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Inhibition of DNA Excision Repair in Human Cells by Arabinofuranosyl Cytosine: Effect on Normal and Xeroderma Pigmentosum Cells

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SUMMARY

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The antineoplastic agent arabinofuranosyl cytosine (ara-C) produces an inhibition of the pyrimidine dimer excision system of human DNA repair. Alkaline sucrose gradient analysis of DNA from normal human skin fibroblasts exposed to 20 J/m^2 of ultraviolet radiation (254 nm) shows an accumulation of DNA single-strand breaks when DNA repair is attempted in the presence of 10 μM ara-C. Cells from complementation groups of xeroderma pigmentosum that are defective in early steps of excision repair show reduced numbers of DNA single strand breaks/10^8 daltons when compared with normal cells. Cesium chloride gradient analysis of radioactive precurser uptake during repair replication indicates that ara-C causes a 6-56% reduction in the number of nucleotide bases inserted into the DNA at concentrations of 1 and 10 μM, respectively. These concentrations result in the substitution for deoxycytidine (dCyd) by ara-C of 40 and 100%, respectively, in repaired regions. Repair inhibition is reversed by 50% upon removal of ara-C and by >95% with the addition of 100 μM dCyd. Chromatography of digested DNA shows that incorporated ara-C is not removed during dCyd reversal, suggesting that ara-C incorporation per se does not play a significant role in the inhibition of repair synthesis. The repair inhibition observed here is dependent on 2 mM hydroxyurea, presumably due to reduction in the intracellular pool of dCyd. The overall results suggest the possibility that ara-C is a weak competitive inhibitor of DNA polymerases associated with ultraviolet-induced excision repair.

INTRODUCTION

Ara-C,[1] used in the treatment of some forms of leukemia (1, 2), is a strong inhibitor of DNA replication in bacterial and mammalian cell systems (3-8). Ara-CTP is a competitive inhibitor of DNA polymerase II in *E. coli* (9) and inhibits DNA polymerase in mouse L-cells (10). Other studies have indicated that ara-C is incorporated into newly synthesized DNA (10-13), suggesting that the compound might act as a chain terminator as a result of configura-

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[1] The abbreviations used are: ara-C, arabinofuranosyl cytosine; ara-CTP, arabinofuranosyl cytosine triphosphate; UV, ultraviolet; XP, xeroderma pigmentosum; dThd, thymidine; dCyd, deoxycytidine; BrdUrd, bromodeoxyuridine; FdUrd, fluorodeoxyuridine; dGuo, deoxyguanosine; dAde, deoxyadenosine.

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tional distortion of the growing DNA strand (11, 13).

Little work on the possible effect of ara-C on UV-type excision repair has been reported. Studies on various polymerase-deficient mutants in *E. coli* (14, 15) have given some evidence that ara-CTP causes a reduction in repair replication, but this inhibition was far less than for semiconservative replication and required relatively high concentrations of ara-CTP (>100 μM). Stenstrom *et al.* (16), in studies on the effect of ara-CTP on DNA replication and repair in isolated rat nuclei, showed that K_i for the compound was 0.5 μM for semiconservative replication and 0.5 mM for repair replication. The present article describes a hydroxyurea-dependent, reversible inhibition of DNA repair by ara-C in normal human skin cells and in cells derived from individuals with xeroderma pigmentosum, a genetic disorder characterized by deficient repair of DNA damaged by UV radiation (17, 18). The usefulness of ara-C inhibition as an assay for the characterization of excision repair capacities of cell lines is discussed, as is the mechanism of the inhibition.

METHODS

Cell lines and culturing methods. Normal human skin fibroblasts were obtained through Oak Ridge Associated Universities. XP cell lines were obtained through the American Type Culture Collection. Cells were grown in 60 mm plastic Petri dishes (Falcon Plastics) in Eagle's minimal essential medium containing 15% fetal calf serum. The medium for all XP cell lines also contained nonessential amino acids (Gibco). Prior to experimental treatments, cells were grown at 37° in humidity- and CO_2-controlled incubators at pH 7.3 for 48 hr from an initial inoculum of 7.5×10^4 cells. During radioactive labeling and all experimental treatments, cells were maintained in Eagle's MEM containing 10% calf serum (E-90/cs).

Alkaline sucrose gradient analysis. Cell cultures grown for 48 hr were then labeled for 24 hr with 4.0 μCi/ml of [^{3}H]dThd (sp act 3 Ci/mmole; Schwarz/Mann) or 0.5 μCi/ml of [^{14}C]dThd (sp act 500 mCi/mmole; Amersham/Searle). The label was removed, and the cells were incubated for 2 hr in E-90/cs medium to deplete the radioactive pools. Cultures prelabeled with [^{3}H]dThd were exposed to 254 nm radiation from a germicidal lamp at an incident dose of 5–20 J/m^2 and incubated for 2–20 hr in medium containing 2 mM hydroxyurea and different concentrations of ara-C (Calbiochem). Some cultures received a chase of 100 μM dCyd (Calbiochem) following exposure to ara-C. Cells prelabeled with [^{14}C]-dThd served as unirradiated controls with hydroxyurea and ara-C. The control and experimental cells were suspended together in cold saline − 0.12% ethylenediaminetetraacetic acid, and 50 μl (about 1×10^4 cells) were lysed for 1 hr at room temperature in 200 μl of 1 N NaOH overlayed on 5–20%, 4 ml alkaline sucrose gradients containing 2 M NaCl. The gradients were centrifuged in a Beckman SW-56 rotor for 155 min at 3×10^4 rpm. Eight-drop fractions were collected on filter paper strips, which were given a 1-hr wash in 5% trichloroacetic acid and two 30-min washes in 95% ethanol. Radioactivity in acid-insoluble material was measured in a Packard liquid scintillation spectrometer. Molecular weight data were analyzed by means of a Digital PDP-11 computer system.

Density gradient centrifugation. Cells were treated by the method of Cleaver *et al.* (19) to separate semiconservatively-labeled DNA from repair-labeled DNA. Cells were suspended in 2.0 ml of saline-citrate buffer (10 mM Tris, 10 mM ethylenediaminetetraacetic acid, 150 mM NaCl, and 5 mM sodium citrate) pH 7.4, and 1.0% sarkosyl was added to a final concentration of 0.1%. The lysate was incubated for 1 hr at 37° with RNase (100 μg/ml) and for an additional hour with proteinase K (100 μg/ml), then shaken with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed against saline-citrate buffer for 24 hr at 4°, diluted to 5 ml with saline-citrate, and mixed with 6.426 g of CsCl.

Centrifugation was performed in a Beckman Model L ultracentrifuge with either a type 40 or type 50 Ti fixed-angle rotor at 3.4×10^4 rpm for 60 hr. Twenty-drop frac-

tions were collected, made 1 N with NaOH, and incubated at 37° for 1 hr; a 2× volume of 10% trichloroacetic acid was added to precipitate acid-insoluble DNA. Each fraction was filtered onto 2.3 cm filter paper discs (Whatman 3MM), then washed twice in 95% ethanol. Radioactivity in each sample was measured as described previously.

Identification of incorporated ara-C. Cells labeled with [^{3}H]ara-C during repair were centrifuged and washed twice in ice-cold phosphate-buffered saline. The pellet was extracted three times with ice-cold 0.2 N $HClO_4$, and the acid-insoluble pellet was washed twice with 0.2 N $HClO_4$. The pellet was dissolved in 0.5 N NaOH (0.5 ml), incubated for 36 hr at 37° (RNA hydrolysis), and acidified by the addition of 150 μl of 6 N $HClO_4$. The resulting precipitate was removed by centrifugation and redissolved in 0.5 ml of 4 mM Tris buffer (pH 8.6) containing 2 mM $CaCl_2$. The DNA was digested to the nucleoside level by successive incubations with micrococcal nuclease, spleen phosphodiesterase, and alkaline phosphatase (Worthington) by the method of Josse *et al.* (20). To the hydrolysate were added 50 μg each of deoxynucleoside and ara-C standards (see Figs. 2 and 5). Samples were chromatographed on Whatman 3MM paper for 72 hr with 1-butanol:5% sodium tetraborate in water (86:14) to identify incorporated radioactivity with ara-C (21). Chromatograms were cut into 1-cm strips and counted in a Searle Analytic 92 scintillation counter with a dioxane:Omnifluor (New England Nuclear) counting solution.

RESULTS

Effect of ara-C on UV-induced repair in normal and XP human cell lines.

Ara-C (10 μM) in the presence of 2 mM hydroxyurea was found to inhibit UV-induced DNA repair in normal human cells by preventing closure in incised, dimer-containing regions following 20 J/m^2 of UV radiation (Table 1). Alkaline sucrose gradient profiles demonstrated weight-average molecular weights smaller (24.33×10^6 daltons) than those of control cells (240.37×10^6 daltons). The number of incised regions equals the number of single-strand breaks/10^8 daltons (7.39) and correlates well with BrdUrd photolysis data for excision repair routinely performed in this laboratory (22). The observed repair inhibition was dependent on hydroxyurea. Experiments performed as above without hydroxyurea resulted in the appearance of only 0.30 strand break/10^8 daltons or 4.1% of the inhibition observed with hydroxyurea.

In cells from excision-defective XP complementation groups, decreases in the number of strand breaks were observed (0.22, 1.09, 0.55, 0.58, and 4.57, respectively). In SGL, an XP cell line as yet uncharacterized as to a complementation group, the number of strand breaks (0.40) indicated highly defective excision of dimers similar to a group A cell line. The XP-variant cell lines, which exhibit normal dimer excision and repair replication (23), produced a greater number of strand breaks (11.92) than normal fibroblasts. In addition, the XP-variant showed signs of cytotoxicity in the irradiated cultures (granular appearance and some floating cells).

Effect of ara-C on repair synthesis in irradiated cells. Ara-C (10 μM) caused a significant reduction in repair synthesis in cells exposed to 20 J/m^2 of UV radiation and allowed to repair for 18–20 hr (Fig. 1A). In a typical experiment, the number of pmoles of [^{3}H]BrdUrd incorporated per cell during repair, measured on CsCl gradients (fractions 12–15), was reduced from a control (no ara-C) value of 2.89×10^{-5} pmole to 2.74×10^{-5} pmole (1 μM ara-C) and 1.27×10^{-5} pmole (10 μM ara-C). Incorporation of [^{3}H]ara-C was demonstrated (Fig. 1B, fractions 13–16) during the repair period. In cells exposed to 1 μM [^{3}H]ara-C, 0.74×10^{-5} pmole/cell was incorporated, compared with 1.07×10^{-5} pmole/cell with 10 μM [^{3}H]ara-C. Chromatography of hydrolyzed DNA demonstrated that incorporated radioactivity was due to [^{3}H]ara-C (>90% of total counts) and not to dCyd or dThd derived from uracil following metabolic deamination of [^{3}H]ara-C (Fig. 2).

The relation of ara-C incorporation to inhibition of repair synthesis and resulting strand break formation is given in Fig. 3. The total number of bases inserted/10^8 daltons in cells exposed to 1 and 10 μM ara-C

TABLE 1

Measurement of DNA repair by means of inhibition by ara-C in normal and XP cells following UV irradiation

Cell cultures grown for 48 hr were then labeled for 24 hr in E-90/cs containing [^{3}H]dThd or [^{14}C]dThd. The label was removed, and cultures were incubated in unlabeled medium for 2 hr. Cells were exposed to 20 J/m^2 of 254-nm radiation and allowed to undergo repair for 18–20 hr in medium containing 2 mM hydroxyurea and 10 μM ara-C. Cultures labeled with [^{14}C]dThd served as unirradiated controls. Control and experimental cultures were suspended together in saline – 0.12% ethylenediaminetetraacetic acid, and the cellular DNA was analyzed on alkaline sucrose gradients (see METHODS).

Cell line	Description	XP complementation group	DNA weight average molecular weight (M_w) × 10^6 daltons		DNA single-strand breaks/10^8 daltons[a]	Percent of normal incision
			without UV	with UV		
HSBP	Normal	—	240.37	24.33	7.39	100.00
	human skin	—	259.81[b]	187.75[b]	0.30[b]	—
	fibroblasts	—	272.48[c]	254.56[c]	0.05[c]	—
SGL	XP	?	252.65	167.91	0.40	5.41
CRL 1223 (Jay Tim)	XP	A	293.77	221.33	0.22	2.98
CRL 1199 (PoCo)	XP	B	281.27	110.93	1.09	14.75
CRL 1158 (PeAr)	XP	C	310.16	166.87	0.55	7.44
CRL 1157 (CayWen)	XP	D	303.59	161.63	0.58	7.85
CRL 1159 (XP-2)	XP	E	264.86	37.54	4.57	61.84
CRL 1258 (PeHay)	XP	XP-variant	221.87	15.60	11.92	161.30

[a] Number of DNA strand breaks = $[2(1/M_{w(\text{with UV})} - 1/M_{w(\text{without UV})})]$.

[b] Cells received no hydroxyurea during repair.

[c] Cells received no ara-C during repair.

was calculated to be about 1350 and 640 bases, respectively, compared with a control value of about 1450. The number of ara-C bases incorporated at the above concentrations was about 100 and 150, respectively. The amount of substitution by ara-C for dCyd based on these calculations is 37.9 and >100% (dCyd-based nucleotides = 19.8% of total nucleotides). The number of DNA strand breaks observed with a 20 J/m^2 UV dose remained constant at ara-C concentrations of 10 μM and higher.

Effect of UV dose and dCyd on ara-C repair inhibition. The number of strand breaks observed in normal cells was also dependent on the UV dose. Irradiated cells given a 2-hr repair period while exposed to 10 μM ara-C showed maximum strand break accumulation at a UV dose of about 10 J/m^2 (Fig. 4). Repair inhibition was reversed following removal of ara-C. For UV doses up to 5 J/m^2 the number of strand breaks was reduced by 55% in 3 hr and by 73% in 15 hr. The amount of reversal decreased with doses of 10 J/m^2 (48 and 54%) and 20 J/m^2 (25 and 42%) in 3 and 15 hr, respectively. If ara-C was removed after 2 hr and cells were incubated in medium containing 100 μM dCyd, the inhibition was reversed at all UV doses by 85% in 3 hr and by >95% in 15 hr.

A chromatographic assay of [^{3}H]ara-C incorporated in 2 hr following exposure of cells to 20 J/m^2 of UV radiation (Fig. 5A) showed that [^{3}H]ara-C was still present in the DNA following a 15-hr chase with 100 μM dCyd (Fig. 5B).

DISCUSSION

Inhibition of repair replication by ara-C following the excision of pyrimidine dimers as demonstrated in normal and XP human cell lines may provide a useful assay of excision capacities in mammalian cells. The

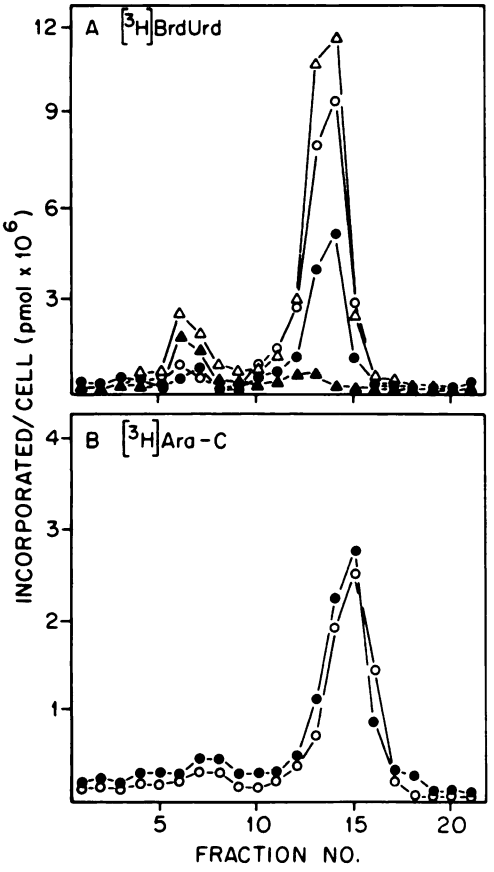

FIG. 1. *Cesium chloride density sedimentation analysis of the effect of ara-C on repair replication in normal human skin fibroblasts*

Cells were incubated for 2 hr in the presence of 10 μM BrdUrd and 1 μM FdUrd, then given a UV dose of 20 J/m². Repair was allowed to take place for 19–20 hr in the presence of 2 mM hydroxyurea, 1 μM FdUrd, and either (A) 12.5 μCi/ml [³H]BrdUrd (20 Ci/mmole) with or without ara-C or (B) 10 μM BrdUrd and [³H] ara-C. The DNA was extracted and analyzed on CsCl gradients as described by Cleaver (19). (A) ▲, no UV; △, UV without ara-C; ○, UV 1 μM ara-C; ●, UV 10 μM ara-C. (B) ○, UV 1 μM [³H]ara-C (15 Ci/mmole); ●, UV 10 μM [³H]ara-C (3 Ci/mmole).

results observed here correlate well with previously reported excision repair capacities in normal human cells (22), excision-deficient XP-groups (17, 24), and XP-variant cell lines (23). This correlation in cells with known repair capacities strongly suggests that the number of DNA single-strand breaks observed is truly a function of excision repair capacity and is not due to different sensitivities of the cell lines to hydroxyurea or ara-C. Preliminary studies indicate that ara-C inhibition is also a useful tool for detecting excision of lesions in DNA damaged by certain carcinogenic compounds. In DNA damaged by benzo(*a*)pyrene diol-epoxides which is repaired by UV-type excision repair in normal cells but not in XP group A (25), an effect similar to that of UV radiation is seen when cells are exposed to ara-C during repair (data not shown).

As with semiconservative replication, the actual inhibitory mechanism of ara-C on excision repair remains unclear (1). The data presented in this study suggest that inhibition occurs immediately following the initial (incision) step of repair. Single-strand breaks in the DNA are observed in as little as two hours after UV-irradiation

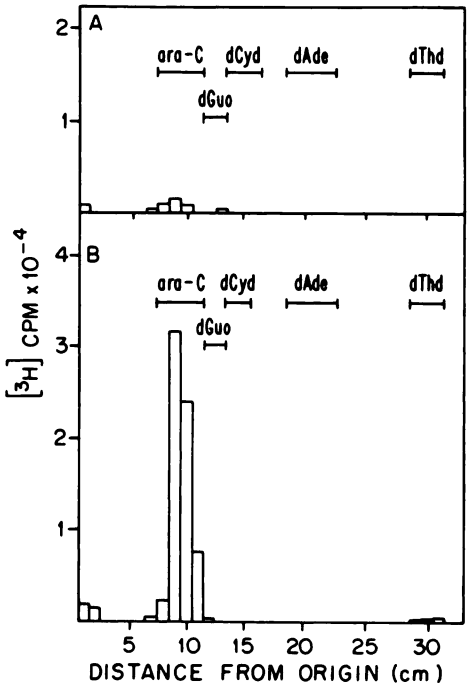

FIG. 2. *Chromatographic identification of [³H]-ara-C in the DNA of normal human skin fibroblasts*

Confluent cultures of normal human fibroblasts were exposed to 20 J/m² of 254 nm radiation followed by an 18–20 hr repair period in E-90/cs medium containing 2 mM hydroxyurea and 2 μM [³H]ara-C (sp act 24 Ci/mmole). The DNA was extracted, subjected to enzymatic hydrolysis, and chromatographed on Whatman 3MM paper as previously described. (A) Without UV, (B) with UV.

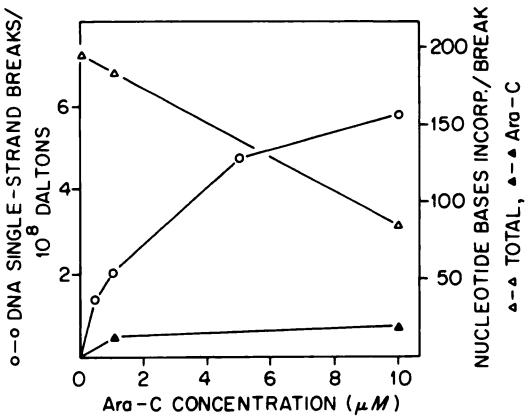

FIG. 3. *Relationship of repair synthesis and ara-C incorporation to the accumulation of DNA strand breaks in normal human skin fibroblasts*

Cells labeled overnight with [^{3}H]dThd were exposed to 20 J/m^2 of 254 nm radiation and allowed to undergo repair for 18–20 hr in the presence of 2 mM hydroxyurea and different concentrations of ara-C. Sucrose gradient analysis was performed as previously described. M_w of DNA repaired in the presence of ara-C were compared with those for unirradiated controls. Values are given as the number of DNA single-strand breaks/10^8 daltons for a given ara-C concentration (○). The total number of incorporated nucleotides/10^8 daltons (△) was calculated from the pmoles of [^{3}H]BrdUrd incorporated per cell (Fig. 2) assuming 100% substitution for dThd. Substitution of ara-C for dCyd (see RESULTS) was calculated from the number of [^{3}H]ara-C bases incorporated/10^8 daltons (▲) compared with the total bases incorporated during repair.

and accumulate as incision continues in dimer containing regions. Maximum strand break accumulation has occurred in 18–20 hr.

Few additional studies have been done on the effect of ara-C on DNA repair. Inhibition has been observed in bacterial repair systems by use of high ara-C concentrations (0.15 to 2 mM) in *E. coli* mutants deficient in specific DNA polymerases (14, 15). In isolated rat hepatocytes, 1 mM ara-C caused inhibition of DNA repair synthesis with kinetics suggesting noncompetitive inhibition (16). However, irreversible inhibition of semiconservative synthesis occurred as well. In addition, several other effects on mammalian cells have been observed at this concentration, including cytotoxicity, inhibition of RNA synthesis, and damage to membrane transport systems (26, 27).

The incorporation of ara-C into repaired DNA as a major inhibitory mechanism is not supported by this study. However, it cannot be completely ruled out, as up to 5% of the DNA strand breaks remain following a 15-hr chase with dCyd. It is imporant to note that incorporated ara-C does not appear to be removed during the dCyd chase (28, 29), even though repair inhibition is reversed by >95%. Additionally, a comparison of the amount of [^{3}H]ara-C incorporated during 3 and 15 hr of repair indicates that chain elongation continues slowly as the amount of labeled ara-C is significantly increased between the two time points.

The requirement for hydroxyurea in the system, which lowers the deoxynucleotide pools by inhibition of ribonucleotide reductase (30, 31), further suggests that at best

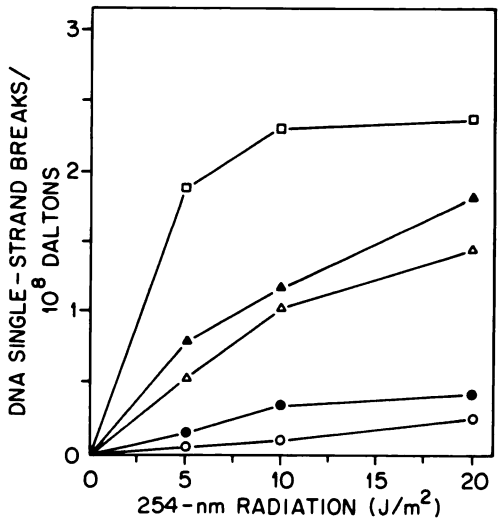

FIG. 4. *Effect of dCyd on DNA repair inhibition by ara-C in UV-irradiated human skin fibroblasts*

Cells labeled with [^{3}H]dThd were exposed to different doses of 254 nm radiation followed by a 2 hr repair period in the presence of 2 mM hydroxyurea and 10 μM ara-C. The ara-C was removed, and repair was allowed to continue for either 3 or 15 hr in E-90/cs medium with hydroxyurea with or without 100 μM dCyd. M_w's of DNA were analyzed on alkaline sucrose gradients. Experimental molecular weight values were compared with those of unirradiated control cells labeled with [^{14}C]dThd. □, ara-C for 2 hr; ▲, ara-C + 3 hr in E-90/cs; △, ara-C + 15 hr in E-90/cs; ●, ara-C + 3 hr in E-90/cs with dCyd; ○, ara-C + 15 hr in E-90/cs with dCyd.

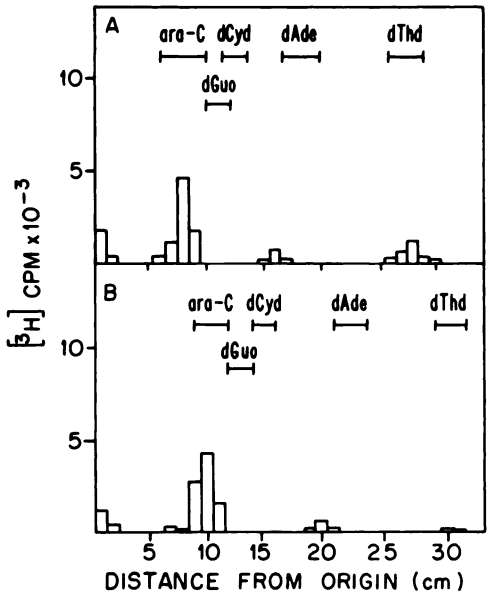

FIG. 5. *Effect of dCyd reversal on incorporated [^{3}H]ara-C in the DNA of normal human skin fibroblasts*

Cultures were exposed to 20 J/m^2 of 254 nm radiation followed by a 2 hr repair period in medium with 2 mM hydroxyurea and 2 μM [^{3}H]ara-C. In addition, some cultures received a 15 hr chase with medium which contained dCyd. DNA isolation, hydrolysis, and chromatography were performed as previously described. DNA for an experiment was pooled from monolayers on three 100 mm tissue culture dishes. Radioactivity detected between dCyd and a dAde is due to free-base cytosine. Radioactivity at the origin (samples 1 and 2) is due to oligonucleotides resulting from incomplete hydrolysis of the DNA. (A) Without dCyd chase; (B) with dCyd chase.

ara-C (or ara-CTP) is a poor substrate for polymerases associated with repair replication and is likely to be a weak competitive inhibitor of repair synthesis at noncytotoxic doses.

Johnson and Collins (32) reported the qualitative appearance of single-strand breaks in HeLa cell DNA following UV radiation and repair in the presence of ara-C, and its reversal by dCyd. The findings suggested inhibition of repair polymerase(s), although no repair replication studies were performed. Also, no evidence was given for ara-C incorporation into the DNA. The accumulation of strand breaks was not dependent on hydroxyurea as we have described here. This discrepancy suggests differences in repair capabilities and DNA precursor metabolism in different cell types (e.g., normal vs. malignant) and further points out the complexity of DNA repair systems.

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