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EFFECT OF ARA-C INCORPORATION ON DEOXYRIBONUCLEIC ACID SYNTHESIS IN CELLS*

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Abstract—Recent work using isolated DNA polymerase–template complexes has shown that arabinofuranosyl derivatives can slow DNA synthesis by being incorporated into DNA. Our results suggest that these agents act by a similar mechanism in L1210 cells. The results demonstrate that inhibition of cellular DNA synthesis by cytosine arabinoside (ara-C) was significantly related to the extent of ara-C incorporation in DNA over a wide range of drug concentrations and times of exposure. Furthermore, treatment with increasing concentrations of ara-C resulted in a greater proportion of ara-C residues at the 3′-terminus of the elongating DNA chain. These observations suggest that ara-C incorporation results in poor primer termini for further chain elongation.

Adenine arabinoside (ara-A) and cytosine arabinoside (ara-C) are anti-leukemic agents [1, 2]. Their cytotoxic effects probably result from disruption of DNA replication with reinitiation of synthesis in previously replicated segments [3, 4]. These agents slow both chain initiation and chain elongation [5, 6]. However, the precise mechanism of inhibition of DNA synthesis remains unclear [7].

Two hypotheses, which are not mutually exclusive, have been formulated previously on the basis of *in vitro* data to explain the effects of these agents on DNA synthesis [7]. Studies using isolated DNA polymerase–template preparations have shown that the active metabolites of the arabinofuranosyl nucleosides, the triphosphate derivatives, act as competitive inhibitors of the replicative DNA polymerase [8, 9]. This is currently the most widely accepted hypothesis to explain the cellular effects of these drugs. Other work using isolated nuclei [10] or DNA polymerase–template preparations [11, 12] has also suggested that incorporated ara-C molecules behave as relative chain terminators which slow DNA synthesis by serving as poor primer termini for further chain elongation [13].

We have demonstrated previously using cesium sulfate density gradient centrifugation that ara-C is incorporated exclusively in DNA and that there is a highly significant relationship between the formation of (ara-C)DNA and loss of clonogenic survival [14, 15]. The misincorporation of ara-C residues in cellular DNA could also result in poor primer termini and slow DNA synthesis. The experiments described in the present studies support this hypothesis. We have demonstrated inhibition of L1210 cellular DNA synthesis as a function of the extent of ara-C incorporation in DNA. More importantly, ara-C residues were more prevalent at the chain terminus when DNA synthesis was inhibited to a greater degree by exposure to higher concentrations of drug. These findings are consistent with the terminal ara-C residue serving as a poor primer for further chain elongation.

MATERIALS AND METHODS

Cell culture. L1210 cells were grown as a suspension culture in SMEM§ with 10% fetal calf serum (FCS), 100 units streptomycin/ml, 100 μg penicillin/ml, 1% L-glutamine and 0.5 mM 2′-mercaptoethanol at 37° in a 5% CO_2 atmosphere. The cytotoxic effects of ara-C were evaluated by treating cells with various concentrations of drug for 0.5, 1, 3, 6 and 12 hr and determining the number of surviving clonogenic cells using colony formation in methyl cellulose as previously described [14].

Incorporation of labeled precursors into L1210 nucleic acids. L1210 cells in logarithmic growth phase were washed twice with phosphate-buffered saline (PBS) and were resuspended at 1×10^6 cells/ml in SMEM. The cells were incubated in serum-free medium with various concentrations (10^{-7} to 10^{-4} M) of [^{3}H]ara-C (24 Ci/mmole, Amersham Corp., Arlington Heights, IL). In parallel experiments, the cells were treated with ara-C (10^{-7} to 10^{-4} M) for 180 min and exposed to 2 μCi/ml [^{3}H]dThd (2 Ci/mmole, New England Nuclear Corp., Boston, MA) during the last 30 min of the incubation or to 10 μCi/ml of ^{32}P (carrier-free, New England Nuclear Corp.) during the entire 180 min.

Labeled cells were washed twice with 5 ml of PBS, resuspended in PBS at 1×10^7 cells/ml, and digested by the addition of 2.5 mg proteinase-K (Boehringer-Mannheim, Indianapolis, IN) in 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.5%

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§ SMEM, minimum essential medium (Eagle's) for suspension cultures.

sodium dodecylsulfate. Subsequent purification was accomplished by phenol extraction. The nucleic acids were precipitated by the addition of 0.1 vol. of 4 M NaCl and 2 vol. of absolute ethanol. After standing overnight at −20°, samples were centrifuged at 10,000 *g* and the resulting pellets were resuspended in 0.005 M EDTA. The samples were then analyzed either by cesium sulfate gradient centrifugation [14] or by assaying directly for acid precipitable radioactivity after treatment with RNase A (40 μg/ml, 90 min, 37°).

Digestion of labeled nucleic acids to 3′-nucleotides. L1210 cells were incubated with [^{3}H]ara-C at various concentrations (10^{-8} to 10^{-4} M) for 6 hr. In parallel experiments, cells were treated with ara-C (10^{-8} to 10^{-4} M) and incubated with 5 μCi/ml [^{3}H]dThd (52 Ci/mmole, New England Nuclear Corp.). The nucleic acids were purified by pronase digestion, phenol extraction, and ethanol precipitation. The DNA fraction was degraded to 3′-nucleotides using micrococcal nuclease (MN) and spleen phosphodiesterase (SP) [16]. The nucleotides (3′-ara-CMP, 3′dTMP) and nucleosides (ara-C, dThd) were separated by high pressure liquid chromatography (HPLC) using previously described methods [14, 15].

RESULTS

L1210 cells were exposed to [^{3}H]ara-C (10^{-7} to 10^{-3} M) for 3 and 6 hr. The amount of ara-C incorporated in DNA was then determined by cesium sulfate gradient centrifugation. Exposure of cells to higher drug concentrations and longer times results in greater quantities of ara-C incorporated in DNA according to a C × T relationship [14]. To determine the effect of ara-C incorporation on DNA synthesis, parallel experiments were performed with ara-C and [^{3}H]dThd. Figure 1 illustrates that, as the extent of ara-C incorporation in DNA increased, there was a corresponding decrease in DNA synthesis as determined by the incorporation of tritiated thymidine. This relationship was explored further using the same concentrations of ara-C for 1 and 12 hr of exposure.

Figure 2 illustrates the relationship as determined by probit analysis [17] between cytotoxic effect and the incorporation of dThd and ara-C in L1210 DNA over a wide range of concentrations and times of exposure to drug. The loss of L1210 clonogenic survival correlates significantly with the log pmoles of ara-C incorporated in DNA ($P < 0.0001$, $[R] = -0.94640$) and with inhibition of DNA synthesis as measured by log pmoles of [^{3}H]dThd incorporated in DNA ($P < 0.0001$, $[R] = 0.96769$). Furthermore, the relationships between log pmoles of ara-C incorporation in DNA and inhibition of DNA synthesis ($P < 0.001$, $[R] = -0.92764$) and log C × T and inhibition of DNA synthesis ($P < 0.0001$, $[R] = 0.96713$) were highly significant.

The nucleosides ara-C and dThd are transported across the cell membrane by the same carrier system [18] and, due to the high capacity of this system, intracellular levels of these metabolites are expected to reflect extracellular concentrations. To validate the use of [^{3}H]dThd incorporation to monitor DNA synthesis, we performed similar experiments using ^{32}P-incorporation in DNA to monitor DNA synthetic capacity. Similar results were obtained, and the relationship between the loss of L1210 clonogenic survival and inhibition of DNA synthesis as monitored by ^{32}P-incorporation was highly significant ($P < 0.0006$, $[R] = -0.8406$). Thus, either isotope is suitable for monitoring the effect of ara-C on DNA synthesis.

The correlation between the formation of (ara-C)DNA and inhibition of DNA synthesis is consistent with the ara-C residue serving as a poor 3′-OH terminal for chain elongation. Further supportive evidence for this hypothesis would be an increased proportion of ara-C residues in the terminal position upon exposure to increased drug concentrations as

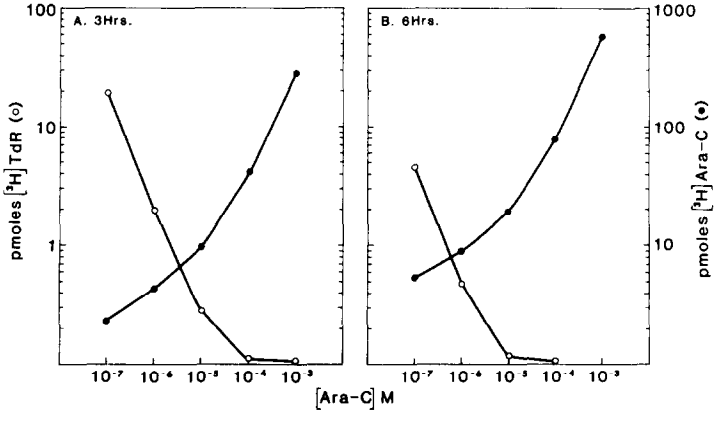

Fig. 1. Effect of ara-C incorporation on inhibition of DNA synthesis. L1210 cells in logarithmic growth phase at a concentration of 1×10^6 cells/ml were incubated with [^{3}H]ara-C (10^{-7} to 10^{-3} M) for 3 and 6 hr. The total cellular nucleic acids were purified and analyzed by cesium sulfate density gradient centrifugation. The [^{3}H]ara-C counts banding in the DNA region of the gradients were determined and used as a measure of the incorporation of ara-C in DNA (●). Similar experiments were performed by exposing L1210 cells to ara-C (10^{-7} M to 10^{-3} M) for 3 and 6 hr with the addition of 2 μCi/ml [^{3}H]dThd during the last 30 min of each incubation. The amount of [^{3}H]dThd incorporated in DNA (○) was then determined as a measure of DNA synthesis.

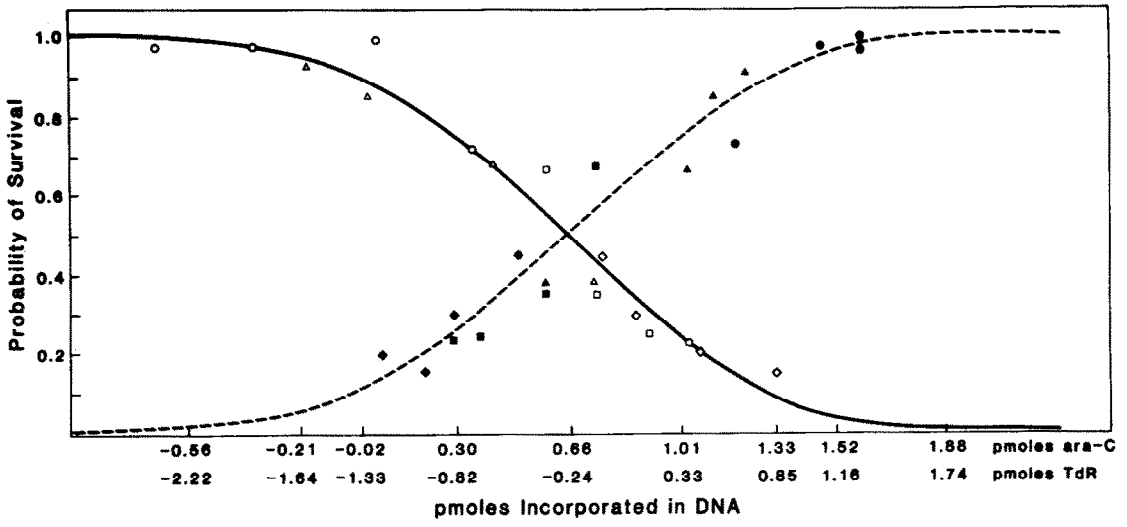

Fig. 2. Relationship between incorporation of ara-C and dThd in L1210 DNA with clonogenic survival. L1210 cells in logarithmic growth phase were exposed to various concentrations of ara-C [10^{-7} M (○, ●); 10^{-6} M (△, ▲); 10^{-5} M (□, ■); and 10^{-4} M (◇, ◆)] for 1, 3, 6 and 12 hr. The amounts of [^{3}H]ara-C (open symbols, solid line) and [^{3}H]dThd (closed symbols, broken line) incorporated in DNA were then determined as described in the legend to Fig. 1. The clonogenic survival of L1210 cells following exposure to ara-C has been described previously [14].

the probability of finding a nucleoside at that position is dependent on the rate of extension at the 3′-terminus. Thus, the proportions of ara-C and dThd residues in internucleotide linkage and at the chain terminus were monitored in cells treated with increasing concentrations of ara-C. After digestion of the labeled DNA with micrococcal nuclease and spleen phosphodiesterase, the ara-C residues in internucleotide linkage were detectable as 3′-ara-CMP, while those residues at the chain terminus were detectable as ara-C. The digests were then analyzed by high pressure liquid chromatography as previously described [14, 15]. The results obtained are summarized in Table 1.

The exposure of cells to increasing concentrations of ara-C resulted in an increasing proportion of ara-C residues at the chain terminus. In contrast, under similar conditions, the proportion of thymidine residues at the chain terminus actually decreased. Thus, greater inhibition of DNA synthesis by ara-C was associated with an increasing proportion of ara-C residues occupying the chain termini. This suggests that ara-C behaves as a poor primer terminus for chain elongation.

DISCUSSION

The precise mechanism(s) of inhibition of cellular DNA synthesis by ara-C remains unclear [7]. Results obtained with isolated DNA polymerase–template preparations demonstrate that ara-C acts as a competitive inhibitor of DNA polymerase with respect to the normal substrate deoxycytidine triphosphate [8]. Other work suggests that incorporation of arabinofuranosyl derivatives in DNA modifies the reactivity of the terminal 3′-hydroxyl leading to chain termination [10–12] or slowing of chain elongation [13]. These hypotheses are not mutually exclusive.

Our experiments were performed to determine if ara-C residues, incorporated in L1210 DNA, would serve as poor primer termini for chain elongation. This hypothesis would be consistent with the finding that inhibition of DNA synthesis was directly related to the extent of ara-C residues incorporated in DNA. Our results show that, over a wide range of concentrations of ara-C and times of exposure, there was a highly significant relationship between inhibition of DNA synthesis and the number of ara-C residues incorporated in DNA. The hypothesis would also

Table 1. Positioning of [^{3}H]ara-C and [^{3}H]dThd residues at different concentrations of ara-C*

	Ara-C		dThd	
Ara-C concn (M)	Internucleotide	Chain terminus	Internucleotide	Chain terminus
Control			97.0 ± 1.7	3.0 ± 1.7
10^{-8}	100.0 ± 0.0	0.0 ± 0.0	96.8 ± 1.3	3.2 ± 1.3
10^{-7}	92.0 ± 2.3	8.0 ± 2.3	97.0 ± 1.1	3.0 ± 1.1
10^{-6}	87.5 ± 3.2	12.5 ± 3.2	98.1 ± 0.5	1.9 ± 0.5
10^{-5}	71.5 ± 1.9	28.5 ± 1.9	99.4 ± 0.5	0.6 ± 0.5
10^{-4}	70.5 ± 2.2	29.5 ± 2.2	99.8 ± 0.2	0.2 ± 0.2

* Values are expressed as mean percent ± standard deviation.

predict that, if ara-C residues in the 3′-position serve as poor substrates for further chain elongation, there will be a greater proportion of ara-C residues at the chain end associated with a greater inhibition of DNA synthesis. The results demonstrate that there was a greater proportion of ara-C residues detectable at the chain terminus when exposure to higher concentrations of ara-C caused an increased inhibition of DNA synthesis. Treatment with ara-C results in expansion of deoxythymidine triphosphate pools [19]. Hence, the increasing proportion of ara-C residues at the chain end cannot be explained by the limited availability of deoxythymidine triphosphate.

It has been suggested previously that ara-C does not act as a poor primer terminus or chain terminator in intact cells because most of the ara-C residues are identified in internucleotide linkage [9]. Similar results have been obtained with studies on the effects of ara-CTP on polyoma virus replication [20]. However, low concentrations of ara-C were used in those studies, and our findings suggest that only exposure to higher concentrations of ara-C results in an increasing proportion of ara-C residues being detectable at the chain terminus.

The degree of inhibition of DNA synthesis over the ara-C concentration range (10^{-7}M to 10^{-3}M) studied was several orders of magnitude greater than the increase in the number of terminal ara-C residues. This could suggest that the effect of ara-C on the template itself results in minimal inhibition of DNA replication *in vivo* when compared to the competitive effect on DNA polymerase. However, the interactions of ara-C terminated template primers with other enzymes of the replication complex, such as the ligating and gap filling activities [21], have not been studied. These enzymes may be inhibited to a much smaller degree than DNA polymerase. The incorporated ara-C residues would serve as poor primers for the polymerase, inhibit DNA synthesis, but nevertheless appear most often in internucleotide linkage. The good correlation between the incorporation of ara-C in DNA and inhibition of synthesis supports this explanation.

Our findings are consistent with the hypothesis that one cellular mechanism of action of ara-C is through incorporation in the DNA strand [14] which results in inhibition of DNA synthesis by ara-C terminated primers that are extended slowly. Further work is directed at determining the importance of this mechanism in inhibiting DNA replication *in vivo* and studying the interaction of ara-C terminated templates with other enzymes of the DNA replication complex.

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