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EFFECTS OF ARABINOSYL-CYTOSINE ON THYMIDINE TRIPHOSPHATE POOLS AND POLYOMA DNA REPLICATION

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SUMMARY: Arabinosyl cytosine at very low concentrations (5-100 nmolar) inhibits the incorporation of [^{3}H]thymidine into polyoma DNA of infected mouse fibroblasts without affecting the labeling of the [^{3}H]dTTP pool. The specific activities of these pools were determined by a new simple method. Inhibition of DNA synthesis affects chain elongation and not initiation of new rounds of replication.

INTRODUCTION

Arabinosyl cytosine (ara-C) is a powerful inhibitor of DNA synthesis of cells grown in culture (1). The nucleoside is phosphorylated to the triphosphate which interferes with the DNA replicating machinery. During polyoma DNA synthesis, ara-C is incorporated into internucleotide linkage of the circular viral DNA (2, 3). The inhibition of DNA synthesis is believed to be caused by the slowing down of the chain elongation process (1). Ara-C has sometimes been thought to affect DNA synthesis by inhibition of ribonucleotide reductase but such a mechanism was excluded by the demonstration that ara-C actually increased the pool sizes of deoxynucleoside triphosphates (4).

Fridland (5) recently reported a dual effect of ara-C on the incorporation of [^{3}H]thymidine into DNA of cultured human lymphoblasts. At the high concentrations of ara-C studied by earlier investigators chain elongation was affected. However, Fridland showed that ara-C also inhibited incorporation of thymidine at very low concentrations (5-10 nM) and in this case he interpreted the results to suggest a specific inhibition of new repli-

Abbreviations: ara-C, arabinosyl cytosine; RI, replicative intermediates.

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cating units of DNA, i.e. an inhibition of strand initiation. We have earlier used polyoma DNA synthesis as a convenient model to study different aspects of mammalian DNA synthesis (6). We now describe experiments in which we determine the inhibitory effects of different concentrations of ara-C on the incorporation of short pulses of [^{3}H]thymidine into replicative intermediates (RI) and completed forms (form 1) of polyoma DNA. This permits us to distinguish between effects on chain elongation and initiation of new rounds of replication. We also measured the specific radioactivities of the dTTP pools in order to establish that alterations in this parameter do not contribute to any observed effects of ara-C on the incorporation of thymidine into DNA.

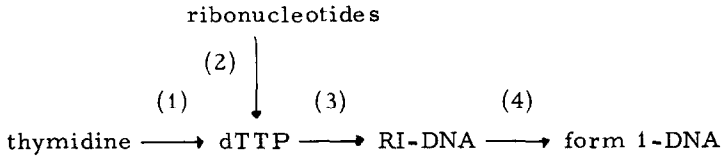
MATERIALS AND METHODS

The cell line (3T6), handling of cells and infection with a high multiplicity of infection of polyoma virus have been described (7). 24 hours after infection of two sets of cultures ara-C was added at different concentrations to the medium (5 ml) of 5 cm dishes containing 1.7×10^6 cells per dish. Incubation was continued at 37° for 60 minutes after which time [^{3}H]thymidine (20 Ci/mmol, final concentration 1 μM) was added. After additional 15 minutes one set of cultures was used for extraction of polyoma DNA by the Hirt (8) procedure while the other set was used for extraction of deoxynucleoside triphosphates by 60% methanol (9). Aliquots of the Hirt extract were used for the determination of total acid precipitable radioactivity (= total DNA synthesis) and for analysis by alkaline sucrose centrifugation (55,000 rpm at 4° for 2.5 hours in a Beckman SW 60 rotor). This procedure separates labeled progeny strands present in RI from labeled form 1 DNA (7) and gives the percentage of radioactivity present in each of the two fractions.

The methanol extract served to determine the specific activity of [^{3}H]dTTP. After evaporation in a vacuum the dry residue containing the deoxynucleoside triphosphates from one dish was dissolved in 0.2 ml of 10 mM Tris, pH 7.8 - 1 mM EDTA. Aliquots of this solution (0.5 and 1 μl) were used to determine the amount of dTTP in the cell extract (10) using the incorporation of [^{14}C]dATP (900 cpm/pmol) into poly d(A-T) by DNA polymerase as a measure of dTTP. In the same determination the amount of ^{3}H incorporated into the polymer was measured by dual channel counting. The specific activity of dTTP could then be obtained directly by division of the [^{3}H] value by the amount of dTTP derived from the [^{14}C] value.

RESULTS AND DISCUSSION

General plan of experiment. Thymidine is incorporated into polyoma DNA by the following sequence of reactions:



The specific radioactivity of the dTTP pool is determined by the balance of pathways (1), representing a series of kinase reactions, and pathway (2), representing de novo synthesis via ribotide reduction. Any change in this balance can cause an altered incorporation of radioactivity via pathways (3) and (4) without necessarily reflecting altered rates of DNA synthesis. Pathway (3) represents the initiation of new rounds of replication while pathway (4) represents chain elongation. The relative amounts of radioactivity recovered after a short pulse of [^{3}H]thymidine in RI and form 1 reflect the rates of pathways (3) and (4). Inhibition of chain elongation results in a preferential decrease in the amount of labeled form 1, while inhibition of initiation should affect labeling of both RI and form 1 to about the same extent.

Ara-C does not affect the specific activity of dTTP. Before evaluating the inhibitory effects of ara-C on pathways (3) or (4) it was necessary to investigate a possible influence on the balance of pathways (1) and (2). Table 1 describes the effects of low to moderate concentrations of ara-C on the amounts and labeling of the dTTP pool in polyoma infected cells. The slight decrease in the total amount of dTTP seen at the highest concentration of ara-C is not considered to be significant since it was not observed in other experiments where the cells were inhibited for 120 min (data not shown). The specific activity of dTTP was clearly not influenced by the addition of ara-C and the incorporation of labeled thymidine into RI and form 1 is thus a true reflection of the synthesis of polyoma DNA at all concentrations of ara-C. We would like to point out that the method used for the determination of the specific activity of dTTP simultanously measures the amount of nucleotide and its radio-

Table 1. Pool size and specific activity of dTTP after addition of arabinosyl cytosine to polyoma infected 3T6 cells

Arabinosyl cytosine added	Pool size	Specific activity
μM	pmoles dTTP/10^6 cells	cpm/pmol
0	580	810
0.005	580	860
0.01	550	810
0.05	410	560
0.1	340	790

activity and thus avoids some of the experimental errors inherent in such measurements.

Ara-C inhibits the synthesis of polyoma form 1 DNA. Alkaline sucrose centrifugation separates the labeled growing progeny strands of RI from form 1 DNA and thus makes possible a quantitation of the radioactivity present in these two forms of polyoma DNA. Fig. 1 depicts three such gradients; panel A gives the results from the control experiment carried out in the absence of inhibitor, panels B and C show data obtained at low and high concentrations of ara-C, respectively. In the control about 50% of the radioactivity was present in each class of DNA molecules. At the high concentration of ara-C, essentially all label was present in RI. Furthermore, in this case the growing progeny strands sedimented more slowly and a shoulder of radioactivity was present in the position of Okazaki fragments. All these effects are explained by a pronounced inhibition of chain elongation. Also at the low concentration of ara-C (panel C) some radioactivity was shifted from the form 1 peak to RI but the effect on the length of labeled progeny strands was not discernible.

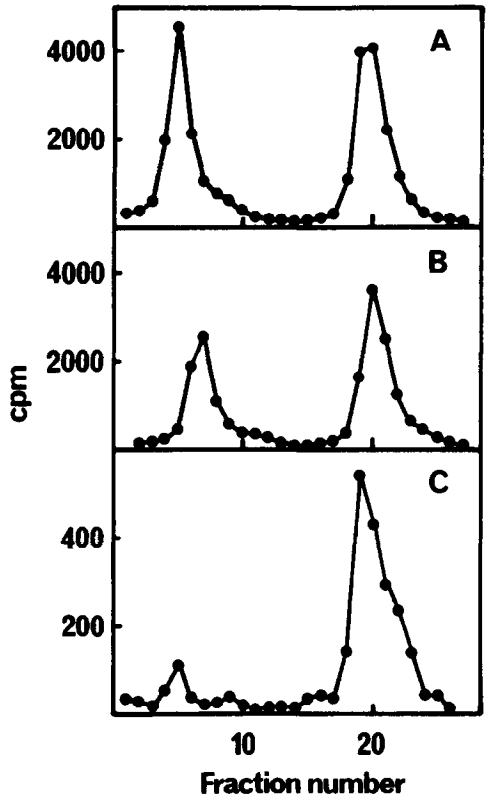


Fig. 1. Analysis by alkaline sucrose gradient centrifugation of polyoma DNA from 3T6 cells labeled for 15 min with [^{3}H]thymidine after 60 min exposure to arabinosyl cytosine. The fractions are numbered from the bottom of the gradient. The first peak corresponds to form 1 DNA while the second peak corresponds to the labeled progeny strands of replicative intermediates. Panel A is a control without ara-C, panels B and C represent experiments with 0.01 and 1.0 μM ara-C, respectively.

Alkaline sucrose centrifugation was then used to analyze the effects of several concentrations of ara-C on the synthesis of total polyoma DNA and form 1 DNA. The results of these experiments (Table 2) show that even at the lowest effective concentration of ara-C (5 nM) the synthesis of form 1 DNA was more severely inhibited than that of total polyoma DNA. With increasing concentrations of the nucleoside the preferential inhibition of form 1 DNA synthesis increased. These results indicate that at all concentrations of ara-C pathway (4) was inhibited.

Table 2. Inhibition of synthesis of total polyoma DNA and form 1 DNA by different concentrations of arabinosyl cytosine

Arabinosyl cytosine added	Total polyoma DNA	Form 1 DNA[a]
μM	% synthesis	
0	100	100
0.005	94	75
0.01	74	60
0.05	70	53
0.1	46	28
0.3	31	17
1.0	9	1.5
10.0	2	0.3

[a] In the absence of added ara-C the radioactivity in form 1 DNA accounted for 48% of the radioactivity of total polyoma DNA.

Our results thus confirm the finding of Fridland (5) that DNA synthesis is inhibited already at very low concentrations of ara-C. However, with polyoma DNA no preferential inhibition of strand initiation was found. Instead, inhibition by both low and high concentrations of ara-C appears to occur by inhibition of chain elongation.

We would finally like to point out that the method described here for the determination of the specific activity of dTTP should be generally applicable to other deoxynucleoside triphosphates.

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