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Determinants of Sensitivity to 1-β-D-Arabinofuranosylcytosine in HCT 116 and NCI-H630 Human Colon Carcinoma Cells

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SUMMARY

The cytotoxicity and metabolism of 1-β-D-arabinofuranosylcytosine (AraC) and its effects on DNA synthesis and integrity were studied in HCT 116 and NCI-H630 human colon cancer cells. In 116 cells, 0.1 μM AraC decreased colony formation by ~50%, whereas 1 μM was required in H630 cells. AraCTP levels after a 24-hr AraC exposure were 2.3- to 3.5-fold lower in H630 cells due to increased ability to deaminate AraCMP. AraC DNA levels increased in proportion to AraCTP pools (r = 0.99) and were 2-fold higher in 116 cells after a 24-hr exposure to 0.1 and 1 μM AraC. Although the half-life of AraCTP was <1 hr in both lines, >80% of AraC DNA was retained at 24 hr after drug removal. Clonogenic capacity was inversely related to the extent of AraC DNA incorporation. Interference with nascent DNA chain elongation increased with increasing AraC concentration × time. A 24-hr AraC exposure produced a dramatic shift in the elution profile of nascent DNA during a 15-hr elution at pH 12.1; these effects were greater in 116 cells (DNA retained on filter [percentage of control]): 78%, 23%, and 9% with 0.1, 1, and 10 μM AraC versus 84%, 42%, and 18% in H630 cells, respectively. The extent of nascent DNA damage was proportional to AraC DNA content. Net DNA synthesis was potently inhibited during AraC exposure in both lines. H630 cells had partial recovery of DNA synthesis at 24 hr after drug removal, whereas persistent inhibition was noted in 116 cells. A slight excess of double-strand breaks in parental DNA was detected after a 24-hr exposure to 10 μM AraC in 116 cells. The extent of DNA fragmentation was more pronounced 16 hr after drug removal and was greater in 116 cells: 8.5%, 19%, and 21% with 0.1, 1, and 10 μM AraC versus 2.3%, 9%, and 15% in 630 cells, respectively. Thus, AraC DNA content, magnitude of nascent DNA damage, duration of DNA synthetic inhibition, and induction of double-stranded DNA fragmenation appeared to be the crucial determinants of lethality.

The deoxycytidine antagonist AraC is an effective agent against acute leukemias but is inactive against human epithelial malignancies (1). AraC enters cells through the facilitated nucleoside transport mechanism, and phosphorylation to AraCMP by dCyd kinase is generally the rate-limiting step in anabolism. The 5′-triphosphate metabolite AraCTP competes with dCTP for incorporation into DNA by DNA-directed DNA polymerase (EC 2.7.7.7) (2–5). On incorporation of AraCTP into DNA, it functions as a potent inhibitor of DNA polymerase-α (involved in Okazaki fragment synthesis on the lagging strand of the replication fork), DNA polymerase-δ (the leading strand replicase), and DNA polymerase-β (involved in the repair of chemically induced DNA damage) (6–14). Inhibition of DNA polymerases in turn interferes with DNA chain elongation during both semiconservative DNA replication and DNA repair (7, 15–20). The incorporated AraCMP residue functions as a relative DNA chain terminator, and interference with chain elongation is influenced by sequence-specificity of the DNA template (6–8, 12, 13, 18, 20). Initiation of new DNA replication intermediates continues, however, leading to accumulation of nascent DNA fragments (18, 20). Over time, the nascent DNA strand can be extended beyond the arabinosylnucleotide residue, and digestion of DNA reveals the presence of AraCMP in the internucleotide linkage (6–8, 12, 13, 18, 20). The incorporated AraC residues are labile under alkaline conditions (17, 21). AraC exposure produces endonucleolytic cleavage of DNA in leukemic cells in a pattern similar to that observed in cells undergoing programmed cell death (22). Cytotoxicity associated with AraC in human leukemias correlates with the extent of DNA incorporation and the intracellular retention of AraCTP after drug exposure (23–28). Resistance in leukemic cells has been attributed to deletion of dCyd kinase, expansion of CTP and dCTP pools associated with increased

ABBREVIATIONS: AraC, 1-β-D-arabinofuranosylcytosine; AraCMP, 5′-monophosphate of AraC; AraCTP, 5′-triphosphate of AraC; dCyd, deoxycytidine; dThd, thymidine; AraU, 1-β-D-arabinofuranosyluracil; AraUMP, 5′-monophosphate of AraU; PBS, isotonic phosphate-buffered saline without calcium and magnesium, pH 7.4; PCA, perchloric acid; HPLC, high-performance liquid chromatography; dCTPase, dCTP pyrophosphatase; TPAH, tetrapropylammonium hydroxide.

CTP synthase activity, increased Cyd deaminase activity, decreased capacity for nucleoside transport, and decreased intracellular half-life of AraCTP after drug removal (1). To gain insights into intrinsic or inherent determinants of sensitivity to AraC, we examined the intracellular metabolism and DNA incorporation of AraC in two human colon cancer cells that had not been selected *in vitro* for resistance. Such information may suggest new strategies to circumvent resistance or increase sensitivity to AraC and other agents with similar metabolism and mechanism of action.

Experimental Procedures

Materials. Moravek Biochemicals (Brea, CA) supplied the [6-^{3}H]AraC (20 Ci/mmol), [5-6-^{3}H]AraCMP (10 Ci/mmol), [2-^{14}C]-dCMP (50 mCi/mmol), [methyl-^{3}H]dThd (25 Ci/mmol), [methyl-^{14}C]dThd (50 mCi/mmol), [8-^{3}H]dGTP (15 Ci/mmol), [5-^{3}H]dCTP (25 Ci/mmol), [5,6-^{3}H]uridine (20 Ci/mmol), and [2-^{14}C]cytidine (60 mCi/mmol). PBS was purchased from Biofluids Inc. (Rockville, MD). Unless otherwise stated, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Inhibition of colony formation. HCT 116 and NCI-H630 cells, anchorage-dependent human colon adenocarcinoma cell lines (29, 30), were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine and 7% fetal bovine serum (Biofluids Inc.). Cell counts and volumes were determined after trypsinization with a Coulter Multisizer II (Coulter Corporation, Miami, FL). For clonogenic assay, 300–500 cells were replicately plated in six-well tissue culture plates. The following day, various concentrations of AraC were added. After a 24-hr drug exposure, the medium was aspirated, the cells were gently washed twice with RPMI medium, and fresh drug-free medium was replaced. Seven days later, the colonies were stained and enumerated. The plating efficiency for the controls averaged 41 ± 4% for HCT 116 cells and 31 ± 2% for H630 cells. The doubling times averaged 18 hr (HCT 116) and 22 hr (H630).

HPLC analysis of AraC metabolites. Exponentially growing cells were exposed to [^{3}H]AraC (0.1, 1, and 10 μM) for 24 hr. The cells were then washed 3 times with iced PBS and extracted with 0.5 N PCA; the acid-soluble fraction was neutralized with 2 vol of 1,1,2-trichlorotrifluoroethane/tri-*n*-ocytlamine (2.3:1, v/v) and lyophilized. AraC metabolites were analyzed according to a previously reported anion-exchange HPLC method with in-line liquid scintillation detection (31, 32), called HPLC method 1. The retention times were as follows: AraC, 1.9 min; AraCMP, 6.0 min; AraCDP, 11.6 min; and AraCTP, 28.2 min.

To resolve any possible AraU nucleotides from AraC nucleotides, a protracted gradient was used (HPLC method 2): Buffer A was 0.001 M ammonium phosphate (pH 3.0), and buffer B was 0.75 M ammonium phosphate (pH 4.5) (33). Buffer A (100%) was run for the initial 11 min, and then the following linear gradients were run in sequence: to 16 min, 94% A/6% B; to 60 min, 92% A/8% B; to 66 min, 80% A/20% B; to 70 min, 70% A/30% B; an isocratic gradient was continued at 70% A/30% B for 4 min, followed by a linear gradient to 60% A/40% B by 106 min and then to 100% B by 116 min. The retention times were as follows: AraC, 3 min; AraCMP, 10 min; AraUMP, 20 min; CDP, 42 min; UDP, 66 min; UTP, 91 min; CTP, 94 min; and AraCTP, 99 min.

Aliquots of the neutralized, lyophilized acid-soluble fraction were reconstituted in distilled water and treated with alkaline phosphatase (EC 3.1.3.1) in alkaline phosphatase buffer (Promega Inc., Madison, WI) for 60 min at 37°. The samples were centrifuged through a 0.22-μm Amicon Centrifree micropartition system (W. R. Grace & Co., Danvers, MA) and frozen until analyzed with reverse-phase HPLC (C8 Radial Pak 0.8 × 10 cm, Waters, Milford, MA). With water as the mobile phase, AraC and AraU eluted at 9.7 and 12.7 min, respectively.

[^{3}H]AraCTP formation was determined before and after removal of 10 μM [^{3}H]AraC. For prewashout [^{3}H]AraCTP levels, the medium was aspirated, and the cells were washed twice with ice-cold PBS and then extracted with 0.5 N PCA as described above. AraCTP retention was determined in duplicate flasks. The medium was aspirated, and the cells were washed once with RPMI 1640 medium and placed in fresh, drug-free complete medium. At the desired time after washout, the medium was aspirated and the cells were washed once with PBS before PCA extraction.

Determination of ribonucleotide triphosphate and dCTP pools. Ribonucleotide triphosphate pools were determined according to a previously reported anion-exchange HPLC method (33). dCTP pools were determined by an enzymatic bacterial DNA polymerase assay as previously described (32–34).

Enzyme assays. Cellular lysates for the kinase assays were prepared by suspending the cell pellet in 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 7.4); after sonication and centrifugation at 12,000 × *g* for 30 min at 4°, the supernatant was collected. Protein content of the cell lysates was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). For determination of dCyd kinase (EC 2.7.1.74) and dCMP kinase (EC 2.7.4.14), the reaction mixture (final volume, 150–300 μl) contained 10 mM ATP, 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.4), 1 mM substrate ([^{3}H]AraC or [^{3}H]AraCMP; 200,000 dpm per sample), and cell lysate. The samples were incubated for 10 to 60 min at 37° in a shaking water bath. AraCMP formation by dCyd kinase was determined by the adherence of the nucleotide to Whatman DE-81 ion exchange filter discs as previously described (32, 35). For the dCMP kinase assay, the reaction was quenched by adding an equal volume of iced 100% methanol; AraCDP and AraCTP formations were then determined by HPLC method 1. The formation of AraC from [^{3}H]AraCMP at neutral pH served as an index of catabolism by 5′-nucleotidase (EC 3.1.3.5).

Cytidine deaminase (EC 3.5.3.5) activity with [2-^{14}C]cytidine as substrate was determined by the method of Chabner *et al.* (36). dCMP deaminase (EC 3.5.4.14) activity with [2-^{14}C]dCMP as substrate was determined by a modification of the method described by Drake *et al.* (37); with [^{3}H]AraCMP as substrate, the reaction was carried out in an identical manner, but [^{3}H]AraUMP formation was determined by HPLC method 2.

For the dCTPase (EC 3.6.1.12) assay, the cell pellet was resuspended in 50 mM Tris HCl (pH 7.8), 50 μM dCTP, and 1 mM dithiothreitol (38). In a total volume of 200 μl, the assay mixture contained the following: 25 mM $MgCl_2$, 25 mM Tris-HCl (pH 8.0), 10 mM mercaptoethanol, 1 mM EDTA, 600 μM [5-^{3}H]dCTP, and cell lysate. The samples were incubated for 2 and 4 min, extracted with ice-cold methanol, and analyzed by HPLC method 1. The data were corrected for the amount of dCTP degraded in the absence of enzyme.

Incorporation of AraC into DNA. Cells in logarithmic growth phase were exposed to [^{3}H]AraC (0.1 μM, sp. act., 1.0–2.5 μCi/nmol; 1.0 μM, sp. act., 0.2 μCi/nmol; and 10 μM, sp. act., 0.06 μCi/nmol) for 24 hr. After three PBS washes, the cells were removed with a cell scraper and collected by centrifugation. The cell pellet was digested for 3 hr in a lysis buffer containing 0.4 mg/ml proteinase K (EC 3.4.21.14), 10 mM Tris/10 mM EDTA (pH 7.4), 0.4 M NaCl, and 0.4% SDS at 65°. The cell digest was extracted 3 times with an equal volume of phenol, phenol/chloroform (1:1), and chloroform, respectively, then precipitated at −70° by adding 0.1 volume 3 M sodium acetate (pH 5.4) and 2.5 vol of absolute ethanol. The precipitate was washed once with 70% ethanol and then dissolved in 2 ml Tris-EDTA (10 mM/1 mM, pH 8.0). The sample was treated with DNAse-free RNAse (EC 3.1.26.2, 50 μg) for 60 min at 37° followed by DNA purification as described. An aliquot of the purified DNA was used to determine absorbance at 260 and 280 nm, and the remainder was subjected to 10% TCA precipitation and collection on Whatman HA filters. The filters were washed 3 times with 5% TCA and once with 95% ethanol and then placed in a liquid scinitillation vial.

pH step alkaline elution of newly synthesized DNA. Exponentially growing cells were exposed to AraC for 2, 4, and 24 hr; the cells were pulsed with 10 μCi [^{3}H]dThd (10 ml per flask) for 1, 2, and

3 hr, respectively. The medium was aspirated, and the cells were gently washed twice with ice-cold PBS and then detached by incubation in 20 mM ice-cold EDTA-Na_2 (pH 7.0). Equal numbers of [^{3}H]dThd-labeled cells (approximately 400,000) were placed on a 25-mm Nuclepore filter (1-μm pore size, Costar Corporation, Cambridge, MA) held in an alkaline elution funnel (Millipore) and lysed with 3 ml of a lysis buffer consisting of an equal volume of 40 mM EDTA-Na_2 (pH 10.0)/4 M sodium chloride and 0.6% *N*-lauroyl sarcosine sodium salt (ICN Biochemicals Inc., Cleveland, OH). The DNA was subsequently washed with 20 mM EDTA-Na_2 (pH 10) and then eluted for 1 hr each with progressively more basic solutions consisting of 20 mM EDTA (free acid form) adjusted to pH steps of 11.0, 11.3, 11.5, and 12.1 with TPAH using a Gilson Minipuls 3 peristaltic pump (Middleton, WI) set at a rate of 4.8 ml/hr (18, 39). Fractions were collected at 20-min intervals. The elution fractions and filter were neutralized with glacial acetic acid, and the radioactive counts eluting with each pH step and the radioactivity retained on the filter were determined. The data are expressed as percentage of total DPM for each fraction.

Detection of DNA single-strand breaks by fixed pH 12.1 elution. To determine AraC-mediated induction of DNA single-strand breaks in high-molecular-weight DNA, exponentially growing cells were prelabeled with [^{14}C]dThd (0.05 μCi/ml, 15 ml per flask) for 24–48 hr. After a 6–24-hr "chase" period, AraC was added. The cells were harvested, lysed on Nuclepore filters at a nondenaturing pH, and washed as described above. The DNA was eluted with 20 mM EDTA adjusted to pH 12.1 with 1 M TPAH at a rate of 2.4 ml/hr (40, 41). Fractions were collected at 1.5-hr intervals for 15 hr and processed as described above.

In separate experiments to determine the effects of AraC on the integrity of newly synthesized DNA, we exposed cells to [^{14}C]dThd (0.05 μCi/ml, 15 ml per flask) for the final 8 hr of incubation. The cells were harvested and subjected to fixed pH elution as desribed above. The total radioactivity incorporated into DNA (defined as the sum of DPM for all elution fractions plus the filter, corrected for background) per 1 million cells in drug-treated cells was compared with that in control cells and provided an estimate of the effect of AraC on net DNA synthesis.

Detection of parental DNA double-strand breaks. A filter binding assay was used to assess double-stranded DNA damage resulting from AraC exposure under nondeproteinizing, non-DNA-denaturing conditions (42, 43). Cells were prelabeled with [^{14}C]dThd as described above. Cells were exposed to AraC for 24 hr, after which they were either harvested or washed twice with RPMI medium and incubated in fresh, drug-free complete medium for an additional 16 hr. After drug exposure, detached and adherent cells were deposited on a Gelman Metricel Membrane (0.8 μm pore size, 25 mm) (Gelman, Ann Arbor, MI) held in an alkaline elution funnel. All liquid passing through the filter was collected. The cells were washed with 10 ml PBS and then lysed with 5 ml of lysis buffer; after the lysis buffer had dripped through by gravity, it was washed from the filter with 10 ml of 20 mM EDTA (pH 10), followed by elution with 10 ml of 20 mM EDTA adjusted to pH 9.6 with TPAH. The total radioactivity was determined by combining the counts from the eluting fractions (loading, wash, lysis, and EDTA washes) with that retained on the filter. DNA fragmentation was calculated by dividing the DPM in the eluting fractions by the total DPM.

Detection of cytoplasmic oligonucleosomal DNA fragments. The Cell Death Detection Enzyme-linked Immunosorbent Assay Kit (Boehringer Mannheim, Indianapolis, IN), a photometric enzyme immunoassay method, was used for the *in vitro* determination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) during and after AraC exposure. Exponentially growing cells were exposed to AraC for 24 hr and were harvested immediately or washed twice with medium and then incubated in drug-free medium for an additional 16 hr. HL-60 human promyelocytic leukemia cells treated with camptothecin for 4 hr were used as a positive control. Detached and adherent HCT 116 and H630 cells were combined and resuspended with incubation buffer supplied by the manufacturer at a concentration of 500,000 cells/ml. After incubation on ice for 30 min, the lysate was centrifuged at 12,000 × *g* for 15 min; a portion of the supernatant containing the cytoplasmic fraction was removed. The enzyme-linked immunosorbent assay was carried out according to the manufacturer's specifications. Briefly, a microtiter plate was coated with murine monoclonal anti-histone antibody, and blocking solution was used to saturate nonspecific binding sites. The cytoplasmic sample was added to the wells; any mononucleosomes and oligonucleosomes in the test sample bind via their histone component to the anti-histone antibody fixed to the well walls. A peroxidase-conjugated anti-DNA antibody was then added to bind to oligonucleosomal DNA. A colorimetric substrate, 2,2′-azino-di-[3-ethylbenythiazoline sulfonate(6)], was added, and after a 10–15-min incubation, the absorbance at 405 nm was measured.

Results

Cytotoxicity of AraC. A 24-hr exposure to 0.1 and 1 μM AraC reduced cell growth at 72 hr to 57 ± 16% and 22 ± 11% of control in HCT 116 cells (mean ± standard deviation, n = 3). In H630 cells, however, these values were 73 ± 7% (0.1 μM) and 46 ± 11% of control (1 μM) at 72 hr. In clonogenic assays, a 24-hr exposure to 0.1, 1, and 10 μM AraC reduced colony formation to 35 ± 7%, 4 ± 2%, and 0.1 ± 0.1% of control in HCT 116 cells (mean ± standard error, n = 9). H630 cells were less sensitive (percentage of control colony formation: 0.1 μM, 79 ± 4%; 1 μM, 48 ± 5%; and 10 μM, 20 ± 6%).

Cytidine nucleotide pools. The endogenous CTP pools were similar in both cell lines (nmol/10^6 cells): HCT 116, 3.2 ± 0.1; and H630, 2.6 ± 0.2 (mean ± standard error, n = 6). dCTP pools were 58- to 74-fold lower than the CTP pools and were also comparable in the two lines (pmol/10^6 cells): HCT 116, 43 ± 1; and H630, 45 ± 5 (mean ± range, n = 2). Incorporation of [^{3}H]dCyd into acid-precipitable material in intact cells provides an estimate of salvage capacity and competition with endogenous dCTP pools for DNA incorporation; [^{3}H]dCyd salvage was comparable in the two cell lines (dpm/10^8 cells): HCT 116, 1675 ± 343; and H630, 2083 ± 244 (mean ± standard deviation, n = 3). CTP synthase activity was determined by measuring CTP formation after a 4-hr incubation of intact cells with 1 μM [^{3}H]uridine. CTP synthase activity was similar in both lines expressed either as the absolute amount of CTP formed over time or as the ratio of [^{3}H]CTP to [^{3}H]UTP: HCT 116, 1.55 pmol/hr/10^6 cells, ratio 0.28; and H630, 1.95 pmol/hr/10^6 cells, ratio 0.26.

AraC metabolism. After a 24-hr exposure, AraCTP levels increased in a concentration-dependent manner (Table 1). AraCTP levels after 0.1, 1, and 10 μM AraC were 2.3- to 3.5-fold lower in the H630 cells. In HCT 116 cells, AraCTP was the predominant nucleotide formed, and the ratios of AraCTP to AraCMP were 0.8 ± 0.1, 7.8 ± 1.8, and 13.2 ± 3.8 with 0.1, 1, and 10 μM, respectively. In H630 cells, in contrast, the ratios of AraCTP to AraCMP were much lower: 0.2 ± 0.1, 1.5 ± 0.5, and 1.2 ± 0.2, respectively.

Because this HPLC method does not distinguish between AraCMP and AraUMP, these nucleotides were resolved with the use of a more protracted anion-exchange gradient. AraUMP was not detected in HCT 116 cells after a 24-hr incubation with either 1 μM or 10 μM AraC. In contrast, AraUMP accounted for 1.7 ± 0.4% and 1.7 ± 0.8%, respec-

TABLE 1
AraCTP formation and DNA incorporation after 24-hr AraC exposure

AraC (μM)	HCT 116	H630	HCT 116/H630
	AraCTP (pmol/10⁶ cells)		*ratio*
0.1	7.7 ± 1.5	2.2 ± 13.5	
1	78 ± 23	34 ± 11	2.3
10	788 ± 186	344 ± 66	2.3
AraC (μM)	HCT 116	H630	HCT 116/H630
	AraC-DNA incorporation (fmol/μg DNA)		*ratio*
0.1	23.3 ± 3.8	11.6 ± 2.7	2.0
1.0	74.8 ± 44.6	36 ± 10	2.1
10.0	146 ± 34.0	119 ± 26	1.2

Exponentially growing cells were exposed to 0.1, 1, or 10 μM [³H]AraC for 24 hr. AraCTP formation, determined by HPLC analysis, are presented as mean ± standard error ($n \geq 4$ separate determinations). Total cellular DNA was purified as described in Methods, and the data are expressed as mean ± standard error ($n \geq 4$ separate determinations).

tively, of the total AraC metabolites (mean ± standard error, $n = 5$) in H630 cells. To determine the proportion of AraU relative to AraC, aliquots of cell extract were treated with alkaline phosphatase to convert all nucleotides to the nucleoside level and then analyzed with reverse-phase HPLC. After a 24-hr incubation with 1 μM [³H]AraC, [³H]AraU accounted for an average of 7% and 35% of the total metabolites in HCT 116 and H630 cells, respectively; with 10 μM [³H]-AraC, [³H]AraU accounted for 5% and 18% of the total metabolites, respectively.

AraCTP retention after drug removal was determined after exposure to 10 μM [³H]AraC. One hour after washout, AraCTP levels had decreased by >50% in both lines (mean ± standard error, $n = 4$): HCT 116, 31 ± 4% remained (244 pmol/10⁶ cells); and H630, 45 ± 4% remained (155 pmol/10⁶ cells). By 4 hr after drug removal, only 7 ± 1% and 13 ± 1% of AraCTP remained in HCT 116 and H630 cells (55 and 45 pmol/10⁶ cells), respectively. These data suggest that residual AraCTP levels do not appear to influence relative cell sensitivities to AraC.

To understand the basis for the differences in AraC metabolism, we determined enzyme activities in cell-free assays using saturating concentrations of substrate (Table 2). The activities of dCyd kinase, dCMP kinase, cytidine deaminase, and dCTPase were similar in the two cell lines. The ability of cells to dephosphorylate [³H]AraCMP at neutral pH was slightly (2.4-fold) higher in H630 cells. In each line, the activity of dCMP deaminase was much higher with [2-¹⁴C]-dCMP as substrate compared with [³H]AraCMP. Although the difference in dCMP deaminase activity was not pronounced with the natural substrate, the ability of H630 cells to deaminate [³H]AraCMP was 6.4-fold higher than that of HCT 116 cells. With AraCMP as substrate, dCMP kinase activity exceeded deaminase activity in both lines, although the ratio was much higher in HCT 116 cells. A greater capacity to deaminate AraCMP most likely accounts for the increased AraU levels and reduced AraCTP levels in H630 cells. The catabolic enzymes dCTPase and 5′-nucleotidase undoubtedly contribute to the very short half-life of AraCTP.

AraC DNA incorporation. DNA incorporation of AraC increased in proportion to AraCTP levels ($r = 0.99$). AraC DNA levels were approximately 2-fold higher in HCT 116 cells compared with H630 cells after a 24-hr exposure to 0.1 and 1 μM AraC, but the difference was only 1.2-fold after 10 μM AraC (Table 1). In contrast to the brief intracellular half-life of AraCTP, AraC DNA appeared to be relatively stable. Twenty-four hours after drug removal, AraC levels in purified DNA were >80% of that measured immediately after drug exposure in both lines (data not shown).

Effect of AraC on DNA chain elongation and nascent DNA integrity. The functional consequences of AraC on DNA chain elongation were measured by pulse incorporation of [³H]dThd (1–3 hr). In the pH transition zone (11.6–12.1), molecular weight influences the selective denaturation of newly replicated DNA exposed to alkali. Therefore, stepwise elution of DNA with gradually more alkaline solutions may show relative differences in the single-strand length of newly synthesized DNA. AraC exposure was accompanied by an increase in the proportion of nascent DNA species eluting with pH steps 11.0, 11.3, and 11.5, with a corresponding decreased proportion of DNA retained on the polycarbonate filter in both HCT 116 and H630 cells. The AraC-mediated alterations in the elution profile of nascent DNA increased in a concentration- and time-dependent manner (Fig. 1). Although the proportion of nascent DNA eluting with pH steps ≤11.5 was slighly higher in H630 cells than in 116 cells, the differences were not significant. The similarity of AraC-mediated interference with DNA chain elongation in both cell lines was unexpected. Because AraCTP competes with dCTP for DNA incorporation, a potential explanation may be the relative ratio of AraCTP to dCTP during drug exposure. With 0.1 μM AraC, AraCTP pools were only 18% and 5% of the baseline dCTP pools in HCT 116 and H630 cells, respectively. AraCTP levels after a 24-hr exposure to 1 and 10 μM AraC,

TABLE 2
Enzymes involved in AraC metabolism

Enzyme	Substrate	HCT 116	H630	HCT 116/H630
		pmol/min/mg		*ratio*
dCyd kinase	AraC	37.9 ± 12.3 ($n = 5$)	50.9 ± 13.4 ($n = 5$)	0.74
5′-Nucleotidase	AraCMP	66 ± 30 ($n = 2$)	156 ± 4 ($n = 4$)	0.42
Cyd deaminase	Cytidine	86.2 ± 24.8 ($n = 4$)	104.1 ± 20.3 ($n = 8$)	0.83
Ratio of kinase to deaminase	AraC/cytidine	0.44	0.49	0.90
		nmol/min/mg		
dCMP kinase	AraCMP	10.4 ± 3.5 ($n = 5$)	6.9 ± 2.9 ($n = 7$)	1.51
dCMP deaminase	dCMP	18.1 ± 8.0 ($n = 2$)	30.8 ± 6.5 ($n = 4$)	0.59
dCMP deaminase	AraCMP	0.5 ± 0.2 ($n = 2$)	3.2 ± 1.2 ($n = 4$)	0.15
dCTPase	dCTP	24.5 ± 7.4 ($n = 4$)	28.9 ± 10.8 ($n = 4$)	0.85
Ratio of kinase to deaminase	AraCMP/AraCMP	20.8	2.2	9.45

Enzyme activities in cell lysates were determined as described in Methods. Data are presented as mean ± range ($n = 2$) or standard error ($n \geq 4$).

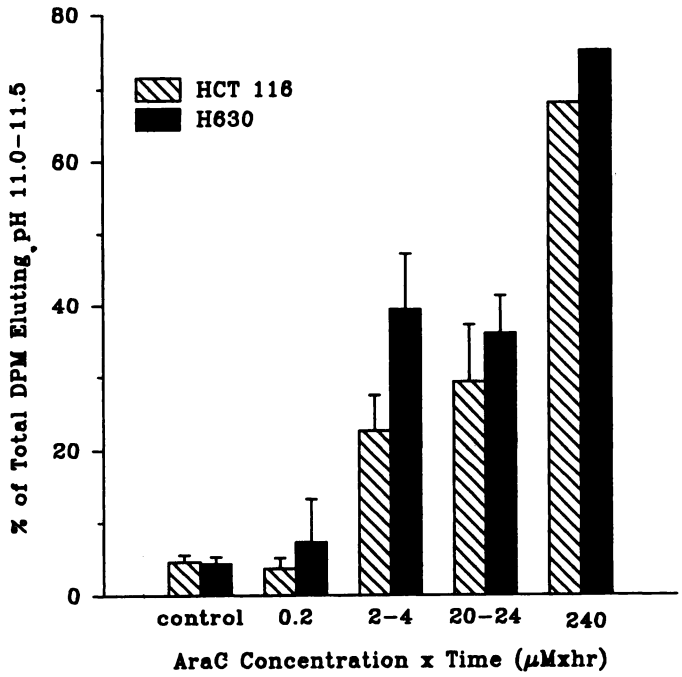

Fig. 1. Effect of AraC on nascent DNA chain elongation by pH step alkaline elution. Exponentially growing cells were exposed to 0.1, 1, or 10 μM AraC for 2, 4, or 24 hr; the cells were pulsed with [^{3}H]dThd (10 μCi/flask) for 1, 2, or 3 hr, respectively. The cells were harvested and lysed on a Nuclepore filter, and the size distribution of single-stranded DNA species was determined by pH step alkaline elution as described in Experimental Procedures. The data are presented as the percentage of total DPM eluting with pH steps 11.0, 11.3, and 11.5 as a function of AraC concentration × time; the mean ± standard error are shown unless otherwise stated (μM×hr): control, n = 13; 0.2, n = 2 (mean ± range); 2–4, n = 6; 20–24, n = 3–7; and 240, n = 1. The median values of the 2–4 μm·hr and 20–24 μm·hr groups differed significantly from that of the control group in both cell lines (HCT 116, $p < 0.02$; H630, $p < 0.001$, Mann-Whitney rank sum test).

however, were 1.8-fold and 18.4-fold higher than endogenous baseline dCTP pools in HCT 116 cells and 0.8-fold and 7.6-fold higher than baseline dCTP pools in H630 cells, respectively. The effects on DNA chain elongation during drug exposure most likely reflect the combined contributions of direct interference by free AraCTP (through competition with dCTP for incorporation into DNA by DNA polymerase) and inhibition of DNA polymerase subsequent to AraC DNA incorporation.

The effect of AraC exposure on the fragility of newly synthesized DNA (8-hr [^{14}C]dThd exposure) was determined by fixed pH alkaline elution. In the presence of pH 12.1 buffer, DNA is expected to be denatured and thus single stranded. During the very slow elution of the DNA over a 15-hr period, the total size of the DNA strand influences its ability to pass through the filter (the smaller the DNA species, the faster it will pass through). DNA fragments caused by preexisting single-strand breaks elute very early (during the initial 1.5–3 hr of elution), and continued exposure of DNA to the alkaline elution buffer may induce DNA breaks in "fragile" DNA. In both cell lines, elution of DNA single-stranded species increased with increasing AraC concentration, and the percentage of total DNA eluting after a 24-hr exposure to 1 and 10 μM AraC was significantly different from control in both cell lines: HCT 116, control, 20 ± 7%; 1 μM, 80 ± 7%; and 10 μM, 93 ± 2% (p = 0.001, Mann-Whitney rank sum test); and H630, control, 14 ± 6%; 1 μM, 62 ± 9%; and 10 μM, 83 ± 6% ($p \leq 0.008$). Because the amount of DNA retained on the filter was slightly different in HCT 116 and H630 controls, the effects of AraC were also expressed as the percentage of DNA retained on the filter relative to control; more pronounced effects were evident with each AraC concentration in HCT 116 cells (Fig. 2, *left panel*) than in H630 cells (Fig. 2, *right panel*). A greater proportion of DNA eluted with the initial elution fraction at each AraC concentration in HCT 116 cells, indicating a larger amount of preexisting low-molecular-weight DNA fragments compared with H630 cells. In addition, a greater amount of nascent DNA eluted in HCT 116 cells over the 15-hr elution period. Persistent abnormalities in newly synthesized DNA were apparent in adherent HCT 116 cells 24 hr after AraC removal (percentage of DNA retained relative to control, n = 4–5): 1 μM, 82 ± 11%; and 10 μM, 53 ± 11%. In contrast, in H630 cells, the elution profile had returned to normal after removal of 1 μM AraC (98 ± 13% retained), whereas the drug effect persisted after 10 μM (66 ± 7%).

Effect of AraC on net DNA synthesis. Comparison of total DNA synthetic rate ([^{14}C]dThd incorporation over an 8-hr pulse) indicated that DNA synthesis was strongly inhibited in both cell lines during AraC exposure (Fig. 3); the effects were somewhat more pronounced in the H630 cell line. A difference emerged, however, 24 hr after drug removal. No significant recovery of DNA synthesis was noted in HCT 116 cells after AraC removal. In H630 cells, however, DNA synthetic capacity at 48 hr (24 hr after drug removal) was significantly higher that that observed during drug exposure (Fig. 3). The extent of recovery was greatest with 0.1 μM, but DNA synthesis remained inhibited by 89% at 24 hr after removal of 10 μM AraC, a concentration associated with 80% reduction in clonogenic capacity. These results suggest that the extent of DNA synthetic inhibition during AraC exposure did not accurately predict ultimate lethality and may, in part, reflect the effects of free AraCTP (similar to the pH step alkaline elution data). In contrast, DNA synthesis at 48 hr (24 hr after drug removal) appeared to correlate closely with cytotoxicity. When the data for both cell lines were combined, nascent [^{14}C]DNA retained on the filter at 24 hr (as percentage of control) demonstrated a very tight linear correlation with DNA synthetic capacity at 48 hr (r = 0.998, data not shown).

Membrane integrity of adherent cells as assessed by trypan blue exclusion was minimally affected during both the initial 24 hr of AraC exposure and 24 hr after drug exposure (≥95% excluded trypan blue). The cell volume for control cells was 2279 ± 81 fl (HCT 116 cells) and 2564 ± 253 fl (H630 cells). Cell volume increased during AraC exposure: HCT 116, 0.1 μM, 1.29 ± 0.07-fold; 1 μM, 1.41 ± 0.07-fold; and 10 μM, 1.27 ± 0.05-fold; H630 cells, 0.1 μM, 1.45 ± 0.16-fold; 1 μM, 1.68 ± 0.28-fold; and 10 μM, 1.61 ± 0.5-fold. The cell volume remained elevated for at least 24 hr after drug removal. AraC exposure was accompanied by expansion of all four ribonucleotide triphosphate pools in both cell lines after a 24-hr exposure. For example, after 0.1 μM AraC, UTP pools increased by 1.3–1.9-fold, CTP by 2.2–3.4-fold, ATP by 1.7–3.4-fold, and GTP by 1.7–2.6-fold. After a 24-hr exposure to 10 μM AraC, ATP content (nmol/10^6 cells) increased from 23 ± 0.1 to 52.5 ± 9.0 in HCT 116 cells and from 13.6 ± 0.4 to 29.1 ± 7.4 in H630 cells. These pool sizes remained elevated

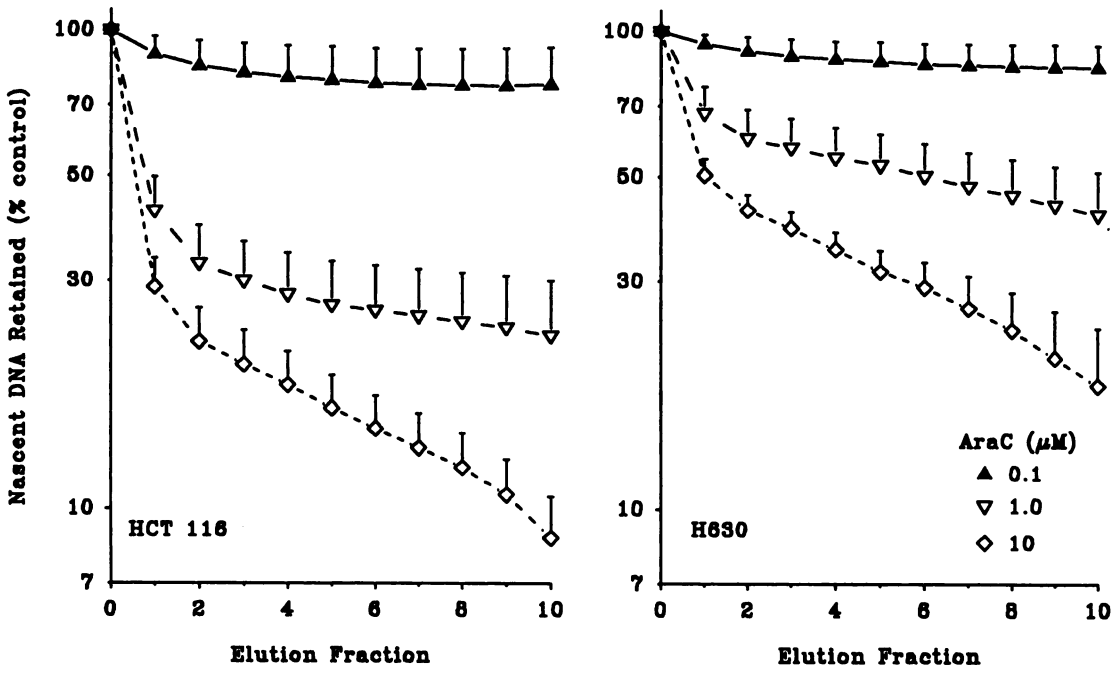

Fig. 2. Fixed pH alkaline elution of newly synthesized DNA. Exponentially growing cells were exposed to 0.1, 1 and 10 μm AraC for 24 hr; the cells were incubated with [^{14}C]dThd (0.05 μCi/ml, 15 ml total) for the final 8 hr of drug exposure. The cells were harvested and lysed on a Nuclepore® filter, and DNA was eluted with a pH 12.1 buffer over a 15-hr period as described in Experimental Procedures. The data are presented as the percentage of the total DNA retained on the filter relative to control (mean + standard error, n = 6 separate experiments) with each 1.5-hr fraction. The percentage of total DNA retained on the filters for the controls was as follows: HCT 116, 80 ± 7%; and H630, 86 ± 6%. Repeated measures analysis of variance was used to test the overall difference in mean values of the elution fractions between the HCT 116 and H630 cells separately for the two higher AraC concentrations; the p value from the F test for differences in cell lines was 0.049 for 1 μM and p = 0.0072 for 10 μM.

even if adjusted for the increase in cell volume accompanying drug exposure. The increase in ribonucleotide triphosphate pools may be a consequence of unbalanced cell growth secondary to the inhibition of DNA synthesis.

Induction of parental DNA damage. Although the effects were less dramatic than those observed for newly synthesized DNA, AraC also affected the integrity of high-molecular-weight DNA (prelabeled with [^{14}C]dThd). Under DNA denaturing conditions (pH 12.1 elution), the proportion of parental DNA retained on the filter in control cells was 86.3 ± 2.5% (HCT 116) and 84.4 ± 3.3% (H630; mean ± standard error, n = 6–7). Immediately after a 24-hr exposure to 1 and 10 μM AraC, a smaller proportion of total DNA was retained on the filter (expressed as percentage of control): 87 ± 4% and 47 ± 11% in HCT 116 cells (n = 5–7) and 84 ± 5% and 56 ± 11% in H630 cells (n = 5 or 6). The lowest concentration of AraC tested, 0.1 μM, did not affect the elution profile of parental DNA in either cell line (percentage of control, mean ± standard deviation, n = 3): HCT 116, 100.3 ± 1.3% retained; and H630, 95.6 ± 3.5% retained.

AraC-mediated induction of double-strand breaks in parental DNA was determined by a filter binding method. This assay technique is conducted at a nondenaturing pH; thus, only DNA fragments with preexisting double-strand breaks are capable of eluting during the brief lysis and wash steps. There was no evidence of double-strand DNA breaks during AraC exposure in H630 cells (Fig. 4). A slight excess of DNA double-strand breaks occurred in HCT 116 cells after a 24-hr exposure to 10 μM AraC. During the 24-hr AraC exposure, virtually all of the cells remained adherent to the tissue culture flask (≥97% cells adherent even with 10 μM AraC). Other investigators have reported that detachment of anchorage-dependent cells portends ultimate cell death (44, 45). In the 24 hr after drug removal, an increased proportion of HCT 116 cells detached from the monolayer. For example, 16 hr after AraC, only 2 ± 0.3% of cells had detached in the control flasks (mean ± standard error, n = 5) compared to 4.2 ± 1.2%, 10.5 ± 1.4%, and 12.0 ± 2.9% after 0.1, 1, and 10 μM AraC, respectively. However, in H630 cells, very few drug-treated cells had detached: 2.9 ± 1.1%, 3.1 ± 0.9%, and 3.6 ± 0.8% after 0.1, 1, and 10 μM AraC, respectively, compared with 2.1 ± 0.9% of control cells. More than 80% of the detached cells excluded trypan blue at 40 hr (16 hr after AraC), indicating intact membrane integrity. By 48 hr (24 hr after AraC), the percentage of detached cells increased. In HCT 116 cells, for example, 17% (1 μM) and 28% (10 μM) of the cells had detached; only 36% of the HCT 116 cells exposed to 10 μM AraC that had detached excluded trypan blue at 48 hr. As recommended by previous authors, we combined the detached and adherent cells for assessment of parental double-strand breaks to avoid underestimating drug effects (44, 45) but limited the time points to those associated with intact membrane integrity of the detached cells (e.g., 24 and 40 hr). The frequency of double-strand breaks at 40 hr was higher at each AraC concentration in HCT 116 cells than in H630 cells (Fig. 4). There was a good correlation between percentage of single-strand breaks in nascent DNA at 24 hr and percentage of parental DNA double-strand breaks at 40 hr (r = 0.946, data not shown).

The Cell Death Elisa Kit was used to determine if the parental DNA fragmentation was accompanied by induction of oligonucleosomal DNA fragments. After a 24-hr exposure

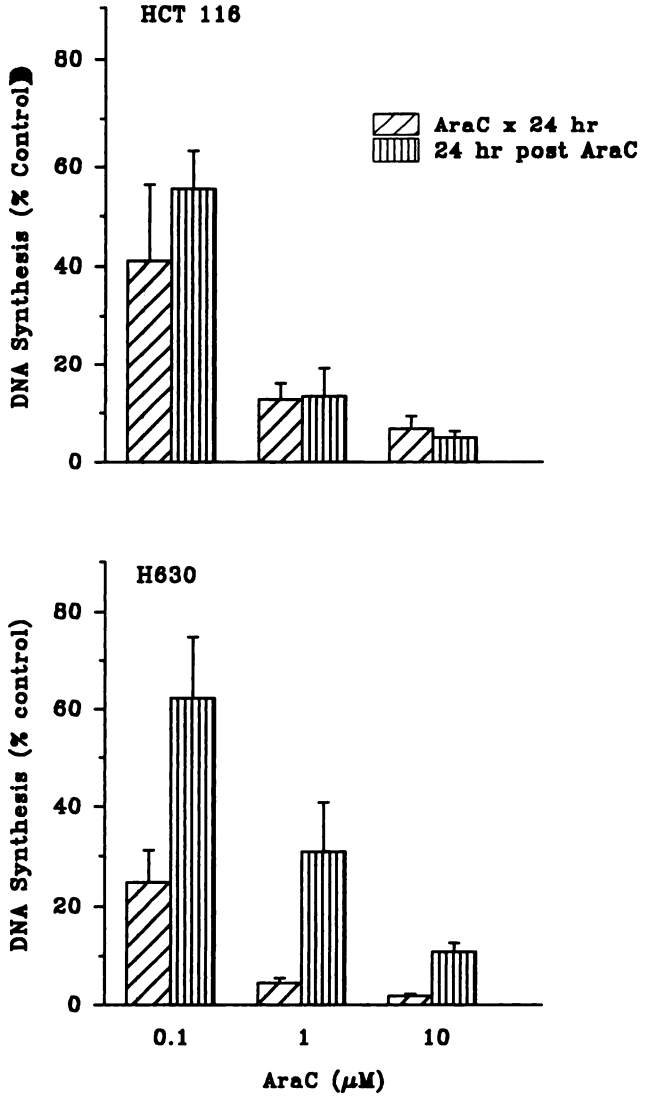

Fig. 3. DNA synthesis during and after AraC exposure. The data for net DNA synthesis in adherent cells were determined in conjunction with the fixed pH alkaline elution experiments. Total [^{14}C]dThd counts less background were determined and expressed as dpm/10^6 cells. The data for AraC-treated cells are presented for DNA synthesis as percentage of control (mean ± standard error, n = 5 or 6). The control values were as follows (dpm/10^6 cells): HCT 116, 143,215 ± 34,825; and H630, 132,607 ± 35,691. The difference between DNA synthesis at 24 hr versus at 48 hr (24 hr after removal of AraC) in H630 cells was significant at each AraC concentration (0.1 μM, p = 0.016; 1 and 10 μM, p = 0.002; Mann-Whitney rank sum test).

to AraC, the optical density of control and drug-treated cells was similar to background in both cell lines (data not shown). At 40 hr (16 hr after AraC), a small increase in absorbance was detected in HCT 116 cells treated with 1 and 10 μM AraC (data are presented as the increase over background, mean ± range of two experiments done in triplicate): control, 1.2 ± 0.1-fold; 0.1 μM, 1.2 ± 0.1-fold; 1 μM, 2.8 ± 0.4-fold; and 10 μM, 5.6 ± 3.7-fold. In H630 cells, the results were as follows: control, 1.4 ± 0.2-fold; 0.1 μM, 1.8 ± 0.4-fold; 1 μM, 2.2 ± 0.5-fold; and 10 μM, 2.5 ± 0.4-fold. HL60 cells treated with camptothecin were included in each experiment as a positive control. In contrast to the two anchorage-dependent human colon cancer cell lines, HL60 cells grow as a suspension culture; the absorbance of the control cells is expected to be higher than control monolayer cells, since each growing suspension culture will contain a certain amount of dead cells. The absorbance of the HL60 control cells was 12.6 ± 6.3-fold above background (mean ± standard error of four experiments done in triplicate). A 4-hr exposure to 5 μM camptothecin produced a dramatic increase in the absorbance: 53.8 ± 17.5-fold over background. These results suggest a fundamental difference in the response to DNA damage between the leukemic and colon cancer cell lines.

Correlations between AraC DNA incorporation and cytotoxic end points. Clonogenic survival decreased in proportion to AraC DNA content at 24 hr (Fig. 5, *top left panel*). There appeared to be a close relationship between induction of single-strand breaks in nascent DNA and increasing AraC DNA content at 24 hr (Fig. 5, *top right panel*); as the extent of AraC DNA incorporation increased, the damage to nascent DNA integrity increased. Furthermore, inhibition of DNA synthesis at 48 hr (24 hr after AraC washout) in the adherent cells was influenced by AraC DNA content (Fig. 5, *bottom left panel*). Delayed induction of parental DNA double-strand breaks 16 hr after drug removal also correlated with AraC DNA content (Fig. 5, *bottom right panel*). Similarly, colony formation decreased as the percentage of single-strand breaks in nascent DNA increased (r = 0.917) and as the extent of double-strand breaks in parental DNA increased (r = 0.991, data not shown).

Discussion

In the present study, to elucidate the determinants of inherent sensitivity to this deoxycytidine analog, we examined the metabolism of AraC and the consequences of its incorporation into DNA in two human colon cancer cell lines. A 24-hr exposure was selected because it slightly exceeded one doubling time in these asynchronous cell cultures. Both cell lines had adequate *de novo* and salvage capacities to form (d)Cyd nucleotides. A 2.3–3.5-fold difference was detected in the amount of AraCTP formed. Although the activities of the enzymes responsible for conversion of AraC to AraCMP and then to AraCDP were comparable in the two cell lines, the ability to deaminate AraCMP was much higher in the less-sensitive cell line. After drug exposure, the intracellular retention of AraCTP was very brief in both cell lines (half-life <1 hr), most likely due to catabolism of AraCTP and, perhaps to a lesser extent, continued incorporation into DNA.

Over the 3-log concentration range studied, there was a close correlation between AraCTP formation and AraC DNA incorporation in both cell lines. In contrast to AraCTP retention, AraC DNA incorporation appeared to be relatively stable. Impaired clonogenic survival after a 24-hr drug exposure was proportional to increasing AraC DNA content. Marked interference with nascent DNA chain elongation (1–3-hr terminal [^{14}C]dThd pulses) was observed during drug exposure, and the accumulation of lower-molecular-weight DNA species eluting with pH steps ≤11.5 increased as the AraC concentration × time increased. The magnitude of these effects during drug exposure were similar in both cell lines and did not correlate with ultimate clonogenic survival. Because AraCTP levels with 1 and 10 μM AraC were comparable to or greatly exceeded the endogenous dCTP pool, it is likely that direct competition of AraCTP with dCTP for incorporation into DNA and inhibition of DNA polymerases-α and -δ by incorporated AraC DNA residues contribute to the profound

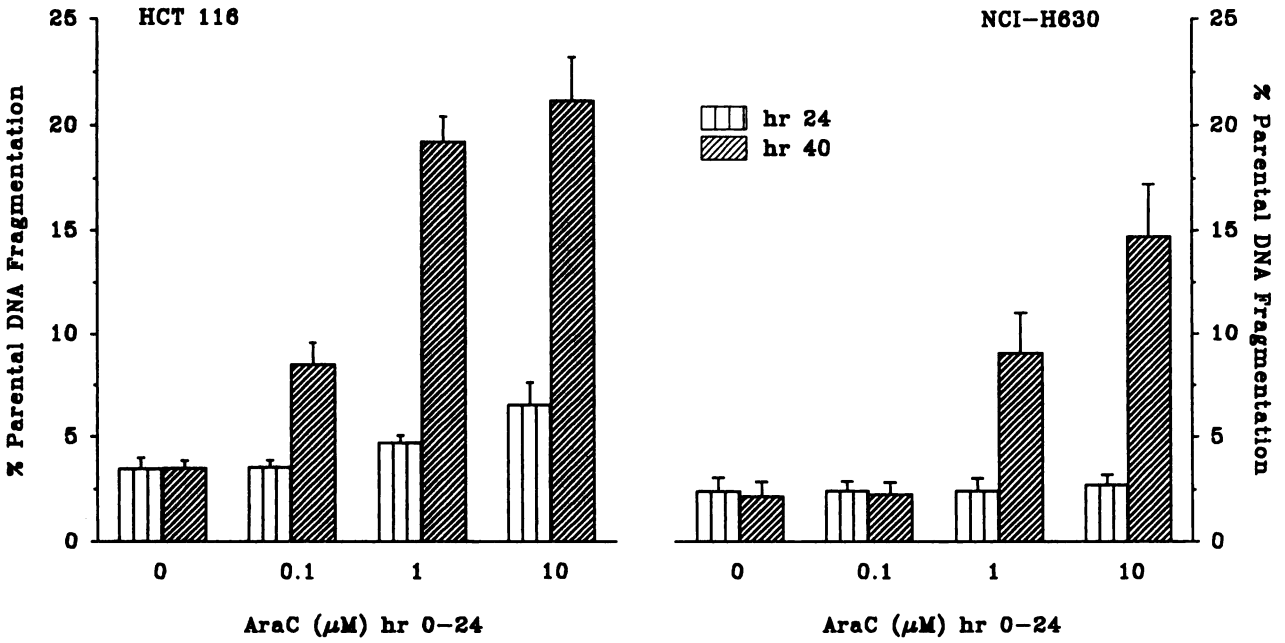

Fig. 4. Induction of parental DNA double-strand fragmentation by AraC. Exponentially growing cells were prelabeled with [^{14}C]dThd for 24–48 hr. After a 6-hr chase period, the cells were exposed to the indicated concentrations of AraC for 24 hr. The cells were either harvested immediately for the filter binding assay or washed twice and incubated in drug-free medium for an additional 16 hr. Adherent and nonadherent cells were collected and lysed under nondeproteinizing, non-DNA-denaturing conditions on Gelman Metricel membranes as described in Experimental Procedures. DNA fragmentation was determined as the percentage of [^{14}C]DNA in the lysis fraction divided by total cellular [^{14}C]DNA. The data, shown as the mean ± standard error from 4 (24 hr) or 5 (40 hr) separate experiments, are expressed as the percentage of fragmented DNA as a function of AraC concentration. The differences in drug-treated cells compared with control were significant for the following conditions: HCT 116, 10 μM × 24 hr, $p = 0.021$; 40 hr, $p \leq 0.006$ for 0.1, 1, and 10 μM AraC; H630 cells, 40 hr, $p \leq 0.008$ for 1 and 10 μM AraC (paired t test).

hindrance of nascent DNA chain elongation. Although incorporated AraC residues function as relative chain terminators, with extended incubation, deoxynucleotides can be added to the 3′-terminal AraCMP residue (4, 6–13, 18, 20). DNA digests have shown that AraCMP may be detected in an internucleotide position, suggesting that longer periods of incubation permit extension of the DNA chain past the fraudulent nucleotide (6, 7, 18, 20).

With fixed pH alkaline elution, however, HCT 116 cells appeared to be more sensitive to AraC-mediated nascent DNA damage. The methodology for fixed pH elution of newly synthesized DNA differed in several respects from that for pH step alkaline elution. The period of [^{14}C]dThd labeling was much longer (8 hr), the DNA was exposed to a sufficiently alkaline buffer (pH 12.1) such that the DNA was single stranded, the elution rate was 2-fold slower, and the duration of elution was longer (15 hr versus 4 hr). The biphasic elution profile is believed to reflect two events: elution of DNA over the initial several hours represents preexisting shorter DNA strands as a consequence of drug exposure. With extended elution, two phenomenon may occur. First, larger but prematurely terminated DNA species may have sufficient time to penetrate the filter, and second, continued exposure to the alkaline elution buffer may induce breaks in abnormal DNA (alkaline-labile sites). Increased DNA fragility is a consequence of drug exposure to many agents that directly incorporate into or intercalate with DNA and to drugs that have secondary effects on DNA (e.g., through effects on nucleotide pools, or the content and activity of DNA repair machinery) (40, 41, 46). In HCT 116 and H630 colon cancer cells, the effects of drug exposure on nascent DNA integrity were proportional to ultimate lethality in clonogenic assays. At each AraC concentration, a smaller proportion of nascent DNA was retained on the filter in HCT 116 cells than in H630 cells, reflecting greater DNA damage. At concentrations associated with ≥80% decrease in clonogenic capacity (≥1 μM in HCT 116 cells and 10 μM in H630 cells), ≥80% of the total nascent DNA eluted over the 15-hr period.

Net DNA synthesis was strongly inhibited during AraC exposure, even with 0.1 μM AraC, in both cell lines, and the magnitude of inhibition did not predict ultimate lethality. In contrast, inhibition of DNA synthesis at 48 hr (24 hr after drug removal, when essentially no free AraCTP was present) was interrelated to both the extent of AraC DNA incorporation and the damage to nascent DNA integrity at 24 hr. H630 cells had a significantly greater ability to recover DNA synthetic capacity after drug removal, whereas persistent inhibition of DNA synthesis was observed in HCT 116 cells. These data imply that the duration of DNA synthetic inhibition may be a more accurate determinant of clonogenic survival than inhibition during drug exposure.

AraC exposure was associated with damage to parental (high-molecular-weight) DNA. Induction of single-strand breaks in parental DNA was evident after a 24-hr exposure, although the effects were not as striking as that observed for nascent DNA. In contrast, induction of double-strand breaks in parental DNA was delayed: although minimal or no double-strand DNA fragmentation was evident after 24 hr, it was detected 16 hr after drug removal. Thus, the results of the present study are consistent with previous reports suggesting that effects of AraC on parental DNA occur subsequent to induction of nascent DNA damage (17, 47). Because such double-strand parental DNA fragmentation cannot be

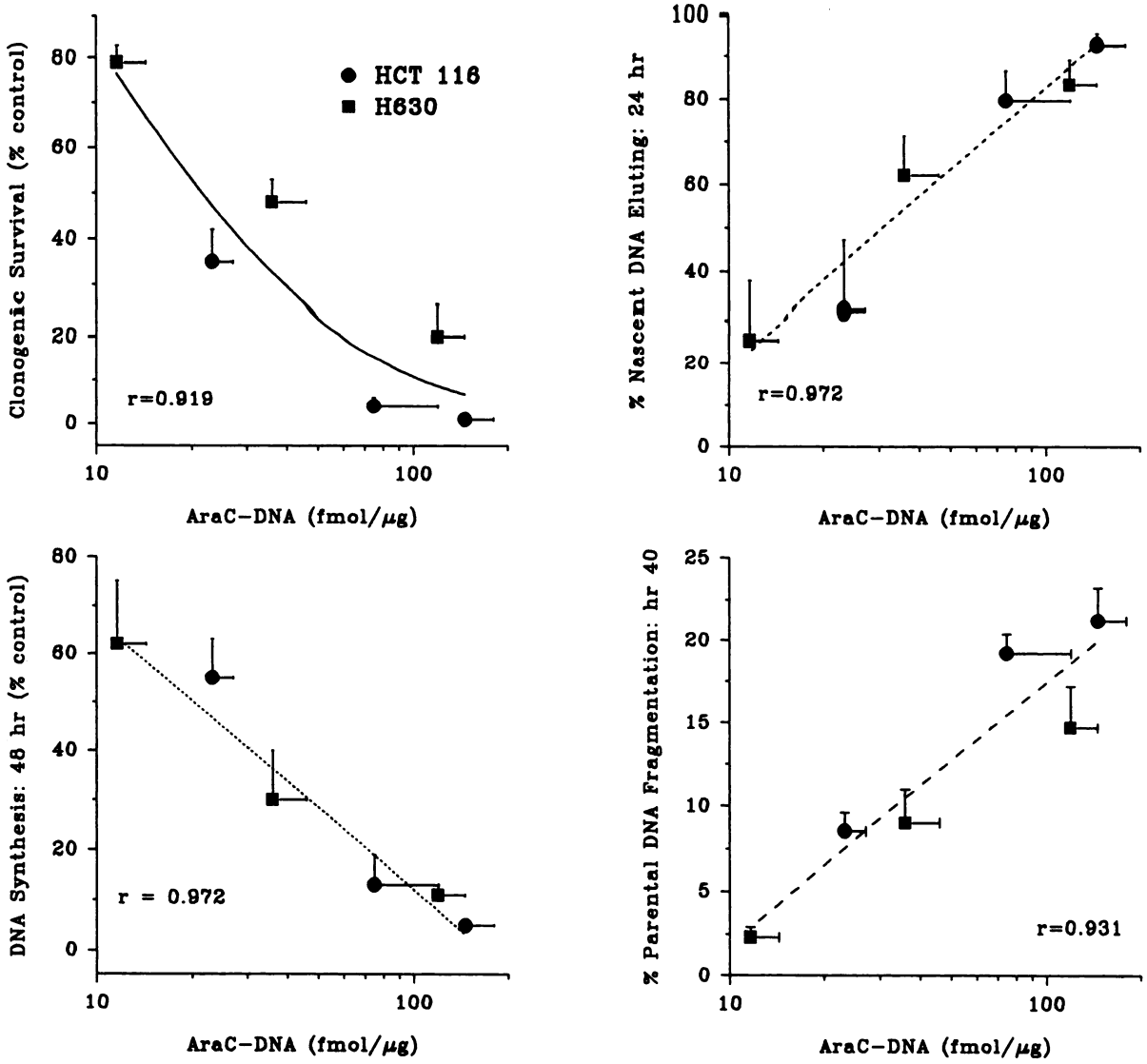

Fig. 5. Correlations between AraC DNA content and cytotoxic end points. For all four panels, AraC DNA content at 24 hr (mean ± standard error) is plotted on the *x*-axis. *Top left*, *y*-axis clonogenic survival (mean ± standard error) after a 24-hr AraC exposure. *Top right*, *y*-axis nascent DNA damage at 24 hr (mean ± standard error) (absolute percentage of total DNA eluting during pH 12.1 elution). *Bottom left*, inhibition of DNA synthesis (mean ± standard error) at 48 hr is depicted on the *y*-axis. *Bottom right*, induction of double-strand fragmentation in parental DNA at 40 hr is shown on the *y*-axis ().

repaired, a good correlation was evident between induction of double-strand DNA fragmentation and lethality.

In leukemic cell lines, induction of programmed cell death has been noted during the initial 24 hr of AraC exposure (22). The morphological features of apoptosis include condensation of the nucleus and a decrease in cell volume. In addition, extensive DNA damage may be accompanied by ATP depletion (believed to be secondary to increased poly(ADP-ribosyl)ation (48, 49). The cell volume in the two colon cancer cell lines increased for at least 24 hr after drug removal, and ATP pools were higher than control, contrary to that expected in cells undergoing apoptosis. Our results suggest that the time course and pattern of parental DNA double-strand fragmentation in these two human colon cancer cells appear to be distinct from those associated with exposure of HL60 cells to camptothecin. Exposure of epithelial cancer cell lines to DNA-damaging agents, such as fluorodeoxyuridine, has been reported to produce high-molecular-weight DNA fragmentation, ranging from 50–300 kb to 5 Mb as detected by pulsed field gel electrophoresis in the absence of nucleosomal laddering (multiples of 180–200 bp) (44, 50). Additional studies will be required to further characterize the pattern of DNA fragmentation in HCT 116 and H630 cells. Human colon cancers have been reported to have a high incidence of mutations in the *p53* gene (51). Such cells appear to have an impaired ability to recognize and/or to respond to extensive DNA damage and may have a diminished capacity to induce programmed cell death. Overexpression of *Bcl-2* antagonizes *p53*-mediated induction of apoptosis, and the ratio of *Bcl-2* to *Bax*, a closely related protein, influences whether a genotoxic stress invokes programmed cell death (52, 53). Clarification of the molecular phenotype of HCT 116 and H630 cells with respect to *p53* and *Bcl-2* will be the subject of future studies. In addition, microsatellite instability and aberrant nucleotide mismatch-DNA repair mechanisms have been identified in hereditary nonpolyposis colorectal cancer and in a subset of sporadic colorectal cancers (54); such cell lines are insensitive to alkylating agents. Of note, it has been reported

that the HCT 116 cell line expresses microsatellite instability (55); similar information concerning the status of the H630 cell line should prove to be informative.

Dose-intensification strategies have been promulgated as an approach to increasing tumor sensitivity. Unfortunately, high-dose approaches with antimetabolites are limited by unacceptable host toxicities due to widespread end-organ effects. An additional consideration for antimetabolites is the potential for saturation of intracellular metabolism. In the case of AraC, increasing the extracellular concentration above 10 μM may be fruitless, since higher concentrations exceed the ability of the cell to phosphorylate the drug (56). If our hypothesis that the duration of DNA synthetic inhibition is an important determinant of ultimate lethality in these colon cancer cells, one might predict that increasing the duration of AraC exposure would result in increased cytotoxicity at lower concentrations. Preliminary data from our laboratory suggest that this occurs. Extending the AraC exposure to 96 hr increased its cytotoxicity: in HCT 116 cells, 0.1 μM reduced clonogenic capacity to zero; in H630 cells, 1 μM AraC was 100% lethal, and 0.1 μM produced >60% lethality (data not shown). Additional studies are under way to determine the extent of DNA damage and inhibition of DNA synthesis after prolonged exposure to submicromolar concentrations of AraC in these cell lines. The clinical challenge will be to develop therapeutic schedules that allow an effective assault on the cancer cell without producing unacceptable host toxicity.

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