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Effects of 5-Fluoro-2′-deoxyuridine on DNA Metabolism in HeLa Cells

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SUMMARY

5-Fluoro-2′-deoxyuridine at a concentration of 0.1 μM completely inhibited incorporation of radioactivity from [^{3}H]dUrd into DNA in HeLa S_3 cells within 5 min of exposure. The dTTP pool size decreased from 800 to 300 pmoles/10^7 cells by 1 hr, and then gradually increased after 3 hr of exposure. Although the incorporation of radioactivity from [^{32}P]H_3PO_4 into DNA was inhibited, the relative incorporation into smaller fragments compared with larger fragments of DNA was found to be increased in the drug-treated cells. When the cells with [^{14}C]thymidine-prelabeled DNA were exposed to 5-fluoro-dUrd, shifts of labeled DNA peaks to smaller sizes were observed between 5 and 18 hr after exposure, as analyzed by alkaline sucrose gradient centrifugation. Exposure of cells to 1 and 10 μM 5-fluoro-[^{3}H]dUrd caused incorporation of radioactivity into DNA. By high-performance liquid chromatographic analysis of nucleosides, it was confirmed that almost all of the radioactivity was incorporated as 5-fluoro-dUrd.

INTRODUCTION

The cytotoxicity of 5-fluoro-dUrd, an anticancer agent (1, 2), has been ascribed to inhibition of thymidylate synthetase (EC 2.1.1.45) by 5-fluoro-dUMP (3–5) or the incorporation of 5-fluoro-Urd into RNA after conversion of 5-fluoro-dUrd to the ribonucleotide analogue (6–8).

However, as reported previously (9), 5-fluoro-dUrd is transformed to 5-fluoro-dUTP and incorporated into DNA *in vitro* using purified enzymes. Although detection of the *in vivo* incorporation of 5-fluoro-dUrd into DNA has been impeded by the high activities of dUTP nucleotidohydrolase and uracil-DNA glycosylase, Kufe *et al.* (10) and Ingraham *et al.* (11) recently reported the identification of 5-fluoro-dUrd incorporated into DNA in 5-fluoro-dUrd-treated cells.

We describe in this paper the natures of the effects of 5-fluoro-dUrd on DNA, and correlate them with the incorporation of 5-fluoro-dUrd into DNA.

EXPERIMENTAL PROCEDURES

Materials. 5-Fluoro-[^{3}H]dUrd (18 Ci/mmole, 98% pure) and [6-^{3}H]dUrd (20 Ci/mmole) were purchased from Moravek Biochemicals (City of Industry, Calif.), [^{3}H]dTTP (73 Ci/mmole) and [^{3}H]dATP (7 Ci/mmole) from ICN Pharmaceuticals (Irvine, Calif.), and [^{14}C]dThd (58 mCi/mmole) from New England Nuclear Corporation (Boston, Mass.). RPMI 1640 media and horse serum were obtained from GIBCO (Grand Island, N. Y.). Cesium sulfate was obtained from Alfa Products (Danvers, Mass.), sucrose from Schwarz/Mann (Orangeburg, N. Y.), ACS scintillant from Amersham Corporation (Arlington Heights, Ill.), and *Escherichia coli* DNA polymerase from Worthington Biochemicals (Freehold, N. J.). All other biochemicals were purchased from Sigma Chemical Company (St. Louis, Mo.), and chemicals from Fisher Scientific Company (Pittsburgh, Pa.).

Cell culture. HeLa S_3 suspension culture was maintained in log phase between concentrations of 1–8 × 10^5 cells/ml in RPMI 1640 medium supplemented with 5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The HeLa cell monolayer was grown with the same medium in an atmosphere of 5% CO_2. Cells were determined to be free of mycoplasma using the diaminophenylindole fluorescence assay.

dNTP pool size determination. Cells (10^7) were washed twice with ice-cold PBS,[1] and extracted with 100 μl of 0.5 N $HClO_4$ for 5 min at 0°. The precipitate was removed by centrifugation, and the supernatant was neutralized by the addition of 12.5 μl of 4 N KOH. After the addition of 12.5 μl of potassium phosphate buffer (pH 7.5), the precipitate formed was removed by centrifugation. The concentrations of dTTP, dCTP, dGTP, and dATP in the extracts were determined by DNA polymerase methods (12, 13).

Alkaline sucrose gradient centrifugation. Cells labeled with [^{32}P]H_3PO_4, [^{14}C]dThd, or 5-fluoro-[^{3}H]dUrd were washed twice with ice-cold PBS, and loaded on top of a 5–20% sucrose gradient containing 0.3 M NaOH, 10 mM EDTA, and 0.66 M NaCl. After 2.5% sucrose containing 0.3% *N*-lauroyl sarcosine (in addition to the same

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[1] The abbreviations used are: PBS, phosphate-buffered saline [0.14 M NaCl/4 mM KCl/0.5 mM Na_2HPO_4/0.15 mM KH_2PO_4 (pH 7.2)]; HPLC, high-performance liquid chromatography.

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reagents as above) was overlaid, centrifugation was performed in a Beckman SW 50.1 rotor for 40 min at 25,000 rpm at 20°. Fractionation was achieved by using a Buchler Auto Densi-Flow IIC from the top to the bottom of a tube. RNA labeled from 5-fluoro-[^{3}H]dUrd was decomposed by incubation of each fraction at 37° overnight. The fractions then were spotted on paper discs, washed with 5% trichloroacetic acid, and counted.

Cesium sulfate density gradient centrifugation. Logarithmically growing cells were incubated in the presence of a variety of concentrations of 5-fluoro-[^{3}H]dUrd for 24 hr, washed twice with PBS, and then treated overnight at 37° with a nucleic acid extraction buffer containing 1% sodium lauryl sulfate, 0.4 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.6), and proteinase K (100 μg/ml). After phenol extraction and ethanol precipitation, nucleic acids were subjected to cesium sulfate (1.548 g/cm^3) density gradient centrifugation in a Beckman SW 50.1 rotor at 25,000 rpm for 65 hr at 20°. A portion (0.1 ml) of each fraction was collected as described above.

Digestion of DNA and HPLC Analysis. The DNA peak fractions after cesium sulfate density gradient centrifugation were combined, dialyzed, and precipitated with ethanol. After heat denaturation, the DNA was digested by incubation with pancreatic DNase I (Worthington) and spleen phosphodiesterase (Worthington) in a total volume of 120 μl, and then with *E. coli* alkaline phosphatase (Sigma) in a total volume of 240 μl. An equal volume of ice-cold 1 M perchloric acid was added to the mixture. The supernatant obtained by centrifugation was neutralized by the addition of 60 μl of 4 N KOH, and lyophilyzed, dissolved in water, and analyzed by HPLC (Micromeritics) using a Partisil PXS 10/25 SCX column (Whatman) in 0.1 M ammonium phosphate buffer (pH 3.5) and a μBondapak C_{18} column (Waters) in 5 mM potassium phosphate buffer (pH 3.5)/5% methanol. Fractions were collected and the radioactivity was measured.

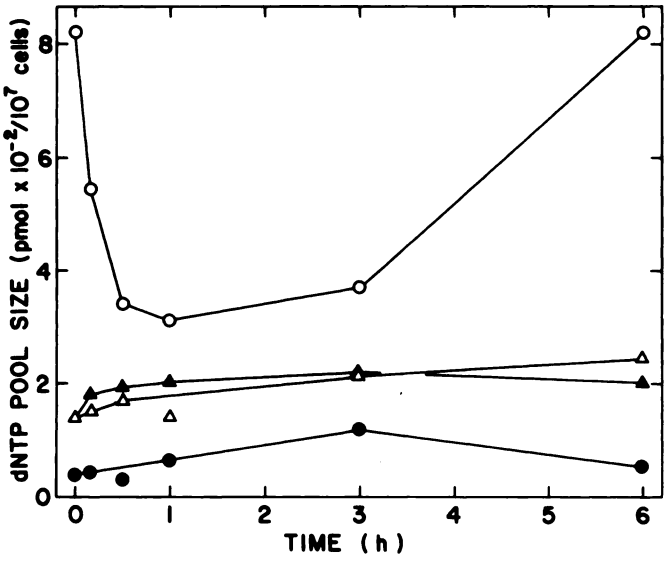

FIG. 1. *Effects of 5-fluoro-dUrd on dNTP pool sizes*

Cells at a concentration of 6 × 10^5 /ml were incubated with 0.1 μM 5-fluoro-dUrd. At the indicated times, 20 ml of the medium containing 1.2 × 10^7 cells were removed, and the dNTP pool sizes were determined as described under Experimental Procedures. ○, dTTP pool size; △, dCTP; ●, dGTP; ▲, dATP.

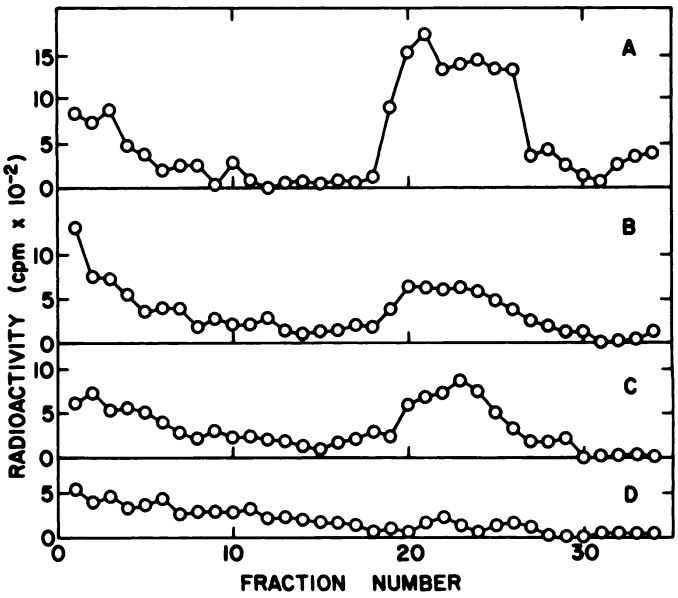

FIG. 2. *Effects of 5-fluoro-dUrd on the incorporation of radioactivity from [^{32}P]H_3PO_4 into DNA*

Cells were incubated with [^{32}P]H_3PO_4 (10 μCi/ml) and a variety of concentrations of 5-fluoro-dUrd [0 (A), 0.1 (B), 1 (C), and 10 μM (D)] for 24 hr at 37°. The cells (5 × 10^5) then were subjected to alkaline sucrose gradient centrifugation as described under Experimental Procedures, and 0.15 ml of each fraction was collected.

RESULTS AND DISCUSSION

Effects of 5-fluoro-dUrd on the dNTP pool sizes in HeLa cells. Using the incorporation of radioactivity from [6-^{3}H]dUrd into DNA as a measure for thymidylate synthetase activity, it was confirmed that exposure of HeLa S_3 cells to 0.1 μM 5-fluoro-dUrd completely inhibited thymidylate synthetase within 5 min (data not shown). When the effects of the inhibition of thymidylate synthetase on the intracellular deoxynucleotide triphosphate pool sizes were examined, it was found that the dTTP pool size dropped from 800 to 300 pmoles/10^7 cells within 1 hr after exposure to 0.1 μM 5-fluoro-dUrd (Fig. 1). Treatment of the samples with dUTPase prior to dTTP pool size determination gave the same results (data not shown), which indicates that there were no significant amounts of 5-fluoro-dUTP or dUTP. The rate of early consumption of dTTP (0.1 fmole/hr/cell) appears comparable to that of the incorporation of dTTP into DNA in HeLa cells. If there were no other source of dTTP, one would anticipate that its pool size would continuously decline until completely depleted. However, as shown in Fig. 1, after a plateau of 1–3 hr the dTTP pool size increased gradually. Therefore, the presence of another source of dTTP is suggested in HeLa cells. This sheds doubt on the idea (3–5) that the cytotoxicity of 5-fluoro-dUrd is due solely to the depletion of dTTP caused by inhibition of thymidylate synthetase. The other deoxynucleotide triphosphate (dCTP, dGTP, and dATP) pools were perturbed slightly.

It should be noted that the concentration of 5-fluoro-dUrd required to inhibit cell growth by 50% (ID_{50}) was 2 nM. The addition of 10 μM dThd, but not Urd, reversed the inhibitory effect, giving an ID_{50} value of 1 μM. These results suggest that the cytotoxicity of 5-fluoro-dUrd is directed at DNA metabolism.

Effects of 5-Fluoro-dUrd on the incorporation of radioactivity from [^{32}P]H_3PO_4 into DNA. The effects of 5-fluoro-dUrd on DNA synthesis was examined using alkaline sucrose gradient centrifugation. This method allows us to determine the degree of single-strand breaks in DNA. In the absence of 5-fluoro-dUrd (Fig. 2A), radioactivity from [^{32}P]H_3PO_4 was incorporated into the DNA which distributed between Fractions 19 and 26. In the presence of 0.1 μM 5-fluoro-dUrd (Fig. 2B), incorporation of radioactivity decreased to nearly one-third that observed in the absence of drug. Although at concentrations between 0.1 and 1 μM (Fig. 2C) a large difference was not observed, 10 μM 5-fluoro-dUrd caused a strong inhibition of the incorporation of the radioactivity into DNA. The proportion of the radioactivity recovered in smaller fractions (Fractions 1–18) to that in larger fractions (Fractions 19–26) increased in drug-treated cells.

Effects of 5-fluoro-dUrd on Hela cell DNA prelabeled with [^{14}C]dThd. The effects of 5-fluoro-dUrd on preexisting DNA were examined. Cellular DNA was first labeled with [^{14}C]dThd for 72 hr, and then the cells were incubated for one generation time without the radioactive compound. When the cells were exposed to 0.1 μM 5-fluoro-dUrd, shifts of single-stranded DNA peaks to smaller sizes, compared with that from control cells receiving no drug, were observed at 5 hr (Fig. 3), 12 hr, and 18 hr (data not shown) after the treatment. At 3 hr after treatment no effect was observed (data not shown). These results suggest that the preexisting DNA suffered lesional damages, nicks or gaps, by 5 hr after exposure to 5-fluoro-dUrd. It is likely that the damages were introduced by incorporation of 5-fluoro-dUrd through repair DNA synthesis and subsequent removal. Between 5 hr and 18 hr little difference in the average size of single-stranded DNA was observed, suggesting that an equilibrium state was established between gap formation and repair synthesis.

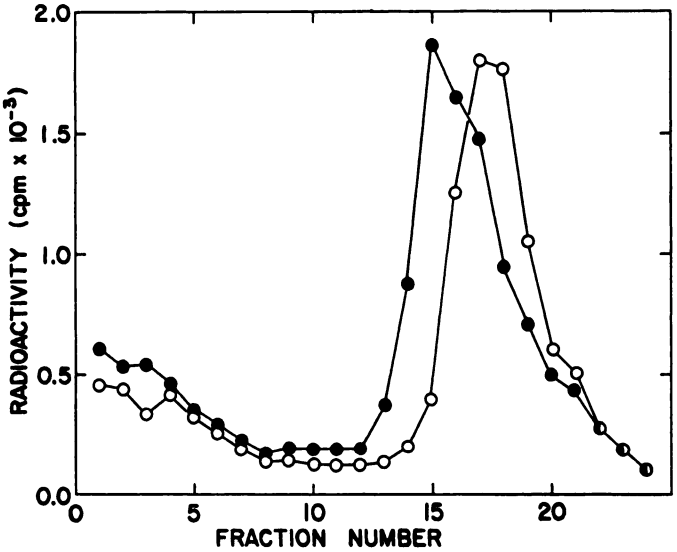

FIG. 3. *Alkaline sucrose gradient centrifugation of DNA labeled with [^{14}C]dThd*

Monolayer cultures of HeLa cells were labeled with [^{14}C]dThd (0.25 μCi/ml). After 72 hr, the medium was replaced with fresh medium without the radioactive compound, and the cultures were incubated for 24 hr. The cells then were incubated in the absence (○) or the presence (●) of 0.1 μM 5-fluoro-dUrd. Cells (10^6) were harvested 5 hr after exposure to the drug. Centrifugation was carried out as described under Experimental Procedures, and 0.2 ml of each fraction was collected.

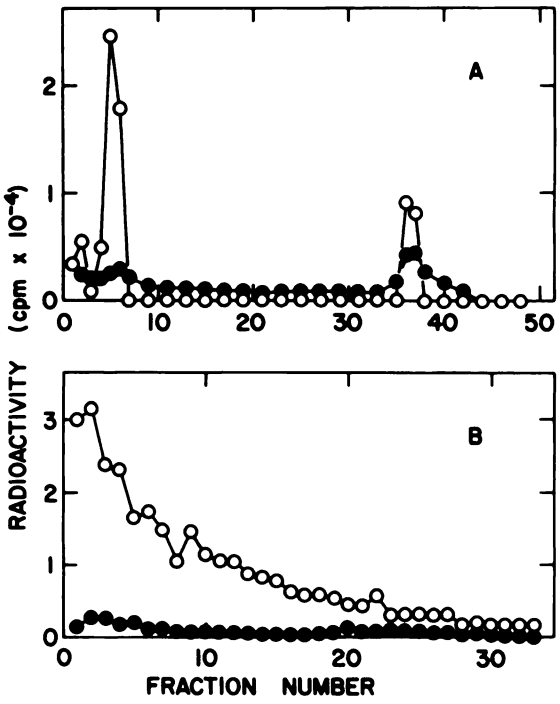

FIG. 4. *Incorporation of radioactivity from 5-fluoro-[^{3}H]dUrd into DNA*

Cells (10^6) were treated with 10 μM (○) or 1 μM (●) undiluted 5-fluoro-[^{3}H]dUrd for 24 hr and subjected to cesium sulfate density gradient centrifugation (A) and alkaline sucrose gradient centrifugation (B), as described under Experimental Procedures. A value of 10^4 cpm corresponds to 1.1 pmoles of 5-fluoro-[^{3}H]dUrd.

Incorporation of radioactivity from 5-fluoro-[^{3}H]dUrd into DNA. The extent of incorporation of 5-fluoro-[^{3}H]dUrd into DNA, under the conditions described above, is difficult to examine, because the 5-fluoro-dUrd incorporated could be efficiently removed by the action of uracil-DNA glycosylase (9). However, after incubation for 24 hr with higher concentrations of 5-fluoro-[^{3}H]dUrd, it was possible to detect the incorporation. As shown in Fig. 4A, when nucleic acids which were extracted from the cells exposed to 10 μM 5-fluoro-dUrd were subjected to cesium sulfate density gradient centrifugation, two peaks of radioactive nucleic acid were observed. The one with lower density (Fractions 4–6, 1.45 g/cm^3) was alkali-stable and was identified as DNA, while the one with higher density (Fractions 36 and 37, 1.68 g/cm^3) was alkali-labile and was identified as RNA. Although more radioactivity was incorporated into RNA than into DNA in the presence of 1 μM 5-fluoro-[^{3}H]dUrd, the major radioactive peak obtained in the presence of 10 μM compound was that of DNA.

The sizes of DNA strands containing the radioactivity from 5-fluoro-[^{3}H]dUrd was examined by alkaline sucrose gradient centrifugation. As shown in Fig. 4B, DNA strands that incorporated radioactivity consisted mainly of small fragments. This was obvious, in particular, when the 10 μM concentration was used. One likely explanation of these results is that nicking or gap formation was going on extensively, through a series of reactions involving uracil-DNA glycosylase. However, we cannot exclude the

possibility that the breakdown of DNA during the exposure to 10 μM 5-fluoro-dUrd was a result of cell death.

The DNA which incorporated radioactivity in the presence of 10 μM 5-fluoro-[^{3}H]dUrd was digested to nucleosides and analyzed by HPLC as described under Experimental Procedures. Almost all of the radioactivity coincided with authentic 5-fluoro-dUrd standard in two different column systems.

In conclusion, a sequence might be depicted, in which cells are busy removing DNA portions containing 5-fluoro-dUrd and repairing them (9, 11). The incorporation of 5-fluoro-dUrd could be facilitated through the inhibition of thymidylate synthetase by 5-fluoro-dUMP. However, during the repair synthesis the analogue could be misincorporated again, and DNA replication would be impeded as a whole.

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