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THE INCORPORATION OF 5-FLUORO-2'-DEOXYURIDINE INTO DNA OF MAMMALIAN TUMOR CELLS

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SUMMARY

Treatment of L1210 mouse leukemia cells with 10^{-6}M [^{3}H]5-fluoro-2'-deoxyuridine resulted in the incorporation of radioactivity into DNA. The DNA hydrolysate was shown to contain [^{3}H]5-fluoro-2'-deoxyuridylic acid by an unequivocal assay involving the formation of a tightly-bound complex with thymidylate synthetase in the presence of 5,10-methylenetetrahydrofolate. The extent of incorporation was estimated to be 1 molecule of 5-fluoro-2'-deoxyuridylic acid per 10^8 DNA nucleotides.

INTRODUCTION

The fluorinated pyrimidines (FUra, FdUrd, and FUrd)[1] are widely used in the treatment of disseminated human cancers, especially of the gastrointestinal tract (1). It is, therefore, important for the maximal development of cancer chemotherapy with these drugs to understand in detail their biochemical mechanisms of cytotoxicity. Treatment with fluorinated pyrimidines produces two major effects in cells: 1) inhibition of DNA synthesis via a powerful inhibition of dTMP synthetase (EC 2.1.1.45) by FdUMP (2,3), and 2) alteration of the processing and function of some types of RNA because of extensive incorporation of FUra in place of Ura (3,4). Recent controversy

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[1] Abbreviations used are: FdUrd, 5-fluoro-2'-deoxyuridine; FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUMP, 5-fluoro-2'-deoxyuridylic acid; dTMP, thymidylic acid; Ura, uracil; FUMP, 5-fluorouridylic acid; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolate; FdUTP, 5-fluoro-2'-deoxyuridine-5'-triphosphate; dUTP, 2'-deoxyuridine-5'-triphosphate; dUMP, 2'-deoxyuridylic acid.

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has swirled over the question of which of these two effects accounts for the majority of the in vivo anti-tumor activity of these drugs. A third possible mechanism of cytotoxic action, incorporation into DNA, has not received serious consideration because until now none has been detected (3,5), except in the case of a bacteriophage that normally has Ura in place of Thy in its DNA (6). The discovery by Goulian et al. (7) that Ura can be misincorporated into DNA of whole cells, and the availability of radiolabeled [6-^{3}H] FdUrd of high specific activity, suggested to us that the extent of incorporation of fluorinated pyrimidines into DNA could now be determined in mammalian cells.

MATERIALS AND METHODS

[^{3}H] FdUrd (specific activity 18 C$_i$/mmol) was obtained from Moravek Biochemicals, City of Industry, California. Fetal calf serum and RPMI-1640 medium was obtained from GIBCO. dTMP synthetase was purified to homogeneity from methotrexate-resistant L. casei as described previously (8). 5,10-CH_2H_4folate was enzymatically prepared as described previously (9). RNase was obtained from Sigma Chemical Co., St. Louis, Missouri, and snake venom phosphodiesterase and bovine pancreatic DNase from Worthington Biochemicals, Freehold, New Jersey.

Cell Culture. L1210 cells were thawed from mycoplasma-free stocks and grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum. A total of 10^9 cells in late logarithmic growth were harvested by centrifugation at 1000 rpm, and placed in 5 ml of sterile PBS. [^{3}H] FdUrd was added to 10^{-6} M and the mixture incubated at 37^{o}C for 4 hr. The cells were then washed with 3 x 10 ml of PBS and immediately processed.

Isolation of DNA. The isolation and purification of DNA, including RNase treatments, was carried out exactly according to Marmur (10). This procedure was followed by 6 deproteinizations with chloroform-isoamyl alcohol, 5 precipitations with ethanol, and 1 with isopropanol until radioactivity in the supernatant was indistinguishable from background. Hydrolysis of the DNA to nucleotides was carried out with bovine pancreatic DNase and snake venom phosphodiesterase according to the method of Singer (11).

Binding to dTMP Synthetase. Aliquots of the DNA hydrolysate were added to a solution containing 1.2 nmol of dTMP synthetase, 0.1 mM 5,10-CH_2H_4folate, 20 mM β-mercaptoethanol in Tris buffer, pH 7.5, to give a total volume of 1.1 ml. After an incubation period of 2 hr at 32^{o}C, 1 ml of a charcoal suspension containing 0.1g of charcoal, 2.5 mg/ml of bovine serum albumin, and 0.1 mg/ml of high molecular weight dextran in 0.1 N HCl was added. The mixtures were centrifuged for 20 min at 4400 x g, and 1.5 ml of the supernatant was removed for counting.

RESULTS AND DISCUSSION

Exposure of logarithmically-growing L1210 mouse leukemia cells to 10^{-6} M [^{3}H] FdUrd continuously for 4 hr resulted in the incorporation of 749 dpm of tritium per mg

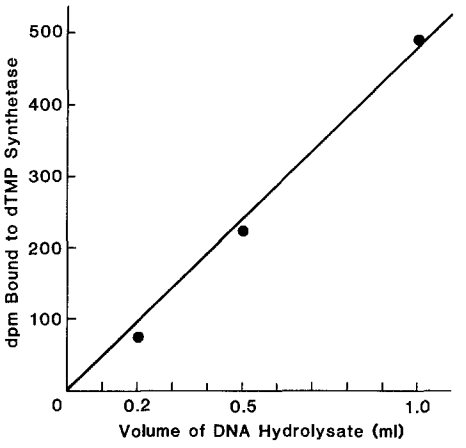

Fig. 1. The binding of radioactive material to dTMP synthetase (L.casei) from a hydrolysate of DNA from L1210 cells exposed to 10^{-6}M [^{3}H] FdUrd for 4 hr. at 37°C.

of DNA. Treatment of the hydrolysate of highly purified DNA from these cells with excess dTMP synthetase and 5,10-CH_2H_4folate resulted in the binding of 87% of the total radioactivity found in the hydrolysate to the protein (Fig. 1). This constitutes an unequivocal identification of the major portion of the radioactivity isolated from the DNA hydrolysate as FdUMP. Using dTMP synthetase as a binding protein is the most specific assay possible for FdUMP, since this enzyme does not form a tightly-bound complex with any other known FUra derivative that would be formed as a product of cellular metabolism. The stoichiometric interaction between FdUMP and dTMP synthetase forms the basis for recently developed ultrasensitive methods for the determination of FdUMP in tumor tissue samples (12). We deliberately avoided chromatographic methods of identification in this study because of the chance of co-migration of one or more metabolites of FdUrd with FdUMP. For example, difficulties have been noted in the separation of FdUMP and FUMP, even by HPLC methods (13). Thus, a small amount of an RNA contaminant (e.g., as in a primer segment) might result in an incorrect identification of FUMP as FdUMP, or at best, give an apparent value for the degree of incorporation much higher than the actual amount of FUra in the DNA.

The level of incorporation that we found amounts to 6.1 x 10^{-9} moles of FdUMP per mole of DNA phosphate. This is equivalent to 1 molecule of FdUMP per 1.6 x 10^8

nucleotides. Assuming 10^{12} daltons of DNA per cell and 3 x 10^{9} nucleotides per genome, there were 19 FUra molecules per mouse cell genome, or about 1 FUra for every 2 chromosomes.

The absence of detectable Ura in DNA under normal conditions has been attributed to the activity of dUTPase, which keeps dUTP levels very low (14), and Ura-DNA glycosylase, which specifically removes any Ura residues that may have been incorporated (15,16). Grafstrom et al. (17) postulated that the small DNA fragments observed in the presence of dUTP arise as a result of the excision and repair processes subsequent to the incorporation of dUMP. Recent studies have shown that FUra nucleotides are processed in a similar manner: FdUTP is a substrate for both dUTPase and DNA polymerase (18), and FUra residues in DNA are substrates for removal by Ura-DNA glycosylase (18), although the kinetic constants are somewhat different than for the corresponding Ura analogs. Provided that concentrations of competing nucleotides such as dTTP remain constant, the number of FUra residues in DNA, after a short initial velocity period while the system comes to equilibrium, should remain constant at a value which would be determined by the relative activities of the aforementioned enzymes.

The question arises as to the possible biochemical effects of the presence of FUra in DNA. Aebersold (19) showed that the frequency of 8-azaguanine-resistant mutants of V79 Chinese hamster cells was increased $\sim$3-fold by treatment of the cells with FdUrd. Jones et al. (20) found that FUra produced oncogenic transformation in C3H/10T1/2 cells. It was postulated that the mutagenic effects of FdUrd may result from DNA replication errors produced by FUra incorporated into the DNA (20). Our data show that the low level of FUra incorporated into the DNA is insufficient to adequately account for the mutagenesis observed by Aebersold (19). However, FdUrd may produce perturbations in cellular deoxyribonucleotide pools that reduce the fidelity of DNA replication (21). The concentration of FdUrd that we found to be the LD_{50} for L1210 cells was 3 x 10^{-10}M (unpublished data). At this concentration we might expect less than 0.02 FUra molecules incorporated per cell genome per 4 hr., or $\sim$ 0.06 FUra molecules incorporated per cell per generation. Thus, at the LD_{50} of

FdUrd, > 90% of the cells would contain no FUra incorporated in their DNA even after undergoing a complete round of DNA replication in medium containing cytotoxic concentrations of FdUrd. Therefore, we conclude that it is unlikely that the low level of FUra incorporated into the DNA plays a significant role in the mechanisms of cytotoxicity of FdUrd in L1210 cells.

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