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Effects of 1-β-D-Arabinofuranosylcytosine Incorporation on Eukaryotic DNA Template Function

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SUMMARY

1-β-D-Arabinofuranosylcytosine (ara-C) incorporates into DNA, and the extent of this incorporation correlates significantly with inhibition of DNA synthesis. The incorporated ara-C residue provides a poor primer terminus for further chain elongation. There is a highly significant relationship between formation of (ara-C)DNA and loss of clonogenic survival. The present studies confirm that incorporation of ara-C into DNA, and not the competitive inhibition of DNA polymerase, is responsible for inducing lethal cellular events. The results also demonstrate that the incorporated ara-C residue is not excised from the DNA strand. Furthermore, the presistence of ara-C residues in DNA inhibits recovery of DNA synthesis following exposure to drug. The relative DNA chain-terminating effect of ara-C provides several mechanisms of action that explain internucleotide and chain terminus positioning of ara-C residues, reinitiation of previously replicated DNA segments, and DNA strand or chromosomal breaks. The precise mechanism of action is dependent upon dose scheduling of this drug.

INTRODUCTION

Ara-C[3] is the most effective agent in the treatment of a human acute myelogenous leukemia (1). The basis for this selectivity against leukemic cells is unclear owing to the lack of a precise understanding of the mechanism(s) of drug action. The active metabolite, ara-CTP, is a potent inhibitor of DNA replication in viruses, bacteria, and eukaryotic cells (2, 3). Although previous studies suggested that ara-CTP inhibits DNA synthesis by competing with binding of dCTP to DNA polymerase (4–6), kinetic studies have demonstrated that ara-CTP is a weak competitive inhibitor of this enzyme and that this competition does not explain the effect of this agent on DNA synthesis (7). In contrast, the inhibition of DNA synthesis by ara-CTP is consistent with the incorporation of this agent into the DNA strand (5, 8, 9).

Ara-C incorporates specifically into DNA and not RNA of murine and human leukemic cells (10, 11). The extent of ara-C incorporation in DNA correlates significantly with inhibition of DNA synthesis (12). Furthermore, the incorporated ara-C residue behaves as a poor primer terminus for further chain elongation. This relative DNA chain-terminating effect results in an increasing proportion of ara-C residues detectable at the chain terminus upon exposure to higher concentrations of drug (12). The inhibition of DNA synthesis by ara-C causes DNA fragmentation (13) as well as alterations in gene expression (14). The extent of (ara-C)DNA formation also bears a highly significant relationship with loss of leukemic cell clonogenic survival (10, 11). These findings suggest that ara-C incorporation into DNA is responsible for inducing lethal cell events. More incisive studies are now required to determine the effect of ara-C incorporation on DNA template function.

Ara-C preferentially inhibited replicon initiation in one study (15), whereas other work has suggested that ara-C slows the rate of chain elongation without altering the site at which DNA replication is initiated within replicons (16). Other studies have shown that ara-C induces rereplication of certain chromosomal segments (17). These events could result in DNA fragmentation (13), chromatid breakage (18), and lethal cellular events. In view of the relative chain-terminator effect of ara-C, cytotoxicity may be included by different mechanisms depending upon the dose scheduling of this drug. The present study thus examines the effects of ara-C incorporation into DNA on template function at varying concentrations of drug. These results may be relevant to the different biological effects observed clinically with ara-C at low (19, 20) and high (21) doses.

MATERIALS AND METHODS

Cell culture. The L1210 cells were grown as a suspension culture in Eagle's minimal essential medium (GIBCO, Grand Island, N. Y.) with

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[3] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, ara-C triphosphate; ara-A, 9-β-D-arabinofuranosyladenine.

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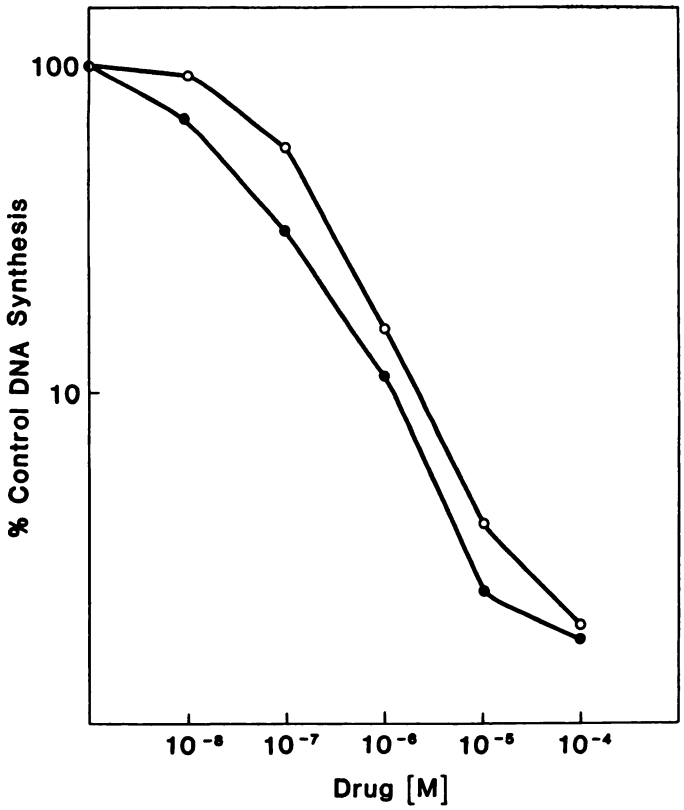

FIG. 1. *Inhibition of DNA synthesis by aphidicolin and ara-C*

L1210 cells in logarithmic growth phase at a concentration of 1 × 10^5 cells/ml were exposed to no drug, 10^{-8} to 10^{-4} M ara-C (●), or 10^{-8} to 10^{-4} M aphidicolin (○) for 3 hr in the presence of ^{32}P (5 μCi/ml). The DNA fraction was purified (14), and the ^{32}P incorporation was determined as a relative measure of DNA synthesis.

10% fetal calf serum, streptomycin (100 units/ml), penicillin (100 mg/ml), 1% L-glutamine, and 0.05 mM 2-mercaptoethanol at 37° and a 5% CO_2 atmosphere.

Incorporation of [^{3}H]ara-C and ^{32}P into nucleic acids. L1210 cells in logarithmic growth phase were washed twice with phosphate-buffered saline and were resuspended at a density of 1 × 10^6/ml in Eagle's minimal essential medium. Cells were incubated with $H_3{}^{32}PO_4$ (5 μCi/ml) (carrier-free; New England Nuclear Corporation, Boston Mass.) for 3 hr in the presence of ara-C (Sigma Chemical Company, St. Louis, Mo.) or aphidicolin (provided by Dr. J. Douros, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.). The L1210 cells were also incubated with 10^{-8} to 10^{-4} M [^{3}H]ara-C (specific activity, 15.5 Ci/mmole; Radiochemical Centre, Amersham, England) and/or $H_3{}^{32}PO_4$ (5 μCi/ml) for 3 hr. The purification of the nucleic acids and the analysis by cesium sulfate gradient centrifugation were performed as previously described (12).

Clonogenic survival of L1210 cells. The clonogenic survival of L1210 cells maintained in logarithmic growth phase was monitored as previously described (12).

Determination of ara-CTP pools. L1210 cells in logarithmic growth phase were exposed to 10^{-7} to 10^{-4} M [^{3}H]ara-C for 3 hr. The cells were then harvested and washed with phosphate-buffered saline at 4°. The nucleotides were extracted by the addition of 12% perchloric acid for 30 min on ice. The supernatant was neutralized with 1 M KH_2CO_3. The nucleotides in the acid-soluble fractions were analyzed on a Varian 5060 (Palo Alto, Calif.) high-pressure liquid chromatograph equipped with an AX-10 anion exchange column using a linear gradient of 2.5 mM KH_2PO_4 (pH 3.0) to 0.5 M KH_2PO_4 (pH 4.4) over 30 min. The eluent fractions were collected, and radioactivity was determined by liquid scintillation counting.

L1210 and synthetic template studies. L1210 cells in logarithmic growth phase were incubated with no drug, 10^{-7} M, or 10^{-6} M ara-C for 3 hr. The DNA was then purified by RNase digestion, sodium dodecyl sulfate/phenol extractions, and ethanol precipitation (12). The DNA (10 μg) was redissolved in 100 μl of reaction mixture (50 mM Tris-HCl, pH 7.5; 8 mM $MgCl_2$) containing 2 × 10^{-6} M [^{3}H]dTTP (specific activity, 17 Ci/mmole; New England Nuclear Corporation), 0.1 mM dCTP, 0.1 mM dATP, 0.1 mM dGTP, and 2 units of *Escherichia coli* DNA polymerase I (Klenow fragment; P-L Biochemicals, Milwaukee, Wisc.). The samples were then incubated for 15 and 30 min. Trichloroacetic acid-precipitable radioactivity was determined by liquid scintillation counting.

Oligo dC-poly(dG) (10 μg) was incubated with no drug, 10^{-7} M, 10^{-6} M, 10^{-5} M, or 10^{-4} M ara-CTP for 5 min at 5° in the presence of 2 units *E. coli* DNA polymerase I. The DNA was precipitated and washed in ethanol. After lyophilizing, the DNA samples were redissolved in 100 μl of reaction mixture containing 4 × 10^{-6} M [^{3}H]dCTP (specific activity, 24.9 Ci/mmole; New England Nuclear Corporation) and 2 units of *E. coli* DNA polyermase I. The samples were incubated for 15 min at room temperature and assayed for radioactivity by collection on filters after trichloroacetic acid precipitation.

RESULTS

Since it is not clear whether slowing of DNA replication per se or the incorporation of ara-C into DNA results in cell lethality, the present experiments were performed with ara-C and aphidicolin, another inhibitor of DNA synthesis, which, like ara-C, competes with dCTP for binding to DNA polymerase but is not incorporated into the DNA strand (22). The effects of ara-C and aphidicolin on L1210 DNA synthesis by each of these agents was similar at concentrations ranging from 10^{-8} to 10^{-4} M. Although both of these agents inhibited DNA synthesis to a similar extent, exposure of L1210 cells to 10^{-6} M aphidicolin for 3 hr had little effect on clonogenic survival, whereas a similar exposure to 10^{-6} M ara-C resulted in nearly 80% loss of clonogenicity (Fig. 2). Furthermore,

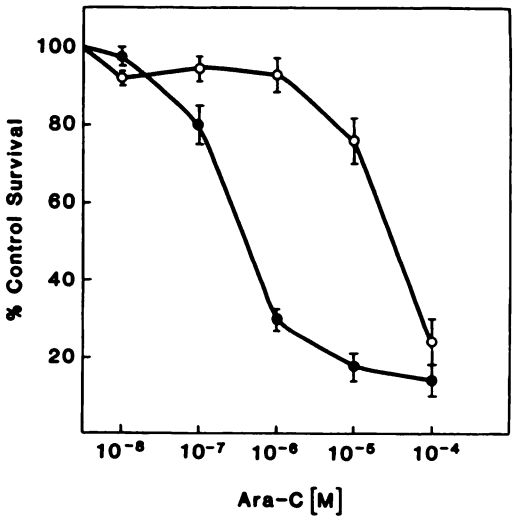

FIG. 2. *Clonogenic survival of L1210 cells following drug exposure*

L1210 cells in logarithmic growth phase were exposed to 10^{-8} to 10^{-4} M ara-C alone (●) or in combination (○) with 10^{-6} M aphidicolin for 3 hr. The cells were washed and monitored for clonogenic survival (12) in the absence of drug. The results are expressed as the mean ± standard deviation of four separate determinations. Percentage control survival of L1210 cells exposed to 10^{-6} M aphidicolin alone for 3 hr: 95.8 ± 7.8%.

the lethal effects of 10^{-8} to 10^{-5} M ara-C were blocked by coincubation with 10^{-6} M aphidicolin. In contrast, 10^{-6} M aphidicolin had less effect on clonogenicity of cells exposed to 10^{-4} M ara-C.

The results obtained with the clonogenic survival experiments suggested that aphidicolin, which binds near the dCTP binding site of DNA polymerase (22), competitively inhibits binding of ara-CTP to this enzyme and thereby formation of (ara-C)DNA. The effect of aphidicolin on the incorporation of varying concentrations (10^{-8} to 10^{-4} M) of [^{3}H]ara-C into DNA was therefore also monitored during 3-hr drug exposures. The results obtained are illustrated in Fig. 3. The formation of (ara-C)DNA was linear for ara-C alone and in combination with 10^{-6} M aphidicolin. However, the extent of (ara-C)DNA formation was decreased in the presence of aphidicolin. This decrease in ara-C incorporation by aphidicolin was less pronounced at higher ara-C concentrations, consistent with a competitive binding of these drugs to DNA polymerase. The relationship between incorporation of ara-C into DNA (Fig. 3) and loss of clonogenic survival (Fig. 2) in the presence and absence of aphidicolin was highly significant ($p < 0.0001$). These findings suggest that the formation of (ara-C)DNA, and not the competitive inhibition of DNA polymerase, is responsible for the loss of clonogenic survival under these experimental conditions.

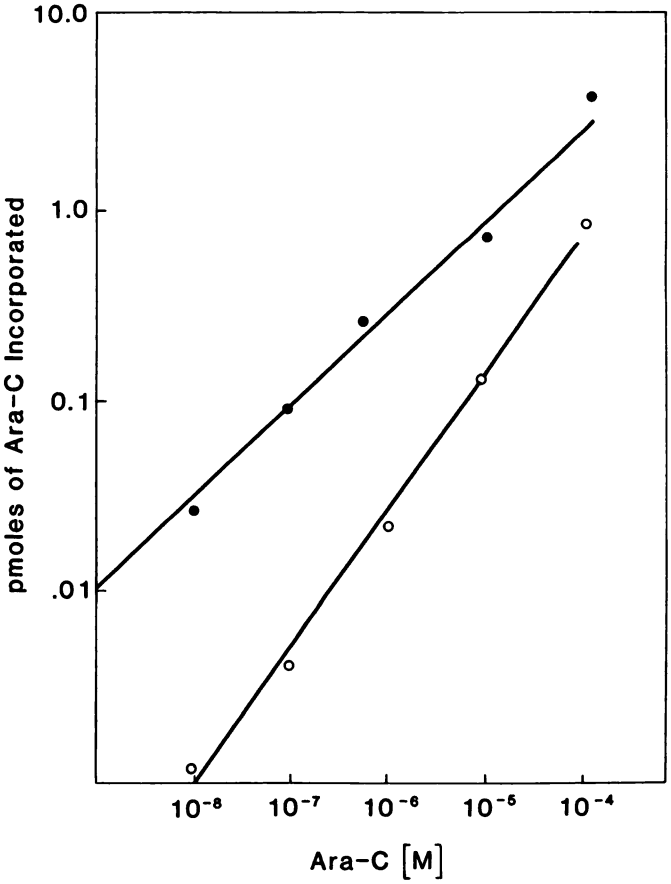

FIG. 3. *Incorporation of [^{3}H]ara-C into L1210 cells*
L1210 cells (1×10^6/ml) were incubated with 10^{-8} to 10^{-4} M [^{3}H] ara-C alone (●) or incombination (○) with 10^{-6} M aphidicolin for 3 hr. The extent of ara-C incorporation into DNA was determined as previously described (14).

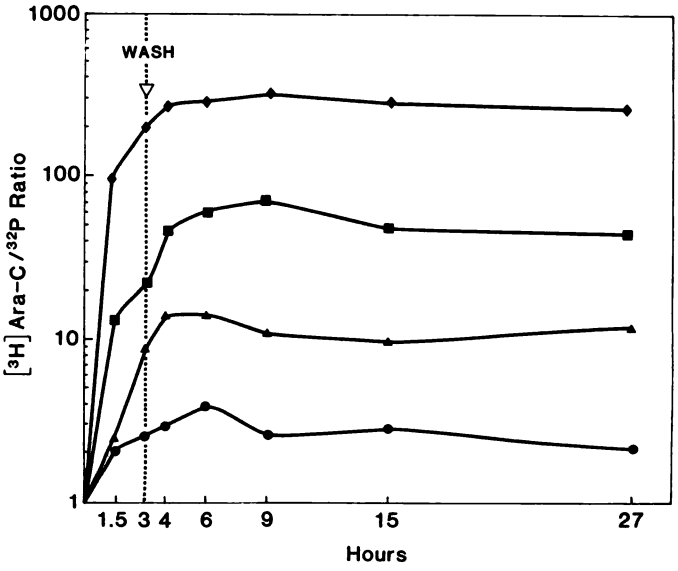

FIG. 4. *Stability of ara-C residues incorporated into DNA*
L1210 cells were exposed to 10^{-7} (●), 10^{-6} (▲), 10^{-5} (■), or 10^{-4} M (♦) [^{3}H]ara-C and ^{32}P (5 μCi/ml) for 3 hr. The cells were then washed, incubated in complete medium without [^{3}H]ara-C or ^{32}P, and then monitored for incorporation of tritium and ^{32}P radioactivity into DNA.

The induction of cell lethality by incorporation of ara-C into DNA could theoretically be reversed by excision of the incorporation ara-C residues. In order to monitor for excision, L1210 cells were labeled with 10^{-7} to 10^{-4} M [^{3}H]ara-C and ^{32}P. The ^{32}P incorporates with each of the nucleotides and thus serves as a relative measure of newly synthesized DNA. A decline in the ratio of [^{3}H] ara-C/^{32}P would therefore be an indicator of selective excision of the ara-C residues. The results obtained in a representative experiment are illustrated in Fig. 4. The [^{3}H]ara-C/^{32}P ratio increased proportionally at each ara-C concentration until the cells were washed free of drug and ^{32}P. However, the ratios remained stable during an additional 24 hr of incubation. These results suggest that selective excision of the incorporated ara-C residues is not detectable following exposure to concentrations that result in ara-C comprising 8% (10^{-7} M)–29% (10^{-4} M) of the residues detectable at the chain terminus (12).

The inability to detect excision of ara-C residues from DNA suggests that the effect of this agent on the DNA template might be irreversible. The inhibition of DNA synthesis was therefore monitored following exposure to drug. The L1210 cells were exposed to varying concentrations of ara-C (10^{-7} to 10^{-3} M) for 3 hr. The cells were then washed and incubated with ^{32}P over a period of 12 hr. The effect of treatment with varying concentrations of ara-C on the recovery of DNA synthesis is illustrated in Fig. 5A. When compared with cells not previously exposed to ara-C, recovery of DNA synthesis decreased following exposure to increasing concentrations of drug. Furthermore, cells exposed to 10^{-5} to 10^{-3} M ara-C demonstrated less than 10% of control DNA synthesis over the 12-hr recovery. These results were not explained by persistence of ara-CTP pools (Table 1), which declined at 12 hr of recovery to levels that do not significantly inhibit L1210 DNA synthesis. Deoxycytidine was also added during the recovery period at a concentration (10^{-5} M) that did not inhibit DNA synthesis. This nucleoside

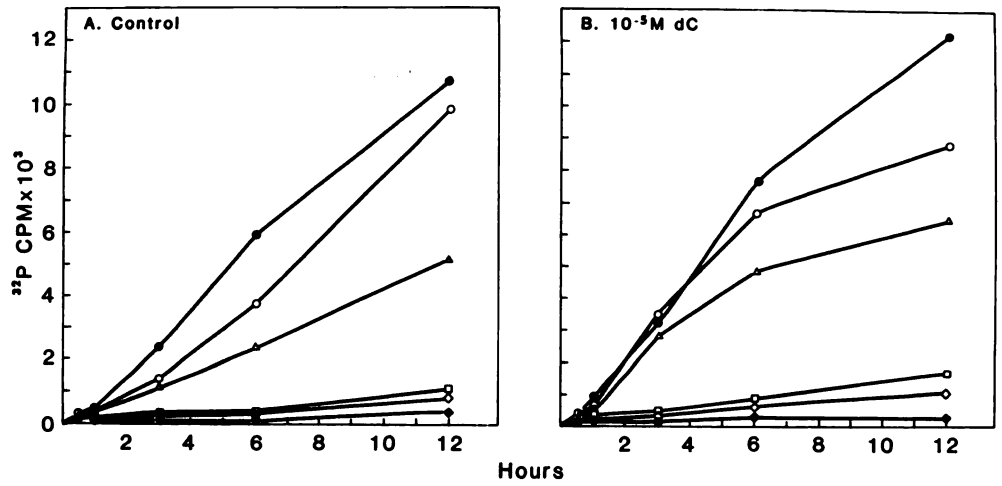

FIG. 5. *Recovery of L1210 DNA synthesis after exposure to ara-C*

L1210 cells in logarithmic growth phase were exposed to varying concentrations of ara-C (control, ●; 10^{-7} M, ○; 10^{-6} M, △; 10^{-5} M, □; 10^{-4} M, ◇; or 10^{-3} M, ♦) for 3 hr. The cells were then washed and resuspended in drug-free medium containing ^{32}P (5 μCi/ml). DNA synthesis was monitored by measuring the incorporation of ^{32}P into DNA at the indicated times. A, No deoxycytidine in the media during the recovery period; B, deoxycytidine (10^{-5} M) present in the media during the recovery period.

TABLE 1

Formation and retention of ara-CTP pools

Results are means ± standard deviation of three determinations assayed after 3 hr of incubation with varying concentrations of ara-C. The ara-CTP $t_{1/2}$ values were determined by a computer-assisted program.

Ara-C concentration	Ara-CTP pool	Ara-CTP $t_{1/2}$
M	*pmoles ara-CTP/10⁶ cells*	*hr*
10^{-7}	1.4 ± 0.1	1.76
10^{-6}	10.1 ± 0.8	1.52
10^{-5}	85.1 ± 4.6	1.18
10^{-4}	246.8 ± 40.1	1.43

had little effect on the reinitiation of DNA synthesis other than causing a somewhat greater initial recovery at 3 and 6 hr for cells treated with 10^{-7} M and 10^{-6} M ara-C (Fig. 5B). Similar effects were observed with 10^{-4} M deoxycytidine. Thus, deoxycytidine does not appear to compete with ara-C once the drug has been incorporated into the DNA strand. These findings are consistent with an irreversible alteration of the DNA template by the incorporated ara-C residue.

The effect of ara-C incorporation on the function of the DNA template was examined further using both the synthetic template oligo(dC)-poly(dG) and purified L1210 DNA. Ara-C residues were incorporated into the synthetic template by incubation with 10^{-7} to 10^{-4} M ara-CTP and *Escherichia coli* DNA polymerase I. The ara-C containing DNA synthetic template was purified free of ara-CTP and therefore competitive effects on DNA polymerase, and then monitored for incorporation of [^{3}H]deoxycytidine. As illustrated in Fig. 6A, the incorporation of [^{3}H]deoxycytidine progressively decreased when using templates exposed to increasing concentrations of ara-C. Similar results were obtained with L1210 DNA purified from cells exposed to 10^{-7} and 10^{-4} M ara-

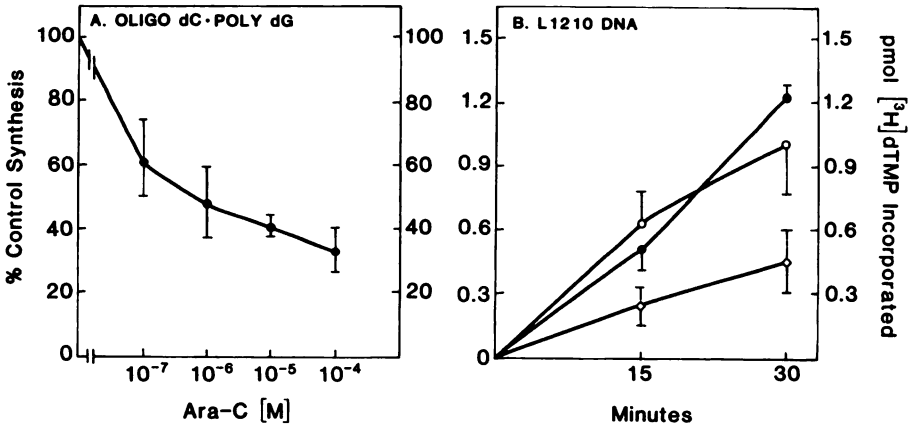

FIG. 6. *Effect of ara-C incorporation into DNA on recovery of DNA synthesis*

A. Oligo dC-poly(dG)-containing ara-C was prepared by incubation of the synthetic template with varying concentrations of ara-CTP and *Escherichia coli* DNA polymerase I for 5 min at 5°. The DNA was purified and incubated with 4×10^{-6} M [^{3}H]dCTP and *E. coli* DNA polymerase I for 15 min at room temperature. The results are expressed as percentage control synthesis (mean ± standard deviation) of two determinations.

B. L1210 (ara-C)DNA was prepared by incubating cells with no drug (○), 10^{-7} M (●), or 10^{-4} M (◇) ara-C for 3 hr. The DNA was purified and used as template in a reaction containing 2×10^{-6} M [^{3}H]dTTP, 0.1 mM deoxynucleosidetriphosphate, and *E. coli* DNA polymerase I. The results are expressed as picomoles of [^{3}H]dTMP incorporated (mean ± standard error for three separate determinations, each performed in duplicate).

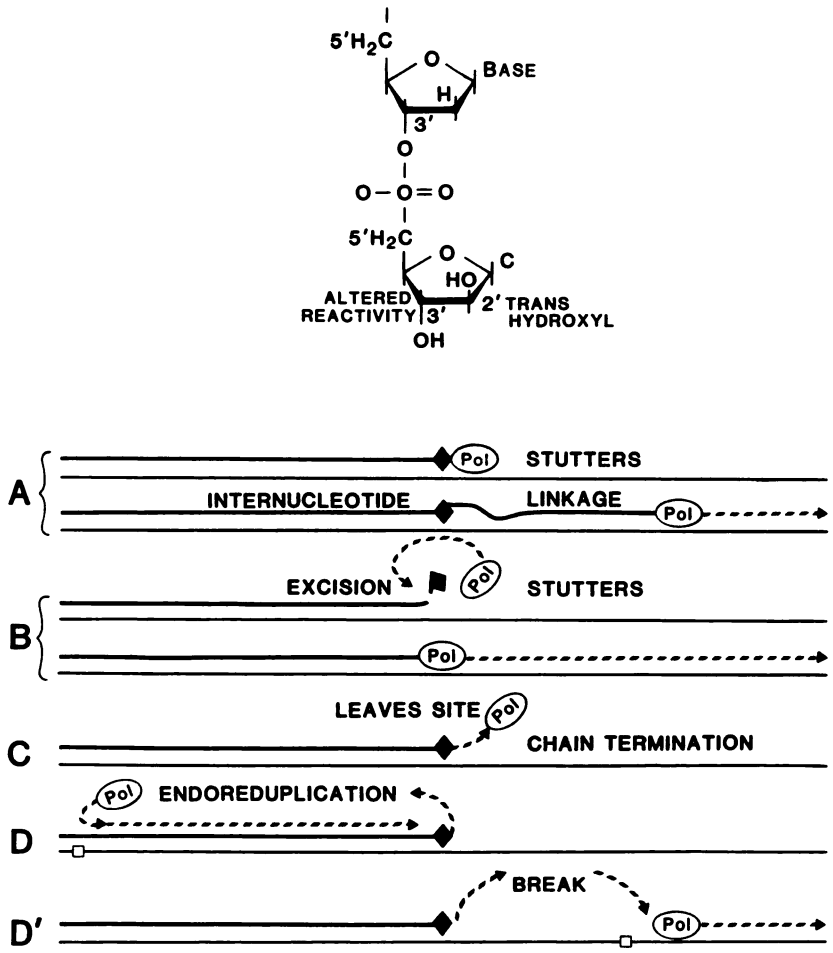

FIG. 7. *Mechanisms of the effects of ara-C incorporation on DNA template function*
Incorporated ara-C residue, ◆; initiation site, □.

C for 3 hr (Fig. 6B). The DNA was purified and monitored for incorporation of [^{3}H]dTMP. The (ara-C)DNA purified from cells exposed to 10^{-7} M ara-C incorporated [^{3}H]dTMP to an extent similar to that obtained with DNA obtained from untreated cells. In contrast, the DNA purified from cells exposed to 10^{-4} M ara-C incorporated significantly less [^{3}H]dTMP than the DNA purified from untreated and 10^{-7} M ara-C-treated cells.

DISCUSSION

Experiments with isolated DNA polymerase and template preparations have indicated that ara-CTP acts as a competitive inhibitor of DNA polymerase (8). However, the inhibitory constant (K_i) of ara-CTP is similar to the Michaelis constant (K_m) of dCTP (7). Although ara-C is an effective inhibitor of cellular proliferation (5), these results suggested that ara-CTP is a rather weak competitive inhibitor of DNA polymerase in cell-free systems and that inhibition of this enzyme is not a significant mechanism by which this agent inhibits DNA replication. In contrast, other studies demonstrated that incorporated ara-C residues behave as relative chain terminators which slow DNA synthesis by modifying reactivity of the terminal 3'-hydroxyl (8, 9, 12). These findings are consistent with conformational and hydrogen-bonding differences of the arabinose sugar moiety altering reactivity of the 3'-terminus and slowing chain elongation.

The demonstration of a highly significantly relationship between the extent of (ara-C)DNA formation and inhibition of DNA synthesis provides further evidence for incorporation of ara-C into DNA as a primary mechanism of drug action. It has also been 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 strands (23). The studies with both ara-C and ara-A have demonstrated highly significant relationships between their incorporation into DNA and loss of clonogenic survival, thus suggesting that the incorporation of arabinosyl derivatives into DNA results in lethal cellular events.

Recent observations with purified mammalian DNA polymerase indicated that excision of the ara-A is necessary for further DNA synthesis (24). Similar observations were made with purified herpes simplex virus DNA polymerase (25). Thus, the altered reactivity of the 3'-terminus at a site of ara-C incorporation could force the polymerase to stutter and then continue to polymerize DNA, leaving the ara-C residue in internucleotide linkage (Fig. 7A). The stuttering effect would slow elongation of DNA strands, rather than lead to an abrupt cessation

of DNA synthesis by chain termination. It is also possible that internucleotide positioning arises through chain extension from the terminal ara-C residue by a polymerase (for example, DNA polymerase β) that is less sensitive to the effects of this drug (26). The results shown in Figs. 5 and 6 could support either mechanism. In contrast, 3′-5′-exonuclease activity associated with DNA polymerase could excise the terminal arabinosyl residue. Although we were unable to demonstrate excision of ara-C residues (Fig. 4), the effects of the exodeoxyribonuclease, if present, could also slow DNA replication, and the arabinosyl residues would be undetectable in the DNA strand (Fig. 7B).

Although previous work suggested that ara-C does not act as a chain terminator because the residues are detectable in internucleotide linkage (9), the altered reactivity of the 3′-terminus by ara-C would predict an association between the presence of a greater proportion of ara-C residues at the chain end and a greater inhibition of DNA synthesis. The previous study demonstrating internucleotide incorporation of ara-C residues (9) employed low concentrations of drug, whereas exposure to higher concentrations of ara-C results in an increasing proportion of ara-C residues detectable at the chain terminus (12). These findings suggest that the polymerase can stutter at a site of ara-C incorporation and then leave that site with the ara-C residue in the chain terminus position (Fig. 7C). This event could occur as a result of overriding the 3′-5′-exonuclease activity or by sufficient distortion of the DNA strand following incorporation of several ara-C residues in close proximity. The effect should be self-limiting, since ara-C would inhibit its own incorporation into DNA. The data in Fig. 3 and in another study (27) support a self-limiting mechanism.

The inhibition of DNA replication results in an aberrant form of DNA synthesis with certain segments of DNA being replicated more than once in a single cell cycle (17, 28). This form of aberrant DNA synthesis (endoreduplication) occurs following inhibition of DNA replication with either ara-C or ara-A (28). In this model (Fig. 7D), the polymerase leaves the site of ara-C incorporation and returns to the site at which DNA replication was initiated within the individual replicons. It is also possible that endoreduplication occurs from the activity of DNA polymerase β when DNA polymerase α is inhibited by ara-C. Endoreduplication has been shown to arise from DNA polymerase β activity in the presence of aphidicolin and in giant trophoblast cells (29, 30). The additional copies of certain segments of DNA might result in the accumulation of DNA fragments (13). Alternatively, DNA strand breaks (13) might accumulate as the polymerase leaves the site of ara-C incorporation to an initiation site in another replicon (Fig. 7D′).

The incorporation of ara-C (or ara-A) into DNA could produce lethal cellular demage through any of the following proposed events: (a) inhibition of chain elongation, (b) chain termination, (c) reinitiation of previously replicated DNA segments, or (d) DNA strand or chromosomal breaks. These proposed events would apply depending upon the dose and schedule of drug administration. Thus, the administration of low doses of ara-C (19, 20) would be expected to slow DNA replication, and ara-C residues would be detectable in internucleotide linkage (Fig. 7A). This partial inhibition of DNA replication by ara-C and ara-A is associated with terminal differentiation of human HL-60 leukemia cells (14). In contrast, the administration of high doses of ara-C (21) results in 0.1 mM plasma levels and more immediate cytoreduction. This effect is probably due to the more absolute chain-terminating effect of this drug at higher concentrations.

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