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A simplified assay for measurement of cytosine arabinoside incorporation into DNA in Ara-C-sensitive and -resistant leukemic cells

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Summary. The assays for the detection of unlabeld 1-β-D-arabinofuranosylcytosine (cytosine arabinoside, Ara-C) incorporation into DNA was simplified. The procedure includes DNA isolation from leukemic cells, quantification of DNA concentrations, breakdown by enzymatic digestion of DNA to nucleosides and a radioimmunoassay (RIA) using an antibody against Ara-C. Different techniques for quantification of DNA concentrations are compared. A fluorimetric technique using Hoechst 33258 is preferred because it is the most specific method. Comparison of this RIA assay with measurement of [^{3}H]-Ara-C/DNA formation under similar conditions in HL-60 cells showed a correlation of 0.99. Ara-C incorporation into DNA of leukemic cells was studied using two rat-leukemia cell lines, one of which is sensitive to Ara-C and the other is an Ara-C-resistant wild type: BNML-Cl/0 and BNML-Cl/Ara-C, respectively. The results showed that Ara-C is incorporated when the cells are incubated at concentrations equal to or higher than the Ara-C concentration that induces 50% growth inhibition after 48 h incubation (IC_{50}). This implies that at lower Ara-C concentration, i.e. levels that do not induce cytotoxicity, Ara-C is not incorporated into DNA. Similar results were obtained with human HL-60 myeloid leukemia cells. The detection limit of this assay is 2 pmol/ml Ara-C; therefore, the assay is more sensitive than measurement of Ara-C triphosphate (Ara-CTP), the only metabolite that can be measured in leukemic cells from patients after in vivo Ara-C administration. On the basis of in vitro studies, the finding of detectable Ara-C/DNA levels in vivo is expected to correlate with cytotoxicity; whether or not the Ara-C/DNA level itself is informative remains to be evaluated.

Introduction

1-β-D-Arabinofuranosylcytosine (Ara-C) is currently the most important drug for treatment of acute myelogenous leukemia (AML). The relationship between intracellular Ara-C metabolism and cytotoxicity remains unclear. To be able to solve the clinical problem of Ara-C resistance, which is a major reason for treatment failure among patients with leukemia, it is important that the intracellular biochemical pathway of Ara-C metabolism that leads to cell death be understood. Similar studies of Ara-C-resistant leukemia might reveal that other biochemical pathways lead to leukemic cell survival after Ara-C exposure.

In experimental and clinical studies, the triphosphate form of Ara-C, Ara-CTP, is considered to be the active metabolite [12, 15]. In this form it is incorporated into DNA, which results in the slowing down of DNA chain elongation [10, 17] and in inhibition of DNA synthesis [3]. The cytotoxic activity of the drug is believed to occur in the S-phase of the cell cycle and to be related to incorporation into DNA [8, 18]. Furthermore, the extent of Ara-C incorporation into DNA appears to correlate better with inhibition of DNA synthesis and loss of clonogenic cell survival than with the intracellular Ara-CTP concentrations [6, 8].

For evaluation of the relevance of Ara-C metabolite concentrations to cell kill in Ara-C-sensitive and -resistant leukemia in vivo, sensitive assays must be designed. In this report we describe a method for the detection of low amounts of Ara-C in DNA after exposure of cells to non-radioactive Ara-C; this method is also applicable for measuring Ara-C/DNA concentrations after in vivo treatment with Ara-C. Consecutive steps in the assay consist of DNA isolation, quantification of DNA content in samples, break-

Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; *Ara-C/DNA,* Ara-C incorporated into DNA; *Ara-CTP,* 1-β-D-arabinofuranosylcytosine triphosphate; *BNML/0,* Brown Norway myeloid leukemia/0 sensitive to Ara-C; *BNML/Ara-C,* Brown Norway myeloid leukemia resistant to Ara-C; *BNML-Cl/0,* Ara-C-sensitive cell line derived from the BNML/0 model; *BNML-Cl/Ara-C,* Ara-C-resistant cell line derived from the BNML/Ara-C model; *CV,* coefficient of variation; *HPLC,* high-performance liquid chromatography; *PBS,* phosphate-buffered saline; *RIA,* radioimmunoassay; *SSC,* sodium saline citrate

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down by enzymatic digestion of DNA to nucleosides and a radioimmunoassay using an antibody against Ara-C [11]. This procedure proved to be more accurate due to the method used for quantification of the DNA concentration; in addition, the method described is easier and more rapid than that described by Spriggs et al. [16] because separation of the nucleosides by HPLC proved to be unnecessary.

The present report concerns the measurement of Ara-C incorporation into DNA in cells from an HL-60 cell line and two rate-leukemia cell lines, the parent cells being sensitive to Ara-C and the wild type being resistant to the drug. Attention was also focused on the relationship between incorporation and sensitivity to Ara-C as indicated by growth curves and clonogenic assays.

Materials and methods

Cells

HL-60 cell culture. The human promyelocytic cell line HL-60 was maintained in culture by growing them in RPMI 1640 with 25 mM HEPES buffer (Gibco, Scotland) supplemented with 4 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (Gibco) at 37° C in a fully humidified atmosphere comprising 5% CO_2 in air. The number of viable cells was determined by counting cells that had not incorporated eosin in a hemocytometer. Cultures were checked for contamination with *Mycoplasma* every 2 months. All experiments were performed using cells in exponential growth. The doubling time of HL-60 cells under these conditions is about 48 h. Ara-C (Sigma, St. Louis, Mo., USA) was added at a final concentration ranging from 0.01 to 100 µM.

Rat-leukemia cell lines. For this study, cell lines derived from two rat-leukemia models were used; one model is the Brown Norway acute myelocytic leukemia that is sensitive to Ara-C (BNML/0), and the second is the Ara-C resistant wild type (BNML/Ara-C). Ara-C resistance was developed in the in vivo model by suboptimal Ara-C treatment and repeated transplantation [4]. Leukemic cells from both of these models grow in vitro, the cell lines being called BNML-Cl/0 and BNML-Cl/Ara-C, respectively; they were generously donated by Dr. Arkesteyn (TNO, Rijswijk, The Netherlands). The doubling time of both rat cell lines is 24 h for cells in exponential growth. Both cell lines can be maintained under conditions similar to those described for HL-60, except that for the resistant cell line, half of the fetal calf serum must be replaced by rat serum.

Patient cells. Bone marrow cells were aspirated from the posterior iliac crest of patients with bone marrow containing >80% leukemic cells at 2 h after cessation of a 2-h Ara-C infusion of 1 g/m^2. The samples were collected in a tube containing 140 units (dry) heparin/10 ml. The red cells were lysed with buffered NH_4Cl [14].

Procedure for Ara-C/DNA measurement

Isolation of cell nuclei. To remove the major part of cellular RNA and protein, cell nuclei were isolated prior to DNA isolation. For this purpose, the cell pellet was resuspended in 0.32 M sucrose buffer (5 mM $MgCl_2$, 1% Triton X-100 in 10 mM TRIS/HCl; pH 7.4) at a concentration of 4×10^7 cells/10 ml buffer. The detergent Triton in this hypotonic buffer partly solubilizes the membranes of the cells. The cells were spun for 10 min at 1,600 rpm. After two washes in buffer, the pellet was resuspended in 2 ml cytoplasm lysis buffer (0.25 M sucrose buffer, 10 mM $CaCl_2$, 0.1% Triton-X-100 in 10 mM TRIS/HCl; pH 7.4); the cells were carefully sheared twice through a 21-gauge needle using a 2-ml syringe; per 4×10^7 cells, the volume was increased to 10 ml with lysis buffer and then incubated for 10 min at 4° C. After centrifugation of the cells for 10 min (2,400 rpm) at 4° C, the pellet can be stored at –20° C until DNA isolation.

DNA isolation. DNA was isolated by a modification of the method described by Davis et al. [1]. Cell nuclei (5×10^7) were lysed overnight in 2 ml 0.5% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM TRIS-HCl (pH 8.0) and proteinase K (Boehringer Mannheim, FRG) at 65° C. The viscosity of the clear solution was reduced by shearing through a 19-gauge needle; 300 µl lysed cells was added to 8 M potassium acetate (final concentration, 1 M), and incubation was allowed to proceed for 15 min at 4° C. Extraction was performed with 3 ml chloroform and the precipitate was centrifuged at 15,000 rpm. The white interphase between the aqueous and chloroform layers can be washed again with water to increase the DNA yield. The aqueous supernatants containing DNA and RNA were collected and treated with 50 µg/ml RNAse I (1 mg/ml PBS, Sigma) and 5 units/ml RNAse T_1 (Boehringer Mannheim, FRG) for 45 min at 37° C. DNA was precipitated with ethanol and dissolved overnight in distilled water.

Quantification of DNA. The absorbance at 260 nm was used to obtain a rough estimate of the DNA concentration in the sample, for accurate measurement of DNA concentration two techniques, both based on fluorometry, were applied. The two methods for quantification of DNA concentrations in samples were compared. If the two methods are equivalent, orthogonal regression analysis of the values of the two assays approaches 1.00.

One method uses ethidium bromide; it is a modification of the technique described by Karsten and Wollenberger [5]. Because ethidium bromide is not a specific dye for DNA, this method includes protein and RNA digestion. For a standard DNA calibration curve, a stock solution of 1 mg/ml thymus DNA (Pharmacia, Uppsala, Sweden) in distilled water is stored in small portions and diluted to 2–10 µg/ml. Tyrode buffer supplemented with 1 mg/ml trypsin (Difco, Detroit, Mich., USA), 8.3 units/ml heparin and 0.05 mg/ml ribonuclease-A is added to the samples and standards (Tyrode buffer: 8 g NaCl, 0.2 g KCl, 0.056 g KH_2PO_4, 1 g $NaHCO_3$, 1.48 g EDTA per liter). The mixture is incubated at 37° C for at least 20 min. After the addition of 1 ml 0.025 mg/ml ethidium bromide in PBS, the fluorescence intensity is measured in a fluorimeter (LS-2 Perkin and Elmer, Norwalk, Conn., USA) at an excitation wavelength of 366 nm and an emission wavelength of 590 nm.

The second method uses Hoechst 33258 as the fluorescent dye. The DNA stock solution is similar to those described above. Samples and the DNA standard curve are prepared in sodium citrate saline buffer (SSC: 0.015 *M* sodium citrate, 0.15 M NaCl). Because of the specificity of Hoechst for DNA, no degradation of protein and RNA is necessary. After the addition of 2 µg/ml Hoechst 33258 in SSC, the fluorescence intensity after excitation at 366 nm and emission at 460 nm can be measured.

DNA digestion to nucleosides. To 50 µg DNA in 500 µl 10 mM TRIS (pH 7.4) were added 10 µl DNAse I [Sigma, 2 mg/ml in TRIS-HCl (pH 7.4) supplemented with 10 mM $MgCl_2$ and 8 mM $CaCl_2$), 5 µl snake-venom phosphodiesterase (Sigma), 5 mg/ml TRIS-HCl (pH 7.4) and 5 µl alkaline phosphatase type III-N bacterial *Escherichia coli* (Sigma) diluted with TRIS (pH 7.4) to 50 units/ml. The samples were incubated at 37° C for varying lengths of time, after which they were ready for radioimmunoassay.

Radioimmunoassay of Ara-C. An antibody against Ara-CMP, raised in sheep, was obtained from Guildhay Antisera LTD. (Guildford, Surrey, UK). The radioimmunoassay (RIA) was performed using a dilution of antibody at which 50% of the added [^{3}H]-Ara-C was bound. Such a serum antibody-dilution curve had to be checked at least every month because the binding capacity decreases with time, resulting in a reduction of the sensitivity of the assay. The measurement of Ara-C in digested DNA samples was carried out as described for serum samples by Piall et al. [11]. In brief, the samples are incubated with diluted antiserum and 0.1 pmol [^{3}H]-Ara-C (see below) per sample for 50 min at 37° C. For termination of the reaction, the samples are put aside at 4° C for 10 min,

after which 0.1 ml continuously mixed Dextran-coated charcoal (Pharmacia) suspension is added; this suspension includes 25 g/l Norit A (Sigma) and 2.5 g/l Dextran T-70 (Pharmacia) in assay buffer. After the samples are maintained for 15 min at 4° C and then centrifuged for 10 min at 4° C and 2,500 rpm, 0.5 ml supernatant is mixed with 10 ml Atomlight (NEN, Boston, Mass., USA) and subsequently counted in an LKB 1217 Rack beta liquid scintillation counter (LKB, Cambridge, UK).

Incubation with [^{3}H]-Ara-C. The results of the RIA were compared with [^{3}H]-Ara-C incorporation into DNA as measured by a scintillation counter. In parallel experiments, HL-60 cells were incubated with 10^{-6} M Ara-C or 10^{-6} M [^{3}H]-Ara-C (specific activity, 28 Ci/mmol; Amersham International, UK). The ratio of [^{3}H]-Ara-C: Ara-C was 1:28, which resulted in ± 5,000 cpm/100 µg DNA. Cell nuclei and DNA were isolated as described above and DNA concentrations were measured. The unlabeled Ara-C samples were assessed by RIA as described above and the labeled samples were estimated by counting of 100 µg DNA using a scintillation counter. The counting efficiency of the scintillation counter under these conditions was 38%.

Results

Isolation of DNA and estimation of DNA concentrations

The isolation of cell nuclei prior to DNA isolation causes cytoplasmic RNA and protein to vanish. HPLC analysis of these DNA samples after enzymatic digestion to nucleosides shows only traces of ribonucleosides (cytidine, <1 ng/µg DNA; data not shown). The DNA concentrations in the samples, as determined by spectrophotometry at 260 nm, were used to calculate the dilution factor for the fluorometric assays. Two methods for DNA quantification were compared: one uses ethidium bromide, which is an intercalating dye [2], and the other, Hoechst 33258; the latter has a high affinity for adenine and thymidine and is not intercalated into DNA [7].

Figure 1 shows the regression line obtained by orthogonal analysis of the concentrations obtained with Hoechst 33258 and ethidium bromide (slope α, 1.023; intercept Y axis, 13.7; correlation coefficient, 0.99; n = 69). The correlation coefficient for the comparison between UV and ethidium bromide is 0.96 and that for UV and Hoechst is 0.96. This indicates that the DNA concentration can be measured with equal accuracy using ethidium bromide and Hoechst 33258 and that the value obtained using UV absorbance is not accurate enough. Because the assay with Hoechst is more specific and less time-consuming, this technique was used throughout these studies.

The many steps leading from intact cell to isolated DNA are accompanied by some cell loss and, thus, DNA loss. In Table 1, the amounts of DNA isolated from the leukemic cells of a patient are listed; two cell concentrations were isolated in duplicate. The DNA yield per number of cells varies considerably; the highest DNA concentration was observed when the cell number was 5×10^7.

DNA breakdown to nucleosides by enzymatic digestion

Ara-C has proven to be stable in the DNA-digesting environment at 37° C for 24 h, as measured by RIA (data not shown). For recovery of known concentrations of Ara-C added to thymus DNA and enzymes, a correlation coefficient of 0.98 was calculated. The precision of the RIA was determined after the addition of 0.5 and 5 ng/ml Ara-C to the assay microenvironment; this yielded a coefficient of variation (CV) of 3.9% (n = 6) and 5.3% (n = 6), respectively.

For investigation of the time needed for total DNA digestion to nucleosides, the experiments must be performed using cells that have been exposed to Ara-C. For this purpose, HL-60 cells that had been incubated with 10^{-3} M Ara-C and leukemic cells from a patient who had received an Ara-C infusion of 1 g/m^2 were used (see Materials and methods). Plateau levels of Ara-C were detected between 4 h after the start of the enzymatic digestion and up to 24 h. An incubation period of 4 h was used for the following studies; however, an overnight incubation period is also acceptable.

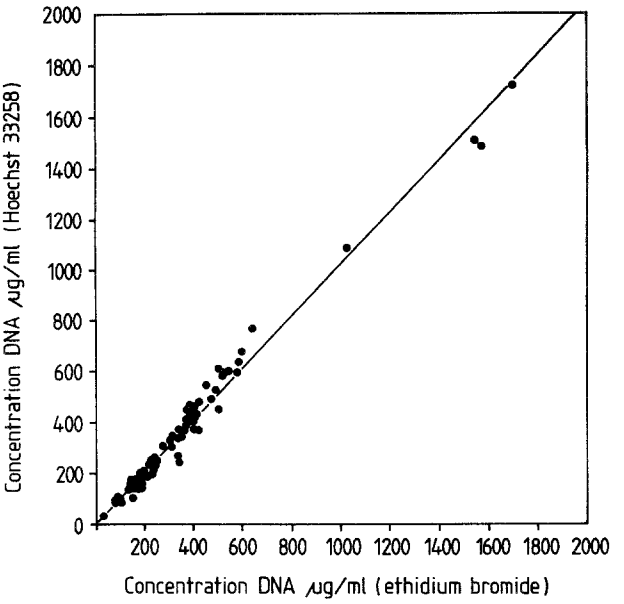

Fig. 1. Comparison between two techniques based on fluorometry for measuring DNA concentrations in samples isolated from leukemic cells. DNA extracts were derived from leukemic cells from patients (HL-60 cells), BNML-C1/0 and BNML-Cl/Ara-C cells

Table 1. Amount of DNA isolated from increasing numbers of cells from a patient with leukemia; DNA concentrations were measured by the Hoechst 33258 technique

		Cell number	DNA (µg/ml)	
			Per total cell number	Per 10^7 cells
Sample	A	2×10^7 cells	102	51
	B	5×10^7 cells	484	97
	C	5×10^7 cells	440	88
	D	1×10^8 cells	770	77
	E	1×10^8 cells	680	68

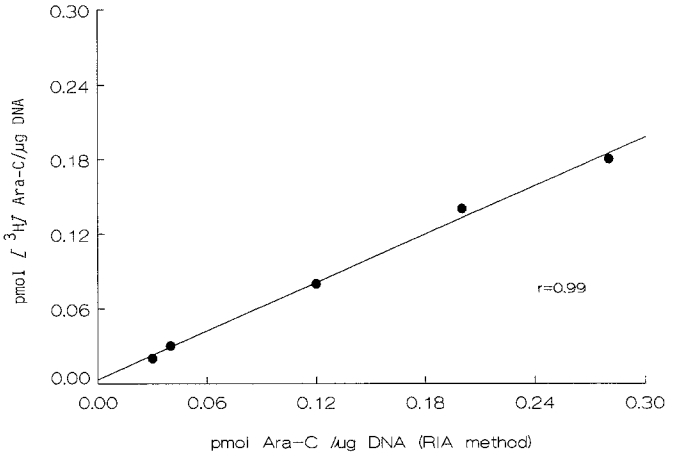

Fig. 2. Relationship between two methods for measuring Ara-C/DNA concentrations: a radioimmunoassay of unlabeled Ara-C versus a technique for measuring radiolabeled Ara-C incorporation into DNA by means of a scintillation counter. HL-60 cells were exposed to 1 µM Ara-C or [^{3}H]-Ara-C as described in Materials and methods. They were incubated for 3 h (in duplicate) and for 6, 12 and 24 h, respectively. The values for the measurement of unlabeled Ara-C represent those from the series in Fig. 3 using 1 µM Ara-C. The data for [^{3}H]-Ara-C incorporation represent the mean values of separate experiments performed in quadruplicate. The equation of the regression line is y = 0.67 X + 0.085

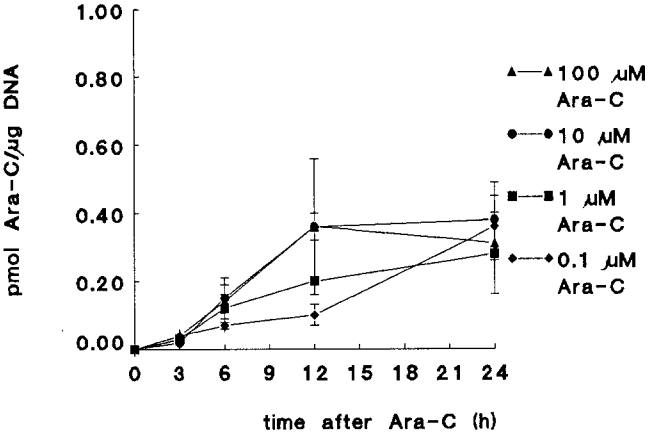

Fig. 3. Incubation of exponentially growing HL-60 cells with Ara-C at concentrations varying from 0.1 to 100 µM Ara-C during the indicated points in time. The values at 3 h for all 4 concentrations and at 12 h for 1 µM represent the mean values of two experiments; at other time points the values represent the mean ± SD of 6 identical experiments

Ara-C incorporation into DNA

Measurement of Ara-C incorporation into DNA in leukemic cells harvested 2 h after the cessation of a 2-h infusion of Ara-C (1 g/m^2) resulted in a mean value of 0.05 pmol Ara-C/µg DNA and a CV of 17% measured as replicates (n = 6) in one DNA sample. The plasma Ara-C concentration during the infusion period was estimated to be 10^{-6}–10^{-5} M. In a DNA sample isolated from HL-60 cells after incubation with 10^{-5} M Ara-C for 6 h, the mean value was 0.12 pmol Ara-C/µg DNA and the CV was

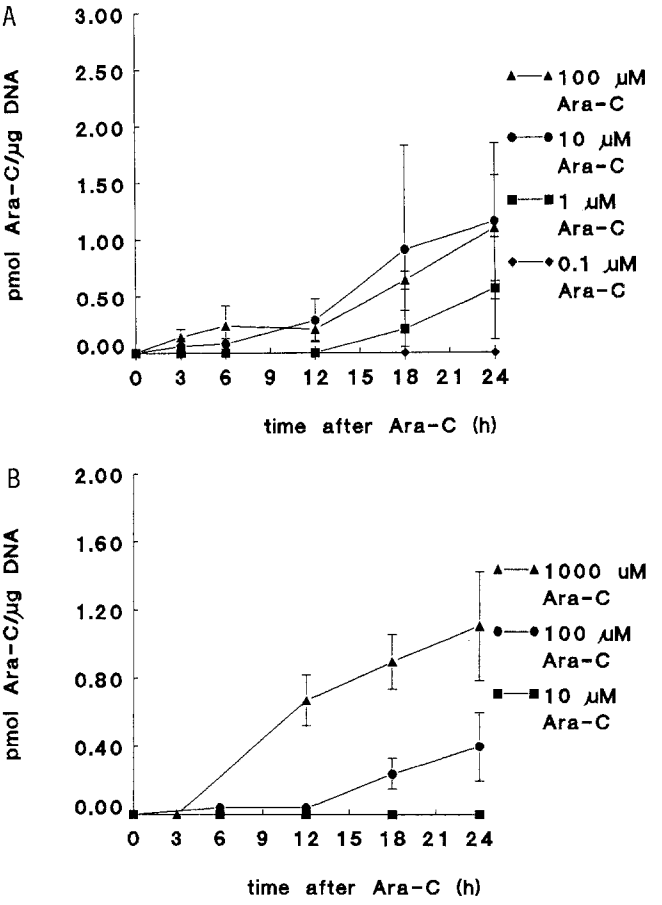

Fig. 4 A, B. Ara-C incorporation into DNA in **A** the Ara-C sensitive cell line BNML-Cl/0 after incubation with Ara-C at concentrations ranging from 0.1 to 100 µM and **B** the Ara-C-resistant cell line BNML-Cl/Ara-C after incubation with Ara-C at concentrations ranging from 10 to 1000 µM. Cells were harvested for the assay at the indicated time intervals. Each point represents the mean ± SD of 3 experiments; the number of experiments carried out at 24 h are shown in Table 2

8.4%, also measured as replicates (n = 6) in one DNA sample. The sensitivity of the assay was 2 pmol/ml. In general, 25–100 µg DNA is dissolved in 1 ml TRIS (10 mM). The sensitivity of the assay can be increased by dissolving more DNA per milliliter; it is possible that this quantum of DNA needs more digesting enzymes or a longer incubation time.

The results of the measurement of Ara-C incorporation by RIA were compared with those obtained by scintillation counter after incubation of the cells in a parallel experiment with [^{3}H]-Ara-C (Fig. 2). The correlation coefficient for the results of the two assays was 0.99. The [^{3}H]-Ara-C/DNA method underestimates the amounts of incorporated Ara-C as compared with the RIA; a similar observation can be concluded from the study by Spriggs et al. [16]. Figure 3 shows Ara-C incorporation into DNA in exponentially growing HL-60 cells after incubation for 24 h with increasing concentrations of Ara-C, varying from 0.1 to 100 µM. An increase in incorporation was observed until 12 h for all concentrations tested; however, cells incubated with the lowest concentrations (0.1 and 1 µM) continued to incorporate Ara-C until the end of the incubation period.

Table 2. Ara-C/DNA concentrations after incubation with Ara-C for 24 h as compared with the sensitivity for Ara-C, expressed as IC_{50}

	HL-60	BNML-Cl/0	BNML-Cl/Ara-C
10^{-8} M Ara-C	±0 ($n = 2$)	0 ($n = 2$)	ND
5.10^{-8} M Ara-C	0.14±0.01 ($n = 2$)	0 ($n = 2$)	ND
10^{-7} M Ara-C	0.30±0.05 ($n = 6$)	0 ($n = 2$)	0 ($n = 2$)
5.10^{-7} M Ara-C	0.20±0.02 ($n = 2$)	0.06±0.01 ($n = 2$)	ND
10^{-6} M Ara-C	0.28±0.12 ($n = 6$)	0.57±0.45 ($n = 5$)	0 ($n = 2$)
10^{-5} M Ara-C	0.37±0.13 ($n = 6$)	1.16±0.69 ($n = 7$)	0 ($n = 2$)
5.10^{-5} M Ara-C	ND	ND	0.10±0.06 ($n = 3$)
10^{-4} M Ara-C	0.36±0.05 ($n = 5$)	1.10±0.48 ($n = 4$)	0.40±0.20 ($n = 7$)
10^{-3} M Ara-C	ND	ND	1.11±0.32 ($n = 5$)
IC_{50}	5.10^{-8} M	10^{-6} M	10^{-4} M

Data represent the mean ± Sd, expressed in pmol/μg DNA; the respective number of experiments is indicated in parentheses. ND, not done; IC_{50}, the Ara-C concentration that induces 50% growth inhibition after 48 h incubation

Ara-C incorporation into Ara-C-sensitive and -resistant cells

The BNML-Cl/Ara-C cell line is derived from the rat Ara-C-resistant leukemia model. Details of the characteristics of the parent BNML-Cl/0 and the BNML-Cl/Ara-C lines will be published elsewhere [13]. In Fig. 4, the incorporation of Ara-C into DNA in both cell lines is shown. A gradually increasing amount of Ara-C was incorporated during 24 h incubation. In the Ara-C-sensitive cell line, we detected Ara-C incorporation into DNA at drug concentrations of >0.1 μM, whereas the resistant cell line required a 100-fold higher concentration before incorporation into DNA could be detected.

Table 2 lists the amounts of Ara-C incorporated 24 h after continuous incubation with Ara-C concentrations varying from 10^{-8} to 10^{-3} M for the three cell lines HL60, BNML-Cl/0 and BNML-Cl/Ara-C. The data represent the mean values of separate experiments ± SD. This table shows that an Ara-C concentration that induces 50% growth inhibition after 48 h incubation in a parallel experiment (IC_{50}) results in detectable Ara-C/DNA levels. When cells are incubated with Ara-C at concentrations lower than the IC_{50} for a particular cell line, very low levels of Ara-C, if any, are incorporated into DNA.

Discussion

On the basis of in vitro studies with human leukemic cells, Ara-C/DNA concentrations are considered to be the most important biochemical parameter for predicting Ara-C-induced cytotoxicity. Measurement of Ara-C/DNA concentrations was performed by incubation of the cells with [^{3}H]-Ara-C [6]. We improved an assay previously described [16] for the detection of non-radiolabeled Ara-C; the sensitivity of the assay enables the detection of 1 molecule of Ara-C among 5,000 molecules of other DNA nucleosides. The many steps leading to DNA isolation imply an unavoidable loss of cells and DNA. It is unlikely that this loss is a serious problem because it is assumed to involve a random loss of cells. Therefore, it is important that the amounts of Ara-C be expressed per microgram DNA and not per cell number, as is clearly shown in Table 1. Working with whole cells, Spriggs et al. [16] found a DNA recovery from HL-60 cells of approximately 200 μg/10^7 cells as measured by absorbance at 260 nm alone. This yield is considerably higher than that found in our study and might be due to protein and RNA contamination. The measurement of DNA concentrations in samples extracted by UV absorption from cell nuclei as opposed to whole cells has proven to be insensitive, as also indicated by the correlation coefficient reported in the Results section of this report.

Separation of the nucleosides by HPLC before RIA was performed on the relevant fractions subsequently collected proven to be unnecessary [16]. One reason for using HPLC might be that the antiserum could exhibit cross-reactivity with deoxycytidine. In the study by Piall et al. [11], deoxycytidine produced a background value of only <0.19%. Because we detected no binding to antiserum in DNA samples isolated from cells that had not been treated with Ara-C, we concluded that possible cross-reactivity with other nucleosides is a negligible factor, since it lies below the detection limit of the assay. No degradation of Ara-C was observed during the whole analytical process, and an excellent coefficient of variation was calculated for the recovery of added Ara-C. Comparison of the RIA and a method based on scintillation counting of [^{3}H]-Ara-C showed a good correlation (Fig. 2). The latter method results in lower values, which indicates a systematic error; in this method many calculations have to be performed, from counts per minute via the specific activity of [^{3}H]-Ara-C to picomoles of [^{3}H]-Ara-C incorporated into DNA. A small decrease in the specific activity of the [^{3}H]-Ara-C used may explain the lower values.

The rather large standard deviation found in these incubation studies, which involved separate experiments (Figs. 3, 4; Table 2), is probably of biological origin, because it was much lower for the assay itself (17% and 8.4%, as mentioned in the Results section). A good explanation for this large interassay variation, which was most pronounced at the IC_{50} concentration in the BNML-Cl/10 line, has not yet been found; in the HL-60 study, the variation was more acceptable.

These studies also show that it takes time before detectable amounts of Ara-C are incorporated (Figs. 3, 4). This finding is in contrast to a previous observation of

Kufe et al. [6]; using radioactive Ara-C and HL-60 cells, these investigators showed maximal incorporation 2 h after incubation for all Ara-C concentrations tested (from 10^{-7} to 10^{-4} M). Recent studies by this group [10] have shown that DNA chain elongation is slowed down after Ara-C incorporation and is not totally blocked. In studies focusing on DNA-synthesis activity after incubations with Ara-C, as measured by [^{3}H]-thymidine uptake, we observed some remaining activity. These observations explain the increasing amounts of Ara-C/DNA concentrations during Ara-C incubation (Figs. 3, 4).

The sensitivity of leukemic cells in culture to Ara-C is expressed as the IC_{50} concentration, which is defined as being the Ara-C concentration that induces 50% growth inhibition after 48 h incubation. IC_{50} studies using HL-60, BNML-Cl/0 and BNML-Cl/Ara-C cells in our laboratory showed that 50% growth inhibition was obtained in the HL-60 cell line with 5.10^{-8} M Ara-C and in the BNML-Cl/0 and BNML-Cl/Ara-C lines with 10^{-6} and 10^{-4} M, respectively (Table 2). The patterns of Ara-C incorporation into DNA in these cell lines are in agreement with the IC_{50} for Ara-C; at concentrations below the latter, Ara-C can hardly be incorporated into DNA.

In clonogenic assays, no reduction in colony numbers was observed at the IC_{50} for Ara-C in either rat-leukemia cell line [13]. A more sensitive assay for evaluating drug-related toxicity in cell lines is not available; these results show that incorporation of Ara-C predicts for cytotoxicity. Whether the Ara-C/DNA concentration itself is informative remains to be seen; it might vary from cell type to cell type, as was observed in the HL-60 and BNML-Cl/0 lines.

No good parameter for predicting the clinical response of patients on Ara-C therapy is available. The correlation between intracellular Ara-CTP levels and clinical response remains controversial [9, 12]. Therefore a parameter that predicts clinical response is desirable. At present we are evaluating Ara-C/DNA incorporation in leukemia cells from patients on Ara-C therapy and correlating the clinical response with these data. In conclusion, detection of Ara-C/DNA in cells after exposure to Ara-C is a sensitive assay; our cell-line studies show that detectable Ara-C/DNA levels predict for cytotoxicity. Evaluation of a correlation between Ara-C/DNA levels in patients' cells and clinical response to Ara-C therapy is in progress.

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