EFFECT OF CYTOSINE ARABINOSIDE ON REPLICON INITIATION IN HUMAN LYMPHOBLASTS

Arnold Fridland

Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, 332 North Lauderdale, P.O. Box 318, Memphis, Tennessee 38101

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SUMMARY

Cytosine arabinoside inhibited DNA synthesis in human lymphoblasts by inhibiting the initiation of DNA replication units. This effect was observed by a decrease in the incorporation of (^{3}H) thymidine into low molecular weight DNA analyzed by velocity sedimentation in alkaline sucrose gradients. In contrast, there was no detectable effect on chain elongation and joining of those molecules that initiated replication before addition of the drug. These data indicate that cytosine arabinoside acts preferentially at the level of initiation of DNA replication rather than chain elongation.

Cytosine arabinoside (1-β-D-arabinosylcytosine, Ara-C), a 2-β-OH analog of deoxycytidine, is one of the primary chemotherapeutic agents for treatment of acute granulocytic leukemia (1,2). Although Ara-C has been under active investigation for more than a decade, its mechanism of action is still not clearly understood.

Intracellular kinases convert Ara-C to 5'-nucleotides, and of these derivatives arabinoside cytidine triphosphate (Ara-CTP) is considered the biologically active form of the drug (3,4). Ara-CTP acts competitively with deoxycytidine triphosphate (dCTP) to inhibit mammalian DNA polymerase activity and hence the elongation of DNA chains (5-8). The inhibition constant (K_i) for this interaction between Ara-CTP and the replication enzyme was found to range from 1 to 20 μM depending on the cell type studied and was similar to the affinity of dCTP for the polymerase (6-8), suggesting, therefore, that DNA polymerase is the primary target for Ara-C (5-8). In studies with murine leukemic cells and mouse L cells, however, the inhibition of DNA synthesis

by Ara-C did not correlate with the inhibition of DNA polymerase activity (7,9). The intracellular concentration of Ara-CTP required for 50% inhibition of DNA synthesis was about 0.03 μM, or at least 70 times lower than the K_i value for DNA polymerase derived from the same cells (7,9). The possibility that Ara-C might interfere with RNA synthesis as well as with DNA synthesis was raised by recent studies of Chuang *et al.*, in which Ara-CTP acted competitively with CTP to inhibit RNA polymerase II of chicken leukemia cells (10,11). The K_i for this inhibition, 14.9 μM, was similar to the K_i's for inhibition of DNA polymerase in other systems (5-7).

The discrepancy between the concentration of Ara-CTP required to inhibit DNA polymerase activity and the much lower concentration required to inhibit cellular DNA synthesis could indicate that the drug acts at a more sensitive level of DNA replication than previously thought. Reported herein are the effects of very low concentrations of Ara-C on DNA replication in human lymphoblasts (CCRF-CEM line). The drug was found to act preferentially at the level of initiation of DNA synthesis and did not appear to inhibit chain elongation.

MATERIALS AND METHODS

Cells. Human lymphoblasts (CCRF-CEM line) were grown as suspension cultures at 37°C in modified Eagle's Medium (Flow Laboratories) containing by volume, 10% antibiotic-antimycotic solution (Gibco), 1% nonessential amino acid solution (Flow Laboratories), 0.5% vitamin solution (Flow Laboratories), as well as 1.4 $NaHCO_3$, 2 mM L-glutamine and 10% fetal calf serum (Flow Laboratories).

Equilibrium density gradient analyses. In studies on the effect of Ara-C on the amount of DNA shifted by BrdUrd to hybrid density, DNA was isolated from the cells with RNase, pronase and chloroform-isoamyl alcohol (24:1) as described by Painter and Shaefer (12). The DNA was sheared with a homogenizer (Virtis) to yield a molecular weight of about 2-4 x 10^7 daltons. The preparation was centrifuged to equilibrium in neutral CsCl gradients in a Beckman SW 50.1 rotor operated at 40,000 r.p.m. at 15° for 40 hr. 0.1 ml fractions were collected from the bottom of the tubes, and each fraction was then filtered through GF/A filters (Whatman, New Jersey) that had been previously washed with 5% TCA. The ^{14}C and ^{3}H radioactivity on each filter was determined by liquid scintillation spectrometry (Searle Analytic, Inc.).

RESULTS AND DISCUSSION

The effect of Ara-C on DNA synthesis was determined by exposing cultures of CCRF-CEM cells, prelabeled with [^{14}C]dThd, to 0.03 μM Ara-C in the presence

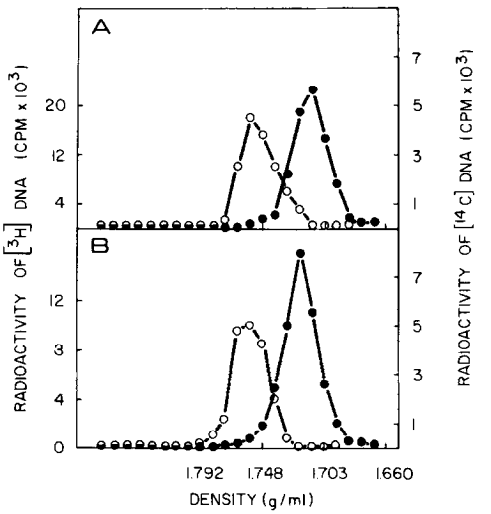

Fig. 1 Incorporation of [^{3}H]dThd and BrdUrd into DNA of CCRF-CEM cells in the absence (A) or presence (B) of Ara-C. Cells (5 x 10^5 ml^{-1}) prelabeled for 24 hr with 0.02 μCi of [^{14}C]dThd (Amersham/Searle, Illinois) were incubated for 1 h with nucleosides at the following concentrations per ml: 2 μCi of [^{3}H]dThd (spec. act. 50 Ci $mmol^{-1}$, New England Nuclear, Mass.), 1 μM BrdUrd and 1 μM FdUrd. The concentration of Ara-C, when present, was 0.03 μM. After incubation, the cells were centrifuged at 600 r.p.m. and resuspended in 0.15 M NaCl, 0.015 M-citrate buffer (pH 7.4) and a solution of 0.5% sodium dodecyl sulfate and DNA isolated as described in Materials and Methods. The ratio of ^{3}H (○——○) to ^{14}C (●——●) radioactivity was 1.2 for cells treated with Ara-C and 3.2 for untreated cells.

of BrdUrd and [^{3}H]dThd for 1 h. The DNA was isolated from the cells and analyzed by equilibrium centrifugation in CsCl gradients. As shown in Fig. 1, the density shift caused by the presence of BrdUrd was the same whether Ara-C was present or not, whereas the ratio of ^{3}H to ^{14}C was reduced by about 60% in cells treated with Ara-C. This finding indicates that the decreased incorporation of exogenous precursors into DNA resulted from inhibition of DNA synthesis and not from changes in the specific activity of DNA precursors in intracellular pools.

To assess the effect of Ara-C on elongation of DNA chains, CCRF-CEM cells either treated with Ara-C or untreated were pulse-labeled for 8 min with [^{3}H]dThd, and the sedimentation properties of labeled DNA were analyzed in alkaline sucrose gradients. In control cells, the profile of ^{3}H activity was associated predominantly with a peak that sedimented at bout 38S; 50% of the total radio-

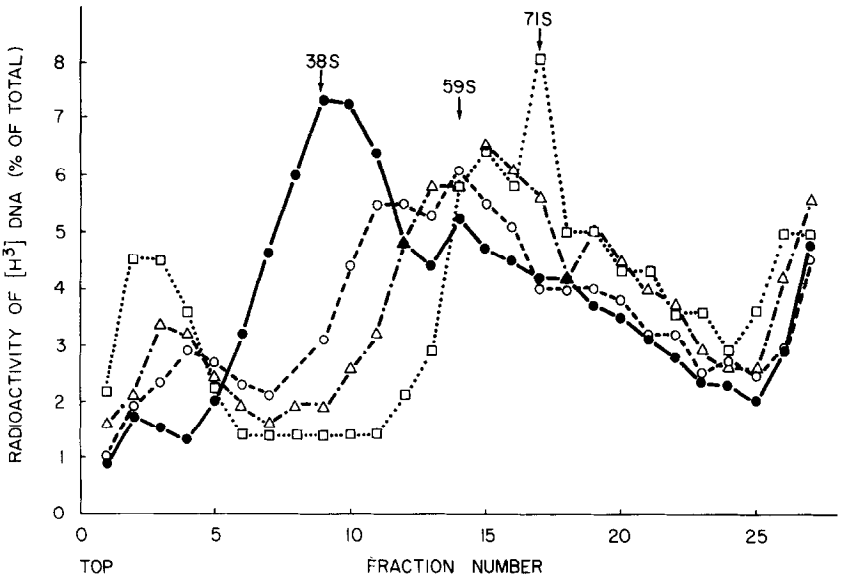

Fig. 2 Effect of Ara-C on sedimentation profiles of pulse-labeled DNA in alkaline sucrose gradients. Cells (5 x 10^5 ml^{-1}) incubated in the absence (●——●) or presence of Ara-C were pulse-labeled for 8 min with 10 μCi of [βH]dThd (spec. act. 50 Ci mmol^{-1}) per ml at different intervals: 15 min (○——○), 30 min (△——△) and 60 min (□——□). The cells were then washed with ice-cold saline-citrate buffer, resuspended in the same buffer and an aliquot containing about 3 x 10^5 cells was layered on top of alkaline sucrose gradients at room temperature in the dark, as described previously (13), and centrifuged 4-5 h later in a Beckman SW 27 rotor for 4 h at 26,000 r.p.m. at 15°. Fractions (1.2 ml) were collected from the tops of the centrifuge tubes and DNA was precipitated on Whatman GF/A filters. Radioactivity was counted as in Fig. 1. This gradient system was calibrated with adenovirus DNA, type 2, and fd DNA as described earlier (13).

activity in the gradient was associated with molecules that sedimented within a range of 21S to 55S (Fig. 2), corresponding to single strand molecules of 10 to 70 μm in length. These data are similar to previous results obtained with this system (13) and with other mammalian cells (14) and are in good agreement with the length of replicons determined by autoradiography (15,16). The ascending part of the curve at low S-values reflect the initiation of synthesis of DNA molecules of replicon size during incubation with [βH]dThd, while the slowly descending part of the curve at higher S-values indicates elongation and joining of replicons to form DNA of parental size.

Pretreatment of cells with Ara-C produced distinct changes in the distribution of radioactivity in sucrose gradients (Fig. 2). In cells pretreated for 15 min, the amount of radioactivity incorporated into DNA of lower molecular weight (21S to 42S) was sharply reduced, while the peak of radioactivity was shifted to a higher S-value (59S) relative to the control. A further reduction in radioactivity in the 21S to 42S region was observed for cells pretreated for 30 min, and the main peak was shifted to 65S compared to 38S for control cells. Sixty minutes after addition of Ara-C the overall rate of DNA synthesis was depressed by nearly 90%, and most of the radioactivity from the ^{3}H-dThd pulse was found in DNA sedimenting at 70S. At all time periods studied, the parental size DNA from both Ara-C treated and control cells was found in the bottom fractions of the gradients, which indicates that Ara-C does not cause cellular DNA to fragment under these conditions. The radioactivity present in the top fractions (1-4) of the gradients may be due to a mixture of Okazaki-type fragments, wall effects and degradation products (17), and was therefore excluded from the analysis of the results.

If Ara-C acted primarily on the synthesis of DNA by reducing the average rate of chain elongation, the sedimentation profiles of pulse-labeled DNA from cells treated with Ara-C would be expected to shift to lower S-values than were observed for DNA from control cells. Since the opposite result was obtained, that is, peaks of radioactivity were associated with progressively higher S-values, it appears that Ara-C inhibited the initiation of new replicons, without effecting the elongation and joining of replicons that had already been initiated.

This interpretation was tested by measuring the density distribution of DNA molecules synthesized in either the absence or presence of Ara-C (0.03 μM) during a 20-min pulse with BrdUrd and [^{3}H]dThd followed by a chase with unlabeled dThd as described by Gautschi *et al.* (18). The DNA was isolated, sheared to fragments of about 2.5 x 10^{7} daltons and analyzed by equilibrium centrifugation in alkaline CsCl gradients. As shown in Fig. 3, the density of

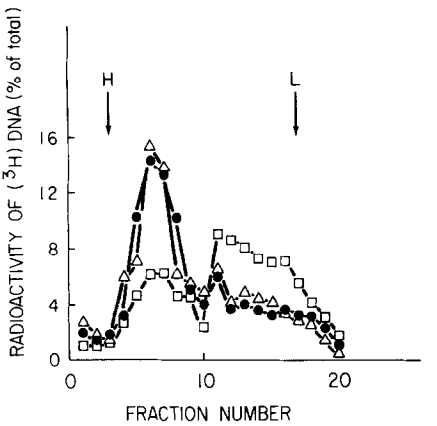

Fig. 3 Density distribution of DNA in cells pulse-labeled with [^{3}H]dThd and BrdUrd. Cells (5 x 10^5 cells ml^{-1}) were incubated with Ara-C (0.03 μM) or hydroxyurea (100 μM) and pulse-labeled with 10 μCi of [^{3}H]dThd (spec. act. 50 Ci $mmol^{-1}$) per ml for 15 min in the presence of 1 μM BrdUrd and 1 μM FdUrd. After incubation, the cells were washed and re-incubated for 5 min in medium devoid of radioactivity and unlabeled nucleosides. Radioactivity in DNA was chased for 90 min in medium containing unlabeled dThd, in the presence of either Ara-C or hydroxyurea. Controls were treated in the same way, except that neither Ara-C nor hydroxyurea was added. The incorporation of [^{3}H]dThd into DNA in cells treated with Ara-C (△—△) or hydroxyurea (□——□) was respectively 63% and 68% of the control (●—●). Arrows indicate the position of normal-density light strands (L) and BrdUrd-containing DNA strands (H) obtained from cells incubated for 8 h with BrdUrd (10 μM) and dCyd (10 μM). DNA, isolation and centrifugation was carried out in alkaline CsCl gradients as described previously (13).

^{3}H-labeled molecules of DNA was the same whether Ara-C was present or not, suggesting that the average rate of chain elongation is not greatly affected by this concentration of drug. By contrast, in the presence of 100 μM hydroxyurea, a known inhibitor of chain elongation (14), the density of the ^{3}H-labeled DNA molecules was substantially reduced (Fig. 3).

Thus, when present at low concentrations, Ara-C exerts its primary effect on DNA synthesis by inhibiting the initiation of replicons. A similar effect on the initiation of DNA replication has been observed with agents other than Ara-C (i.e., 2,4,-dinitrophenol (14) and ultraviolet irradiation (18,19)). Since Ara-C is incorporated into growing DNA chains in cells (3,7,20) one could postulate that it alters the physical state of DNA, which in turn could lead to inhibition of initiation of new replicons within chromosomes. Alternatively, the

presence of Ara-C in DNA might trigger a feedback mechanism involved in the initiation of new rounds of replication. Both these possibilities are being investigated.

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