy incorporated 0.00–0.36 pmol ara-C/μg DNA during 24 h of therapy. These findings thus suggest that this method can be used to monitor the *in vivo* incorporation of ara-C into leukemic cell DNA.

INTRODUCTION

Ara-C[3] is one of the most effective agents in the treatment of AML (1). The combination of an anthracycline with ara-C at conventional doses (100–200 mg/M^2) has resulted in improved rates of remission induction (2). This agent has also been shown to be effective at high (2–3 gm/m^2) and low (20 mg/m^2) doses. The precise mechanism of action of ara-C and the basis for its efficacy in the treatment of leukemia, however, still remain unclear.

The active metabolite of ara-C is the triphosphate derivative (ara-CTP) which constitutes the majority of intracellular metabolites and is a potent inhibitor of eukaryotic DNA replication (3–5). The mechanisms underlying this inhibition of DNA synthesis have been attributed to effects on DNA polymerase and to incorporation of ara-C into the elongating DNA strand. Although initial studies emphasized the inhibition of DNA polymerase α (4–6), kinetic studies have demonstrated that ara-CTP is a relatively weak competitive inhibitor of this enzyme and that this competition does not completely explain the effect of this agent on replicative DNA synthesis (7).

Other studies demonstrated that ara-C is incorporated in internucleotide linkage and at the DNA chain terminus (3, 8). More recent work from our laboratory has shown a significant relationship between the incorporation of ara-C into DNA and loss of leukemic cell clonogenic survival (9, 10). Furthermore, the incorporated ara-C residue acts as a relative chain terminator and the extent of ara-C(DNA) formation correlates significantly with inhibition of DNA synthesis and the loss of clonogenic survival (11, 12). These findings suggest that the incorporation of ara-C into DNA may in part be responsible for the effects of this agent on DNA synthesis.

The effectiveness of ara-C in the treatment of AML has prompted clinical studies designed to examine biochemical parameters predictive of cytotoxicity. Indeed, a significant association has been found among intracellular ara-CTP concentrations, inhibition of DNA synthesis, and leukemic cytoreduction using pharmacologically directed ara-C therapy (13). Myeloblasts from patients with AML have been examined for ara-C incorporation *in vitro*, but these studies have not yielded significant correlations with clinical outcome (14). Similar studies monitoring *in vivo* incorporation of ara-C into leukemic cell DNA have been limited by the unavailability of an assay sensitive enough to quantitate this event. Since ara-C(DNA) formation has been shown to be the single most powerful predictor of leukemic cell lethality *in vitro* (15), we have developed an approach to monitor ara-C incorporation into DNA *in vivo*. The present work describes this approach and its application to clinical samples.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were reagent grade purchased from Fisher Scientific, unless otherwise specified. Nucleoside standards and enzymes (proteinase K, DNase I, RNase A, snake venom phosphodiesterase, bacterial alkaline phosphatase) were purchased from Sigma Chemical Co., St. Louis, MO. All enzyme concentrations refer to final concentrations. Tetrahydrouridine was obtained from Calbiochem.

Cell Culture. HL-60 cells (obtained from the American Type Tissue Culture Collection) were grown in RPMI Medium 1640, supplemented with 15% heat-inactivated fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells in logarithmic growth phase were incubated for 24 h with various concentrations of [^{3}H]ara-C (20 Ci/mmol; Moravek Biochemicals, Brea, CA) or unlabeled ara-C. The cells were washed 3 times with Dulbecco's phosphate-buffered saline and then analyzed for ara-C incorporation into DNA.

Human leukemic myeloblasts were collected by bone marrow aspiration or venipuncture. The blood or bone marrow sample was diluted 1:2 (v:v) with Hanks' balanced salt solution (Gibco, Grand Island, N.Y.) and the mononuclear fraction collected by Ficoll-Hypaque density centrifugation. The contaminating red blood cells were removed by lysis with 0.87% NH_4Cl and the T-lymphocytes removed by E-rosette formation with sheep erythrocytes.

DNA Extraction. Cellular DNA was collected according to previously described methods (16). For each assay 2–4 × 10^7 cells were suspended in 0.5 ml of lysis buffer (0.2 M Tris, pH 8.0, 0.1 M EDTA, and 2% sodium dodecyl sulfate. RNA was removed by digestion with RNase A (50 μg/ml) for 30 min at 37°C. Proteinase K (100 μg/ml) was added and the cell lysate incubated at 50°C for 15 h. The samples were then placed on ice and 0.2 ml of 5 M potassium acetate was added to each sample. After 30 min, the samples were centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was collected and the precipitate washed with 0.2 ml of cold wash buffer (0.10 M Tris, pH 8.0; 0.05 M EDTA, 1.4 M potassium acetate) and centrifuged at 4°C for 15 min. The supernatants were combined. Two and one-half ml of ethanol were then added and the DNA was precipitated overnight at −70°C. The DNA pellet was collected by centrifugation at 3000 × *g* for 30 min at

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[3] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; HPLC, high pressure liquid chromatography; RIA, radioimmunoassay; AML, acute myelogenous leukemia; ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate; ara-C(DNA), ara-C incorporation in internucleotide linkage or at the DNA chain terminus.

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4°C. The pellet was dried for 10 min by lyophilization.

DNA Digestion to Nucleosides. The DNA was resuspended in 0.5 ml of digestion buffer (0.05 M Tris, pH 8.5, 10 mM $CaCl_2$, 8 mM $MgCl_2$). Tetrahydrouridine was routinely added (0.1 mM) to block any contaminating cytidine deaminase activity in the enzyme preparations. DNase I (50 μg/ml) was added and the sample incubated for 1 h at 37°C. Snake venom phosphodiesterase (0.4 U/ml) was added to each sample. Thirty min later, bacterial alkaline phosphatase (0.2 U/ml) was added and the samples were incubated overnight at 37°C. The resulting solution was filtered with Amicon Centriflo Filters (Amicon Corp., Danvers, MA; M_r 10,000 cut-off) and kept at −70°C until analyses.

HPLC Separation of Nucleosides. The nucleosides were separated by reverse-phase HPLC and detected by absorbance at 254 nm. An isocratic separation was performed using an SCX (Waters Associates, Milford, MA) cartridge in a Waters Z module with 0.02 M KH_2PO_4, pH 2.85, at a flow rate of 3 ml/min. Using this separation, the nucleoside retention volumes were: 1-β-D-arabinofuranosyluracil, 2.3 ml; thymidine, 3 ml; deoxyguanosine, 6 ml; ara-C, 9 ml; deoxycytidine, 12 ml; and deoxyadenosine, 18 ml. Fractions of 0.6 ml were collected and fractions 12–20 were routinely assayed for ara-C.

RIA. The pH of each fraction was adjusted to 7.4 by the addition of NaOH. The concentration of ara-C in each HPLC fraction was determined by a previously described ara-C RIA (17). Fraction aliquots (200 μl) were diluted with RIA buffer (0.05 M K_2HPO_4, 0.9% NaCl, pH 7.4) to a volume of 400 μl. Sheep anti-ara-C antibody (Guildhay Antisera, Guildford Surrey, UK) was added to each tube in a concentration sufficient to bind approximately 40% of [^{3}H]ara-C. The appropriate amount of antibody was determined by competitive binding studies. The assay was then initiated by the addition of 0.1 pmol [^{3}H]ara-C (Amersham; 28.8 Ci/mmol). The assay mixture was incubated for 30 min at 37°C and then terminated by the addition of 100 μl of activated charcoal slurry (Wein Laboratories, Succaswana, NJ). The samples were then centrifuged and 500 μl of the supernatant were counted. The results were compared to a simultaneously determined standard curve. All RIA determinations were performed in duplicate. Fractions with ara-C concentrations greater than 5 ng/ml were diluted with RIA buffer and reanalyzed. The limit of detection for the RIA was 0.5 ng/ml. The daily variation was less than 10% for standard samples.

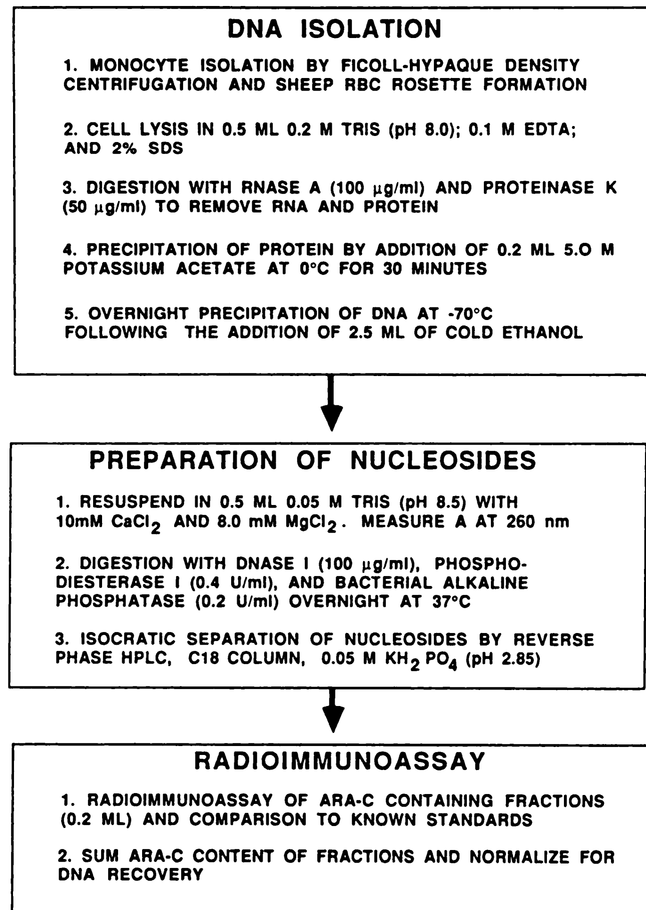

Fig. 1. Scheme for the determinational unlabeled ara-C incorporation into the DNA of HL-60 cells or myeloblasts. For each determination 1–2 × 10^7 myeloblasts were collected from bone marrow or peripheral blood by Ficoll-Hypaque centrifugation. The steps required for DNA isolation, nucleoside preparation, and radioimmunoassay are described in detail in "Materials and Methods."

RESULTS

The analysis of ara-C incorporation into DNA is a multistep process involving DNA extraction, enzymatic digestion to nucleosides, HPLC separation, and quantitation of ara-C residues by RIA. The overall scheme of analysis is shown in Fig. 1. The development and validation of the individual steps are described below.

Ara-C Recovery during DNA Extraction. We first examined the quantitative recovery of DNA from HL-60 cells following treatment with ara-C. Cells were labeled for 24 h with various concentrations (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} M) of [^{3}H]ara-C. An aliquot from each sample was removed and the incorporation of ara-C into DNA determined directly by trichloroacetic acid precipitation. The DNA was then extracted from the remainder of the sample according to the method described in Fig. 1. The purified DNA was resuspended and the amount of labeled ara-C determined for each of the ara-C concentrations. The mean recovery of [^{3}H]ara-C after DNA extraction (compared to direct trichloroacetic acid precipitation) was 84 ± 7% and did not vary substantially with drug concentration.

Recovery of Ara-C during HPLC and RIA. The recovery of ara-C was next examined during the HPLC separation and RIA. A variety of HPLC separations have been described for the determination of ara-C concentrations (18,19). However, none of these approaches provided adequate separation of ara-C from other nucleosides which could interfere with the ara-C RIA. We therefore examined both reverse-phase C_{18} column separations and ion exchange methodologies. The separation chosen is illustrated in Fig. 2*A*. As shown, ara-C elutes as a single sharp peak with a retention time of 180 sec. The results also demonstrate that the [^{3}H]ara-C elutes in 3–5 fractions. Using these conditions, deoxycytidine, the nucleoside most likely to cause competitive inhibition in the RIA, had a retention time of 246 sec and did not contaminate the ara-C fractions. In this regard, tritium was undetectable in the ara-C-containing fractions after adding a 1,000-fold excess of [^{3}H]deoxycytidine. Furthermore, the other deoxyribonucleosides were also separated from ara-C (Fig. 2*A*). Finally, 1-β-D-arabinofuranosyluracil, the product of ara-C deamination, eluted near the void volume and did not interfere with the ara-C purification. The limit of detection for ara-C by UV absorbance is approximately 1 μM.

The pH of the HPLC fractions was adjusted to 7.5 and then analyzed by RIA. Since the HPLC separation buffer and the RIA buffer were similar, it was possible to directly analyze 0.2-ml aliquots for ara-C content. The RIA was standardized at different ara-C concentrations and the inter-assay variation was less than 10%. The standard curve, as determined by 6 independent assays, is shown in Fig. 2*B*.

The recovery of ara-C following HPLC separation and RIA was examined at known concentrations of ara-C. Various quantities of ara-C were mixed with the deoxynucleosides and subjected to HPLC separation and then RIA. Recovery over a 100-fold range of ara-C concentrations is shown in Fig. 3. This recovery of ara-C was linear over this range and exhibited a

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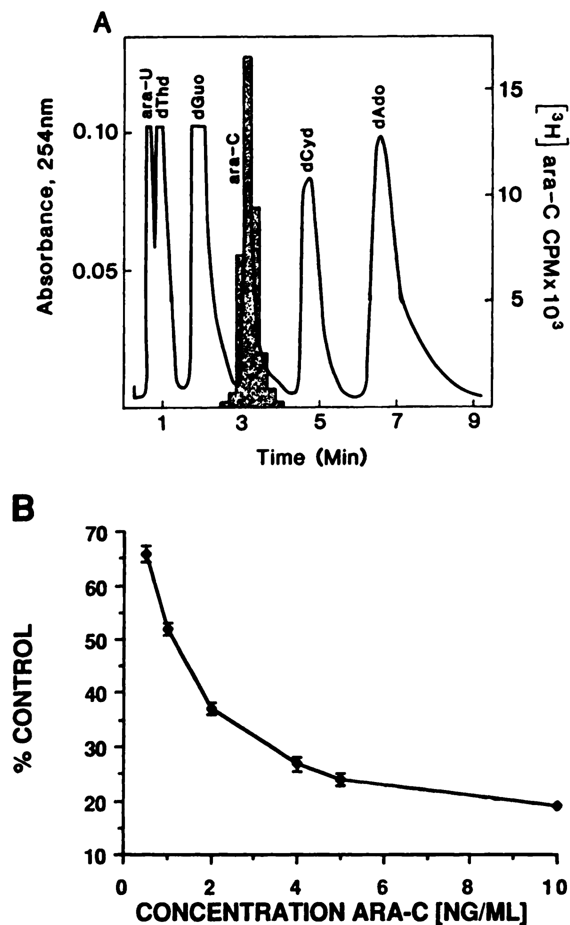

Fig. 2. *A*, nucleoside chromatogram for the isolation of ara-C from nucleoside. *B*, standard curve for radioimmunoassay. Values represent the mean of six determinations each performed in triplicate.

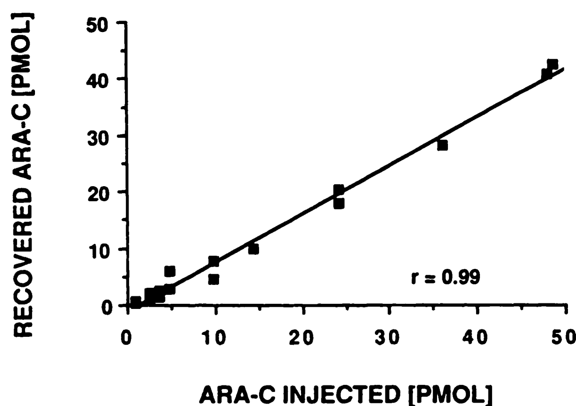

Fig. 3. Recovery of ara-C following HPLC and RIA. Various amounts of ara-C (0–40 pmol) were separated by HPLC and 0.6-ml eluate fractions were analyzed for ara-C by RIA. Each value is the mean of duplicate determinations.

linear regression curve fit of $r = 0.99$. The slope of the linear regression line reflects a mean ara-C recovery of 85%. The limit of detection for ara-C was 5 pmol. Taken together, these findings suggested that ara-C recovery was consistent and sufficient to justify use of this approach for quantitative studies.

Internal Standard for Sample Analysis. An internal standard was also used in developing the assay. Aliquots of [^{3}H]thymidine-labeled DNA (200,000 cpm) were added to cells being analyzed for ara-C incorporation. DNA was purified from the lysed cells and then digested to nucleosides. Recovery was then monitored after filtration (Fig. 1). The results demonstrated tritium recoveries of greater than 80%. No further losses of tritium were obtained after collection of thymidine fractions from the HPLC elute.

Determination of Unlabeled Ara-C Incorporation into HL-60 DNA. The incorporation of ara-C into HL-60 DNA was compared using two techniques. HL-60 cells in logarithmic growth phase were exposed to various concentrations of labeled and unlabeled ara-C. The formation of ara-C(DNA) was first determined by [^{3}H]ara-C incorporation assays. The extent of [^{3}H]ara-C incorporation was log linear over drug concentrations of 10^{-7} to 10^{-4} M. These results were compared to measurements of unlabeled ara-C incorporation as described in Fig. 1. A comparison of these results is shown in Fig. 4. There was a high degree of correlation between the results of these two methods ($r = 0.93$).

We also examined the effect of ara-C concentration on incorporation of unlabeled ara-C into HL-60 DNA. HL-60 cells were exposed to various concentrations of unlabeled ara-C for 24 h and the formation of ara-C (DNA) was determined according to the method described in Fig. 1. The incorporation of ara-C into DNA (normalized as pmol/μg DNA) was log linear for ara-C concentrations of 1 μM to 100 μM (Fig. 5). The DNA recovery for HL-60 cells was approximately 200 μg DNA/10^7 cells.

Application to Clinical Specimens. This technique has been

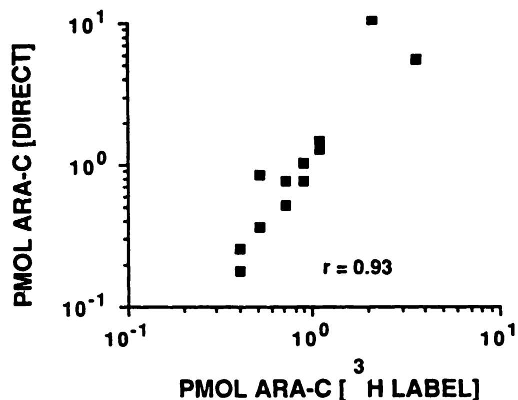

Fig. 4. Comparison of methods monitoring labeled and unlabeled ara-C into DNA. HL-60 cells (10^7 cells/sample) in logarithmic growth phase were exposed to various concentrations of [^{3}H]ara-C or unlabeled ara-C. For each concentration of ara-C, formation of ara-C(DNA) was determined by [^{3}H]ara-C incorporation and by the method described in Fig. 1. The values for [^{3}H]ara-C incorporation are the mean of 5 determinations. The values for the measurement of unlabeled ara-C incorporation (method of Fig. 1) are the mean of duplicate determinations. Values are expressed as pmol/10^6 cells.

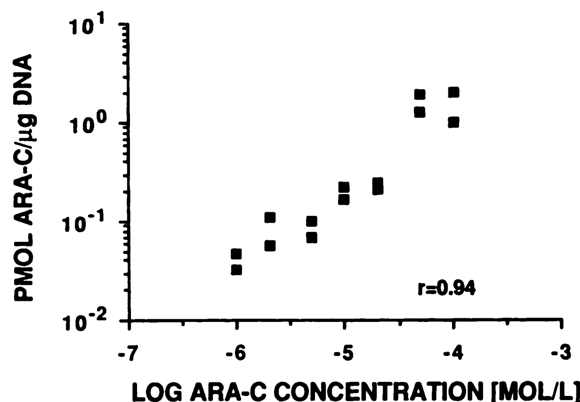

Fig. 5. Incorporation of ara-C into HL-60 cell DNA. HL-60 cells in logarithmic growth phase were treated for 24 h with 1–100 μM ara-C. The incorporation of unlabeled ara-C was measured (method of Fig. 1) and normalized for DNA recovery.

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used to monitor the incorporation of ara-C into the DNA of circulating myeloblasts from patients with refractory leukemia undergoing high-dose ara-C therapy. Patients received 250 mg/m^2/h of ara-C as a continuous infusion. Plasma ara-C concentrations of 5–30 μM are characteristically achieved during these infusions. Circulating leukemia cells were collected prior to treatment and after 24 h of ara-C therapy. The myeloblasts were collected by Ficoll-Hypaque separation and analyzed for ara-C incorporation (Fig. 1). Samples of HL-60 cells exposed to known concentrations of ara-C were analyzed in conjunction with the clinical material as concurrent standards. The results are listed in Table 1. None of the pretreatment myeloblast samples had detectable amounts of ara-C. The ara-C(DNA) formation in leukemic myeloblasts after ara-C treatment as measured on a normalized pmol/μg DNA basis was less than the ara-C incorporation into DNA of HL-60 cells exposed *in vitro* to 5–10 μM ara-C for 24 h.

DISCUSSION

We have previously studied the relationships among ara-CTP pools, formation of ara-C(DNA), and cytotoxicity of human HL-60 promyelocytic leukemia cells (15). Although a correlation was found between ara-CTP pools or continuous cellular exposure to ara-CTP and cell kill, these relationships were less significant than that obtained with formation of ara-C(DNA). Furthermore, the extent of ara-C incorporation into DNA was predicted by the product of the ara-CTP level and time. This finding was in concert with the dependence of ara-C(DNA) formation on continuous exposure to the triphosphate metabolite. These results thus supported the concept that incorporation of ara-C into DNA is a biochemical parameter highly predictive of ara-C induced cytotoxicity (15).

Previous studies have monitored the incorporation of ara-C into leukemic cell DNA *in vitro* by incubating in the presence of various concentrations of [^{3}H]ara-C (10, 20). The extent of *in vitro* ara-C(DNA) formation in blasts from patients with leukemia correlated with loss of clonogenic survival (10). The interpretation of these findings, however, was limited by the nature of this type of experiment, which requires growth of primary leukemic cultures. Consequently, the development of an assay to monitor ara-C incorporation into DNA *in vivo* could provide greater insights into the pharmacodynamics of this agent.

In the present study, we describe an approach which is capable of detecting unlabeled ara-C residues incorporated into DNA.

Table 1 *In vivo incorporation of ara-C into leukemic cell DNA during high-dose (250 mg/m^2/h) continuous-infusion ara-C therapy*

Patients with refractory, relapsed acute leukemia had myeloblasts collected for measurement of ara-C incorporation into myeloblast DNA according to the protocol outlined in Fig. 1. Plasma ara-C levels were measured by RIA as described (16).

Patient	Age/sex	Disease	Plasma ara-C level (μM)	Ara-C incorporation (pmol/μg DNA)	Response
1	49/F	AML	24.2	0.36	NR[a]
2	70/M	MDS/AML[b]	ND[c]	0.29	NR
3	35/M	AML	12.2	0.14	NR
4	37/F	AML[d]	8.3	<0.01	NR
5	74/M	MDS/AML	ND	0.29	NR
6	28/M	CML[e]	4.5	0.08	NR

[a] NR, no response.
[b] MDS, myelodysplastic syndrome.
[c] ND, not determined.
[d] AML following alkylating agent therapy.
[e] CML, chronic myelogenous leukemia.

This assay utilizes a previously described ara-C RIA after separation of ara-C from deoxyribonucleosides. The sensitivity of this approach was determined by comparing incorporation of [^{3}H]ara-C and unlabeled ara-C into HL-60 cell DNA under the same experimental conditions. The results demonstrate remarkably similar levels of ara-C(DNA) formation. The expression of the results as pmol ara-C incorporated per μg DNA represents the frequency of misincorporation and is therefore not affected by variations in sample cell number or losses of DNA during the extraction procedures. We have previously expressed our *in vitro* [^{3}H]ara-C incorporation studies as pmol incorporated into the DNA of a fixed number of cells. Thus, although both approaches express results on the basis of misincorporation frequency, we have used pmol ara-C/μg DNA for monitoring incorporation of this drug *in vivo*.

This method to monitor ara-C(DNA) formation *in vivo* has been applied to samples of leukemic blasts obtained from patients receiving high doses of ara-C by continuous infusion. Plasma ara-C levels (10 μM) achieved with this dose schedule (21) and 24 h of drug exposure would predict 0.5–1.0 pmol ara-C incorporated per μg DNA on the basis of *in vitro* [^{3}H]ara-C incorporation studies. Ara-C(DNA) was undetectable in leukemic blast DNA prior to treatment and reached levels of <0.01–0.36 ara-C/μg DNA in these samples after 24 h. Thus, the amount of ara-C(DNA) formation in leukemic blasts *in vivo* is somewhat less than that obtained from ara-C incorporation studies in HL-60 cells. Greater incorporation of ara-C into HL-60 cells would be expected, since HL-60 cells have a larger S-phase fraction than circulating myeloblasts. The number of patient studied thus far does not permit correlation between clinical parameters and ara-C incorporation into DNA. Although the present studies were conducted on leukemic blasts from patients receiving high doses of ara-C by continuous infusion, this approach may also be applicable during the administration of conventional doses of this agent. In contrast, the level of sensitivity would preclude the detection of ara-C(DNA) formation during low-dose continuous-infusion regimens which achieve plasma ara-C levels of approximately 5×10^{-8} M (22) and result in the incorporation of 0.02 to 0.06 pmol ara-C/10^6 cells (20).

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