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INCORPORATION OF ARABINOSYL CYTOSINE INTO 2-7S RIBONUCLEIC ACID AND CELL DEATH*

MING-YU CHU

Division of Biological and Medical Sciences, Brown University, Providence, R.I., and Department of Medicine, Roger Williams General Hospital, Providence, R.I., U.S.A.

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Abstract—Murine leukemic cells, L5178Y, exposed to arabinosyl-cytosine (ara-C) for brief periods of time during exponential growth undergo exponential cell death and cannot be rescued by deoxycytidine. The increments of cell death correlate with a linear increase of incorporation of the analog into the ribonucleic acid (RNA). Incorporation into DNA ceased after 60 min in cells incubated with [^{3}H]ara-C ($3{\cdot}3 \times 10^{-5}$M, 750 μc/μmole) for 30–240 min (14–93 per cent cell kill). In addition, incorporation of the analog into DNA could also be reduced by pretreatment of the cells with FUdR and methotrexate, although cell kill was increased 4-fold. The incorporation of [^{3}H]ara-C was mostly in the 2-16S RNA when isolated by a sucrose gradient. The 2-16S RNA was fractionated on 5% polyacrylamide gels and the label appeared first in 1-2S RNA, then in the 2-4S RNA and then in heavier RNA. Incorporation of [^{3}H]uridine into 2-7S RNA was markedly decreased by pretreatment of the cells with ara-C; other RNA fractions were unaffected. It is suggested that ara-C incorporation in 2-7S RNA may result in acute cell death.

1-β-D-ARABINOFURANOSYL cytosine (ara-C) has been shown to inhibit the reproduction of a wide variety of organisms, such as bacteria,[1,2] mammalian cells in culture,[3,4] DNA viruses,[5,6] infectious virus,[7] and transplantable tumors in mice and in rats.[8] It is a useful agent in the management of human leukemia[9,10] and for viral infections of the eye.[11,12]

It has been proposed that arabinosyl cytosine inhibits the formation of deoxyribosyl cytosine diphosphate from ribosyl cytosine diphosphate in whole cells.[3,13,14] In another study it was demonstrated that both deoxyribosyl cytosine and ara-C are phosphorylated by one enzyme,[15,16] deoxycytidine kinase. A third and unknown site of inhibition was indicated from studies in which we have shown that cells incubated for brief periods of time (1 hr, $3{\cdot}3 \times 10^{-5}$ M) with the drug underwent rapid cell death and that such treated cells could not be rescued by deoxycytidine,[17] although deoxycytidine was efficiently phosphorylated and the drug rapidly lost from the cell.[17,18] Arabinosyl cytosine triphosphate (Ara-CTP) was found to inhibit DNA polymerase,[19,20] but the relevance of this finding to cell death is uncertain, since we have shown that the DNA polymerase activity of L5178Y cells is rapidly restored after ara-C is removed, even though the cells are largely nonviable.[21]

[^{3}H]Arabinosyl cytosine is incorporated into both the DNA and RNA fractions of mammalian cells.[17,22,23] It has been shown in earlier studies that acute cell death produced by ara-C correlates with incorporation of the analog in the RNA fraction.[17] This

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report extends these findings and describes the distribution of [^{3}H]ara-C in 2–16S RNA of L5178Y cells under different conditions of incubation.

MATERIALS AND METHODS

The techniques for growing murine leukemic cells (L5178Y) in culture have been described.[24] Cells used in all experiments were in the exponential phase of growth and were maintained at 37° in growth medium throughout the experiment. Cell viability was determined by the dilute agar colony method in a medium containing deoxycytidine (5×10^{-5} M).[17] Arabinosyl cytosine was obtained through the courtesy of the Research Laboratories of the Upjohn Company. 5,6-[^{3}H]arabinosyl cytosine, purified by chromatography, and 5,6-[^{3}H]uridine were purchased from Schwarz Biochemicals. Polyacrylamide gels were purchased from Eastman Organic Chemicals.

(1) *Time course study of [^{3}H]arabinosyl cytosine.* L5178Y cells (2×10^7/ml, 20·0 ml) were exposed to [^{3}H]ara-C (750 μc/μmole, $3{\cdot}3 \times 10^{-5}$ M) during exponential growth and incubated for periods ranging from 30 to 240 min. After incubation, an aliquot was removed for the determination of total RNA and DNA,[3] and an aliquot for the determination of cell viability. The remaining cells were centrifuged at approximately 800 *g* for 15 min at 4°, washed once with 10·0 ml of cold medium, and stored at −20° overnight.

(2) *Extraction of ribonucleic acid fraction.* The cell pellet was resuspended in 20·0 ml of 0·01 M sodium acetate (NaAc), pH 5·1, containing 12 mg sodium EDTA, 1·0 ml of 2·6% bentonite and 60 mg sodium dodecyl sulfate (SDS). The cells were allowed to lyse for 15 min at 37°. An equal volume of phenol-saturated NaAc, pH 5·1, was added to the cell suspension and the material was shaken vigorously at room temperature for 20 min. The deproteinized suspension was centrifuged at 800 *g* for 15 min. Two volumes of cold 95% ethanol were added to the aqueous phase to precipitate the nucleic acids at −10° for 10 min. The precipitate was collected by centrifugation and washed twice with 2 vol. of cold 95% ethanol. The resulting pellet was redissolved in 1·0 ml of 0·01 M NaAc. $MgCl_2$ (1×10^{-3} M) and 50 μg DNase (RNase free) were added and incubated at 10° for 10 min. After incubation, the RNA was precipitated with 3 vol. of cold 95% ethanol at −10° for 10 min, and resuspended in 0·5 ml of 0·01 M NaAc.

(3) *Ribonucleic acid separation on sucrose gradient.* RNA separations on sucrose gradient were performed carefully by layering about 0·447 mg (14·0 O.D. units) of the sample in a volume of 0·4 ml on top of a 5–20% linear sucrose gradient and centrifuging at 30,000 rev./min in the SW 65K swinging bucket rotor of a Spinco Model L-2 (65) ultracentrifuge for 17 hr. Fractions of 0·08 ml were collected after the bottom of the centrifuge tube was punctured.

(4) *Separation of ribonucleic acid fractions on polyacrylamide gels.* A stock solution (1 l.) of pH 8·3 buffer containing 108 g of tris buffer, 9·3 g disodium-EDTA, and 55·0 g of boric acid was prepared. This buffer was diluted to 1:10 to fill the buffer reservoir in the electrophoresis cell and was used undiluted in the gel preparation. Five per cent gel (acrylamide and *N,N'* methylene bisacrylamide in a weight ratio of 19:1), 10·0 ml of 6·4% DMAPN (dimethylaminopropionitrile) in water, 16 ml of buffer and water to 150 ml, was prepared. The gel solution was warmed to 25°, ammonium persulfate (10·0 ml of 1·6% in water) was added, and the solution was poured into the electrophoretic cell. The eight-place slot former was put in place and

gelation was allowed to proceed for 30 min before removing the excess gel. The RNA samples were collected, appropriate fractions were pooled and concentrated, and then dissolved in 0·15 ml buffer solution containing 40% sucrose. An aliquot of the RNA sample containing approximately the same amount of radioactivity was applied into the slot of the vertical electrophoresis cell. Markers used were 16S RNA, 4S RNA and bromophenol blue. The electrophoresis was carried out at 150V for 4 hr.

(5) *Determination of radioactivity in gel fractions.* The gels were sectioned into strips of 12 mm wide and 2 mm long in the direction of electrophoresis. The strips were dissolved by autoclaving in 1·0 ml of 0·2 N NaOH. Samples were counted in a Nuclear-Chicago scintillation spectrometer; counting efficiencies were 16–20 per cent, quenching by the samples was corrected.

(6) [^3H]*Uridine uptake after pretreatment with arabinosyl cytosine.* Exponentially growing cells ($3·5 \times 10^5$/ml) were harvested from culture by centrifugation at 37° and diluted to a concentration of approximately $1·5 \times 10^7$ cells/ml in a total volume of 20·0 ml. Half of the cells were pretreated with ara-C ($3·3 \times 10^{-5}$ M) for 1 hr and the other half were used as control. [^{3}H]Uridine (2340 μc/μmole, $1·6 \times 10^{-6}$ M) was added to both the treated and control cells and incubated at 37° for 30 min. The treated and control cells were then harvested at 4° by centrifugation and washed once with cold medium. The cell pellets were extracted for RNA and separated on sucrose gradients and polyacrylamide gels as described in Methods 2, 3 and 4. In some experiments, ara-C ($3·3 \times 10^{-5}$ M) was added after the pulse label of [^{3}H]uridine (2340 μc/μmole, $1·6 \times 10^{-6}$ M) and aliquots were removed at 0, 20 and 60 min time intervals.

RESULTS AND DISCUSSION

L5178Y cells in the exponential phase of growth were exposed to ara-C under different conditions. The rate of cell kill determined by the agar colony method was 52 per cent/hr when ara-C was at a concentration of $3·3 \times 10^{-6}$ M in the intervals of 3–8 hr. When ara-C was at a much higher concentration ($3·3 \times 10^{-5}$ M), the rate of cell kill was also 52 per cent/hr from 1 to 6 hr (Fig. 1). The cell kill during those conditions could not be modified by deoxycytidine.[17] In contrast, cells exposed to ara-C ($3·3 \times 10^{-6}$ M) for 2 hr or ara-C ($3·3 \times 10^{-5}$ M) for 30 min were rescued by subsequent exposure to deoxycitidine. The shoulder was shortened from 2 hr to 30 min by increasing the ara-C level to $3·3 \times 10^{-5}$ M. The effect of this shoulder is presumably because of the deficiency of deoxyribosyl cytosine triphosphate (dCTP) induced by ara-C.

In order to determine if an incorporation of ara-C into nucleic acids could be correlated with irreversible inhibition of cell death, a time-course study was carried out. The data of Fig. 2 indicate that [^{3}H]ara-C at $3·3 \times 10^{-5}$ M was incorporated into RNA and the incorporation was linear and correlated with acute cell death (Fig. 1). At the same concentration incorporation of [^{3}H]ara-C into DNA was terminated after 1 hr of incubation, probably because of inhibition of DNA polymerase by ara-CTP accumulated in the cell, and did not correlate with cell death. However, the incorporation of [^{3}H]ara-C at a lower level, $3·3 \times 10^{-6}$ M, in both RNA and DNA fractions was linear with time. DNA polymerase was active, although cell death continued. In addition, at the lower level of ara-C ($3·3 \times 10^{-6}$ M), there was more ara-C in the DNA but less cell death. Therefore, incorporation in DNA was not

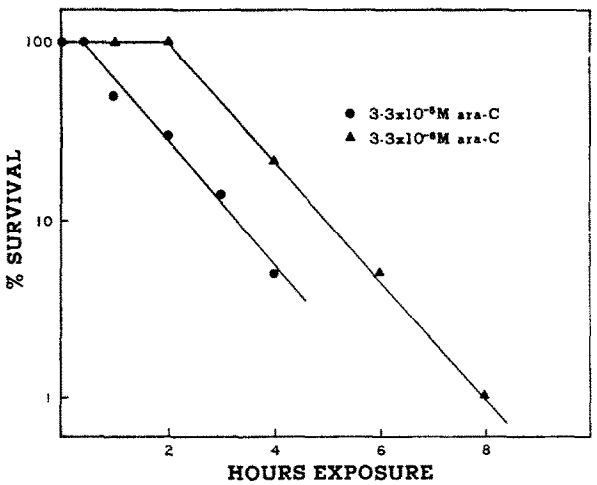

FIG. 1. Kinetics of cell death by the dilute agar colony method. L5178Y cells (approximately 1×10^4/ml) in the exponential phase of growth were exposed to arabinosyl cytosine ($3{\cdot}3 \times 10^{-6}$ M and $3{\cdot}3 \times 10^{-5}$ M) for different periods of time. The cells were removed from the drug and diluted in order to provide 60 colonies per tube (cloning efficiency of noninhibited control cells, approximately 75 per cent), which were treated as described in Methods and normalized to a cloning efficiency of 100 per cent.

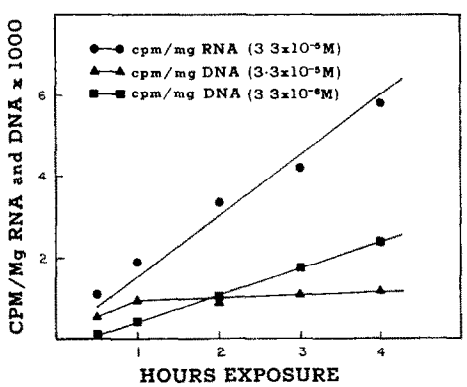

FIG. 2. Time course of [^{3}H]arabinosyl cytosine uptake into RNA and DNA. L5178Y cells (approximately 2×10^7/ml) in the exponential phase of growth were exposed to [^{3}H]ara-C (750 μc/μmole, $3{\cdot}3 \times 10^{-5}$ M). The RNA and DNA were prepared (Methods) from approximately $1{\cdot}0 \times 10^8$ cells at each of the periods of time indicated. The radioactivity was determined by liquid scintillation counting and the concentration of nucleic acids was determined by the absorbancy at 260 and 280 mμ.

chosen for further study at this time because not only did cell death not correlate with DNA incorporation when ara-C was at a higher concentration ($3{\cdot}3 \times 10^{-5}$ M) but also it was found that ara-C incorporation into DNA fraction could be reduced by pre-incubation of cells with methotrexate although much more cell death occurred. Instead, attention was focused on RNA, in which time-dependent events were uniformly correlated with cell death.

Analysis of time-course studies with labeled RNA fractionated on sucrose gradients (5–20%) shows that [^{3}H]ara-C was incorporated mostly into RNA in the 2–16S region, and that the amount incorporated increased during incubation with the analog.

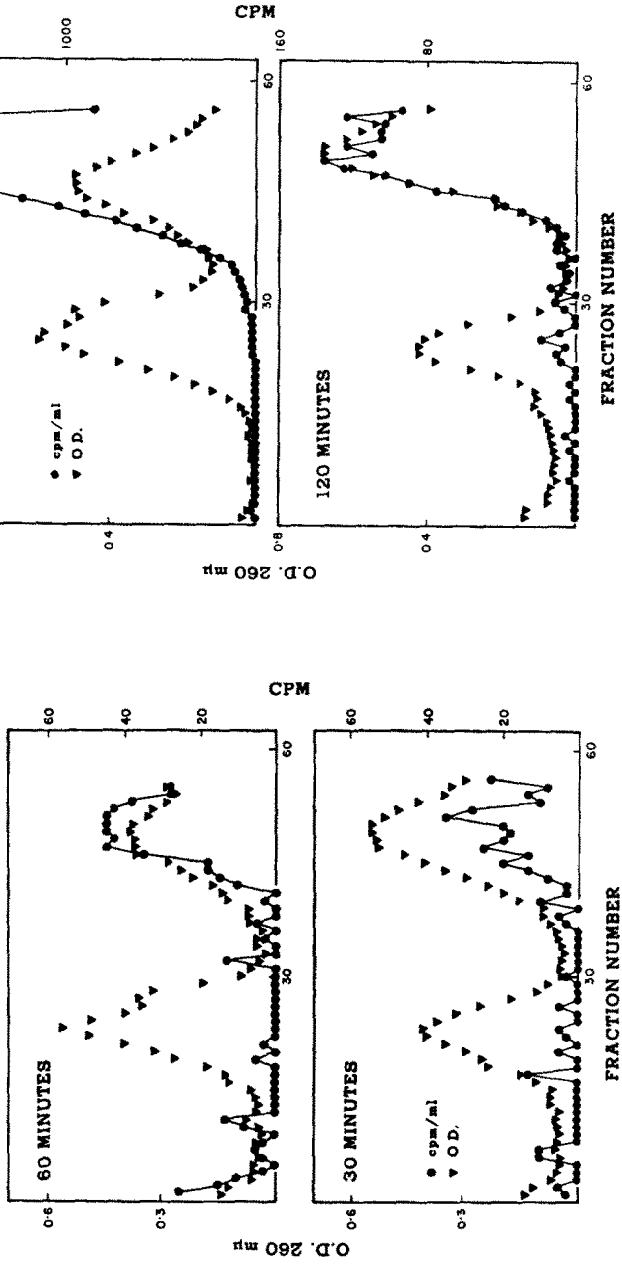

FIG. 3. Separation of [^{3}H]ara-C RNA on sucrose gradient. L5178Y cells in the exponential phase of growth were exposed to [^{3}H]ara-C (750 μc/μmole, $3{\cdot}3 \times 10^{-5}$ M). The RNA was prepared (Methods) from approximately $1{\cdot}0 \times 10^8$ cells at each of the periods of time indicated. 0·447 mg (14·0 O.D. units) of the sample in a volume of 0·4 ml was layered on top of a 5–20 per cent linear sucrose gradient and centrifuging at 30,000 rev./min in the SW 65K swinging bucket rotor of a Spinco Model L-2 (65) ultra centrifuge for 17 hr. Fractions of 0·08 ml were removed after the bottom of the centrifuge tube was punctured. The radioactivity was determined by liquid scintillation counting and the concentration of nucleic acids was determined by the absorbancy at 260 and 280 mμ.

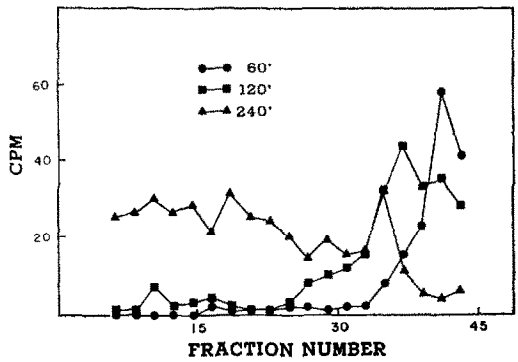

FIG. 4. Separation of RNA time course 2–16S [^{3}H]ara-C on polyacrylamide gels. L5178Y cells in the exponential phase of growth were exposed to [^{3}H]ara-C (750 μc/μmole, $3{\cdot}3 \times 10^{-5}$ M). The RNA was prepared (Methods) from approximately $1{\cdot}0 \times 10^8$ cells at each of the periods of time indicated. RNA of the 2–16S region from sucrose gradients was pooled and an aliquot (0·1 ml) was resolved on 5 per cent polyacrylamide gels.

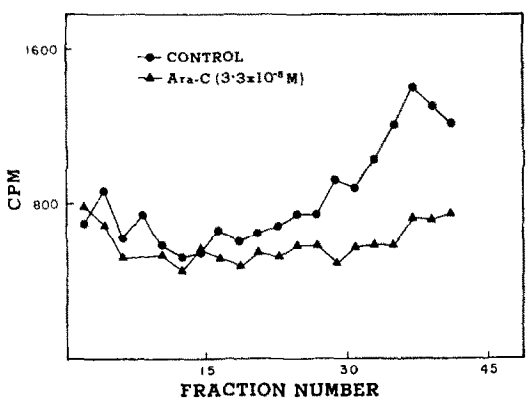

FIG. 5. [^{3}H]UR RNA uptake with ara-C pretreatment. L5178Y cells ($1{\cdot}5 \times 10^7$/ml, 20·0 ml) in the exponential phase of growth were pretreated with ara-C ($3{\cdot}3 \times 10^{-5}$ M) for 1 hr. [^{3}H]Uridine (2340 μc/μmole, $1{\cdot}6 \times 10^{-6}$ M) was added to both the treated and control cells and incubated at 37° for 30 min. The RNA fraction was prepared (Methods) from approximately $1{\cdot}0 \times 10^8$ cells. RNA of the 2–16S region from sucrose gradients was pooled and an aliquot (0·1 ml) was resolved on 5 per cent polyacrylamide gels.

A minor incorporation into heavier RNA was found (Fig. 3). Since incorporation into total RNA was correlated with cell death, RNA of the 2–16S region from sucrose gradients was pooled and resolved on 5% polyacrylamide gels. The time course results indicate that the analog after incorporation into 1–2S moves to 2–4S, and then into heavier RNA (Fig. 4). The increase of the size of RNA molecules may have resulted from chain extension. It is also possible that ara-C after incorporation prevents further synthesis of the low molecular weight RNA fraction. It is apparent that ara-C in the 2–16S RNA is related to the acute cell death which occurs after the first 30 min of incubation. Additional studies concerning this fraction of RNA have been completed. Thus it was found that the incorporation or retention of [^{3}H]uridine and [^{3}H]proline into macromolecules could not be affected by ara-C.* In contrast, in cells

* Unpublished data.

pretreated with ara-C, [^{3}H]UR incorporation into the lower molecular weight RNA (2–7S) was markedly decreased (Fig. 5). This would seem to indicate that ara-C which is incorporated into lower molecular weight RNA during short periods of time (1 hr, $3{\cdot}3 \times 10^{-5}$ M) prevents [^{3}H]UR incorporation and possibly the synthesis of these RNA species, and as a consequence the acute cell death occurred. At present, the low molecular weight RNA is the most proximal biochemical site to be uncovered.

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