Relationships Among Ara-CTP Pools, Formation of (Ara-C)DNA, and Cytotoxicity of Human Leukemic Cells

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Cytosine arabinoside (ara-C) is the most effective agent in the treatment of acute myelogenous leukemia (AML). The mechanism of action of ara-C and the basis for selectivity against leukemic cells, however, remain unclear. The active metabolite, ara-C triphosphate (ara-CTP), is an inhibitor of DNA replication in viruses, bacteria, and eukaryotic cells.6,7 Two hypotheses have been proposed to explain the effects of ara-C on DNA synthesis. One hypothesis is that ara-CTP inhibits DNA polymerase by competing with the binding of deoxy-CTP (dCTP) to this enzyme.8-10 Although this is a widely accepted hypothesis, ara-CTP does not affect the incorporation of deoxy-thymidine triphosphate (dTTP), deoxy-adenosine triphosphate (dATP), or deoxy-guanosine triphosphate (dGTP)11-13 and therefore does not induce functional alterations in DNA polymerase. Kinetic studies have also demonstrated that ara-CTP is a weak competitive inhibitor of DNA polymerase and that this competition does not explain the more potent inhibition of DNA synthesis by this agent.14 Finally, it has not been clear how this mechanism of action produces cytotoxicity during brief periods of exposure, although reinitiation of DNA synthesis after inhibition by ara-C can result in replication of previously replicated DNA segments.15

An alternative hypothesis is the inhibition of DNA synthesis by ara-CTP through its incorporation into the DNA strand.9,11,16 ara-C incorporates only in DNA and not RNA of murine and human leukemic cells.17,18 Furthermore, there is a highly significant relationship between formation of (ara-C)DNA and loss of clonogenic survival. The extent of ara-C incorporation into DNA correlates with inhibition of DNA synthesis, and the incorporated ara-C residue behaves as a relative chain terminator.19 These findings are consistent with conformational and hydrogen bonding differences of the arabinose sugar moieties altering reactivity of the 3'-terminus and slowing chain elongation.20,21

Although these two hypotheses are not mutually exclusive, our data have suggested that the incorporation of ara-C into DNA is one mechanism responsible for producing lethal cellular events. However, to determine the usefulness of the various biochemical markers for monitoring cytotoxicity, we have studied the relationships among ara-CTP pools, formation of (ara-C)DNA, and cytotoxicity of the human HL-60 promyeloblast cell line. The results demonstrate that the single most powerful predictor of cell lethality is the extent of incorporation of ara-C into DNA.

MATERIALS AND METHODS

Cell Culture

HL-60 promyelocytes17 (provided by Dr. Herbert Lazarus, Dana-Farber Cancer Institute, Boston) were maintained in RPMI 1640 (GIBCO, Grand Island, NY) containing 4 mmol/L l-glutamine (GIBCO), 1% penicillin/streptomycin (GIBCO), and 20% heat-inactivated fetal bovine serum (Flow Laboratories, McLean, Va) at a density ranging from 1 x 10^5 to 10^6 cells/mL in 5% CO_2 humidified atmosphere at 37°C. Viable cells were determined by trypan blue exclusion.
**Incorporation of Ara-C Into HL-60 Nucleic Acid**

HL-60 promyeloblasts were washed twice in phosphate-buffered saline and resuspended at $5 \times 10^5$ cells/mL in RPMI 1640 medium. The cells were incubated with $10^{-7}$, $10^{-6}$, $10^{-5}$, or $10^{-4}$ mol/L $^3$H-Ara-C (Radiochemical Centre, Amersham, England; sp act 15.5 Ci/mmol) for 0.5, 1.0, 3.0, 6.0, 12.0, and 24.0 hours. The nucleic acids were then purified for analysis of cesium sulfate gradients as described.13

**Determination of Ara-CTP Pools**

HL-60 promyelocytes were exposed to $10^{-7}$, $10^{-6}$, $10^{-5}$, or $10^{-4}$ mol/L $^3$H-Ara-C (Radiochemical Centre, Amersham, England; sp act 15.5 Ci/mmol) for 0.5, 1.0, 3.0, 6.0, 12.0, and 24.0 hours. Cells were then harvested and washed with phosphate-buffered saline at 4°C. Nucleotides were extracted by the addition of 12% perchloric acid (HClO$_4$) for 30 minutes on ice and then removal of the precipitate. The supernatant was neutralized with 4 mol/L KH$_2$CO$_3$. The nucleotides in the acid-soluble fractions were analyzed by Varian 5060 (Palo Alto, Calif) high-pressure liquid chromatograph equipped with an AX-10 anion exchange column, using a linear gradient of 2.5 mmol/L KH$_2$PO$_4$ (pH 3.0) to 0.5 mol/L KH$_2$PO$_4$ (pH 4.4) over 30 minutes. The eluant fractions were collected, and radioactivity was determined by liquid scintillation counting. A representative profile is illustrated in Fig 1.

**Clonogenic Survival of HL-60 Promyeloblasts**

The HL-60 cells in logarithmic growth phase were washed twice in phosphate-buffered saline and resuspended in RPMI 1640 medium with 10% heat-inactivated, dialyzed fetal calf serum at a concentration of $5 \times 10^5$ cells/mL. The cells were then incubated in varying concentrations ($10^{-7}$, $10^{-6}$, $10^{-5}$, or $10^{-4}$ mol/L) of Ara-C for 3, 6, 12, and 24 hours. After drug exposure, the cells were collected in drug-free medium without serum. After counting in a model Z Coulter Counter (Coulter Electronics, Hialeah, Fla), the HL-60 cells were plated in 0.8% methylcellulose (RPMI 1640) containing 20% fetal calf serum and 10% conditioned media.18 Viability was determined after 7–10 days by scoring colonies containing 20 cells. Percentage colony formation was determined by the ratio of colonies formed by Ara-C-treated cells to untreated cells.

**RESULTS**

We have previously monitored $^3$H-Ara-C incorporation into HL-60 nucleic acids using cesium sulfate density gradient centrifugation. Under these nondegrading conditions, Ara-C was detectable only in the DNA fraction, and all of the tritium radioactivity detectable represented Ara-C.18 A similar analysis has been performed by monitoring the incorporation of $^3$H-Ara-C into HL-60 DNA after exposure to varying concentrations ($10^{-7}$–$10^{-4}$ mol/L) of drug for 0.5, 1, 3, 6, 12, and 24 hours. Figure 2 illustrates the pattern of incorporation under these experimental conditions. Thus, the extent of (Ara-C)DNA formation was dependent on both drug concentration ($C$) and time ($T$) of exposure. The product of $C \times T$ correlated very significantly with pmol of Ara-C incorporated in DNA (coefficient $[R] = .930, P < .0001$).

The formation of intracellular Ara-CTP pools was also monitored under the same experimental conditions used to measure incorporation of Ara-C into

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**Fig 1.** Chromatogram monitoring $^3$H-Ara-CTP formation in HL-60 cells. HL-60 cells ($5 \times 10^5$/mL) were incubated with $10^{-7}$ mol/L $^3$H-Ara-C (sp act 15.5 Ci/mmol) for 3 hours. The acid-soluble fraction was purified and analyzed by high pressure liquid chromatography on a Micropak AX-10 ion exchange column, using a linear gradient of 2.5 mmol/L KH$_2$PO$_4$ (pH 3.0) to 0.5 mol/L KH$_2$PO$_4$ (pH 4.4) at a flow rate of 1.5 mL/min. Appropriate markers were added and fractions (3.0 mL) were collected and assayed for tritium radioactivity.

**Fig 2.** Incorporation of $^3$H-Ara-C into HL-60 DNA. HL-60 cells ($5 \times 10^5$/mL) were incubated with $10^{-7}$ (A), $10^{-6}$ (A), $10^{-5}$ (B), or $10^{-4}$ (C) mol/L $^3$H-Ara-C for 0.5–24 hours. The nucleic acids were then purified for analysis as previously described.17 The results are expressed as the mean ± SD of two determinations performed in duplicate.
HL-60 DNA. The results obtained are illustrated in Fig 3. The formation of Ara-CTP pools was dependent on drug concentration. However, these pools declined with time, and consequently were not as closely correlated with the product $C \times T$ (coefficient $[R] = .552, P < .026$). Computer-based linear modeling can be used to analyze the relationships among concentration, time, clonogenic survival, Ara-C incorporation into DNA, and the Ara-CTP pool levels. If a linear regression model is used to fit the formation of (Ara-C)DNA to the Ara-CTP pool data, it can be shown that the measurement of Ara-CTP pools is useful in predicting incorporation of Ara-C into DNA (coefficient $[R] = .749, P < .008$). However, if a more sophisticated two-variable linear model is used, a combination of Ara-CTP pools and time will explain >90% of the variation in the log Ara-C incorporation into DNA ($[R]^2 = .912$).

Another approach to the analysis of the Ara-CTP pool data is to calculate continuous cellular exposure by monitoring areas under the curves illustrated in Fig 3. These areas were calculated by the trapezoidal rule. The continuous cellular exposure to Ara-CTP correlated significantly with log pmol Ara-C incorporation into DNA (coefficient $[R] = .768, P < .0005$); however, this analysis was of less predictive value than the more simple determination of a single Ara-CTP level $\times T$ (coefficient $[R] = .85, P < .0002$).

The relationships between Ara-C incorporation into DNA and formation of Ara-CTP to drug-induced cytotoxicity were studied by comparing these parameters to the loss of clonogenic survival after drug exposure. The effect of Ara-C on clonogenic survival of HL-60 cells was determined by exposure to concentrations of $10^{-7}$–$10^{-4}$ mol/L for 1, 3, 6, 12, and 24 hours. As we have previously demonstrated, the percentage cell kill was dependent on drug concentration and time of exposure (coefficient $[R] = .698, P < .0026$).

The significance of the relationship between loss of clonogenic survival and intracellular Ara-CTP levels was rather low (coefficient $[R] = .491, P < .053$; Fig 4A). In contrast, the relationship between log percent cell kill and log pmol Ara-C incorporation in DNA was the single most powerful predictor of lethality using the linear model (Fig 4B). Furthermore, an $[R]^2 = .860$ was obtained if the analysis includes concentration and time. This means that >85% of the variation in log percentage kill can be predicted from knowledge of time, concentration, and incorporation of Ara-C into DNA. The relationships among Ara-CTP pools, continuous cellular exposure to Ara-CTP, and formation of (Ara-C)DNA, with cytotoxicity as a function of single and multiple variables (time and concentration), are listed in Table 1.

**DISCUSSION**

Experiments with isolated DNA polymerase-template preparations have demonstrated that Ara-CTP acts as a competitive inhibitor of DNA polymerase. However, the inhibitory constant ($K_i$) of Ara-CTP is similar to the Michaelis constant ($K_m$) of dCTP. These results indicate that Ara-CTP is a rather weak competitive inhibitor of DNA polymerase and that inhibition of this enzyme is not a significant mechanism by which this agent inhibits DNA synthesis. Other studies have suggested that the incorporated Ara-C residues behave as relative chain terminators, which slow DNA synthesis by modifying reactivity of the terminal 3'-hydroxyl. The demonstration of
a highly significant relationship between the extent of (Ara-C)DNA formation and inhibition of DNA synthesis provides rather compelling evidence for incorporation of Ara-C into DNA as a major mechanism of drug action. The formation of Ara-CTP is required for incorporation of Ara-CMP and thereby inhibition of DNA synthesis by altered reactivity of the chain terminus.

Because it is clinically relevant to define biochemical parameters predictive of Ara-C cytotoxicity, the present studies were undertaken to determine the relationships among Ara-CTP pools, formation of (Ara-C)DNA, and loss of clonogenic survival. The results confirm that, as previously demonstrated, there is a highly significant relationship between incorporation of Ara-C into DNA and loss of leukemic cell clonogenic survival. Furthermore, although there is a correlation between Ara-CTP pools and lethality, this relationship is less significant than that of Ara-C incorporation in DNA and loss of leukemic cell clonogenic survival. Therefore, although there is a correlation between Ara-CTP pools and lethality, this relationship is less significant than that of Ara-C incorporation in DNA and lethality. The less significant relationship between Ara-CTP pools and cell kill may be due to a threshold, related to the $K_i$ for DNA polymerase or dCTP pools, above which an increase in Ara-CTP has little effect. The present findings with Ara-CTP are therefore consistent, in part, with previous studies demonstrating that the retention of Ara-CTP correlates with cytotoxicity of murine and human leukemic cells as determined by survival and remission duration.26,27

The results obtained in the present study also indicate that a determination of the continuous cellular exposure to Ara-CTP (area under the curve or $C \times T$ for Ara-CTP) provides a correlate of cell lethality. Similar results have been obtained with total cellular exposure to 9-$\beta$-d-arabinofuranosyladenine 5'-triphosphate (Ara-ATP) and cell death.28 We have shown that Ara-A, like Ara-C, incorporates specifically in DNA and that the Ara-A residue at the 3'-terminus provides a poor primer for elongating DNA strands.29 Thus, the incorporation of arabinosyl derivatives (Ara-C and Ara-A) into DNA would be dependent on the continuous cellular exposure to the triphosphate metabolite, and either parameter would provide significant correlates of cytotoxicity. The interrelationships among Ara-CTP levels, continuous cellular exposure to Ara-CTP, and Ara-C incorporation into DNA found in the present studies support such a hypothesis.

Finally, the present studies demonstrate that monitoring incorporation of Ara-C into DNA is the single most powerful predictor of cell lethality. This finding, the highly significant relationship between extent of (Ara-C)DNA formation and inhibition of DNA synthesis, and the relative chain-terminating effect of this drug strongly suggest that incorporation into the DNA strand is responsible for inducing lethal cellular events. The incorporation of Ara-C (or Ara-A) into DNA could produce lethal cellular damage through any of the following proposed events: (1) inhibition of chain initiation and ligation; (2) faulty processing of DNA; (3) chromosomal breaks; and (4) reinitiation of previously replicated DNA segments.30 Although any of these proposed events may apply depending on the dose and schedule of drug administration, transient inhibition and resumption of DNA synthesis can result in premature reinitiation with certain segments of DNA undergoing multiple replicas.31,32,36,38 This mechanism could result in altered gene expression and in the effective clinical use of low doses of Ara-C to partially inhibit DNA replication.

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