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The Effect of Inhibition of Cytidine Deaminase by Tetrahydrouridine on the Utilization of Deoxycytidine and 5-Bromodeoxycytidine for Deoxyribonucleic Acid Synthesis

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SUMMARY

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The effect of cytidine deaminase activity on the utilization of deoxycytidine and 5-bromodeoxycytidine for DNA synthesis in normal and neoplastic mouse tissues was investigated utilizing tetrahydrouridine to inhibit cytidine deaminase *in vivo*. Tetrahydrouridine increased approximately 3-fold the incorporation of deoxycytidine into the DNA of two transplantable lymphomas, a mammary adenocarcinoma, and bone marrow. The utilization of deoxycytidine for DNA synthesis was also increased by tetrahydrouridine in mouse testes, but not in the spleen or small intestine. The toxicity of 5-fluorodeoxycytidine was similarly increased by inhibition of cytidine deaminase. In contrast to the effect of tetrahydrouridine on deoxycytidine, the incorporation of 5-bromodeoxycytidine into DNA was decreased approximately 70% by inhibition of cytidine deaminase with tetrahydrouridine. This suggests that the incorporation of 5-bromodeoxycytidine into DNA proceeds mainly by deamination of the nucleoside to 5-bromodeoxyuridine, followed by phosphorylation to 5-bromodeoxyuridylate, rather than the alternative pathway proceeding by phosphorylation of 5-bromodeoxycytidine to 5-bromodeoxycytidylate, followed by deamination of the nucleotide to 5-bromodeoxyuridylate.

INTRODUCTION

Catabolism of pyrimidine nucleosides may limit their utilization for nucleic acid synthesis in mammalian tissues and diminish the effectiveness of pyrimidine nucleoside analogues used clinically as antineoplastic or antiviral agents. Neil *et al.* (1) have reported that tetrahydrouridine, a potent inhibitor of cytidine deaminase (EC 3.5.4.5), increased the antileukemic activity of cytosine arabinoside in mice. In the present investigation we have utilized tetrahydrouridine to study the effect of cytidine deaminase activity on the incorporation of deoxycytidine and 5-bromodeoxycytidine into the DNA of normal and neoplastic mouse tissues.

Deoxycytidine and its 5-halogenated analogues are not substrates for mammalian

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nucleoside phosphorylases (2, 3). Cytidine deaminase therefore catalyzes the initial step in deoxycytidine catabolism. 5-Bromo- or 5-iododeoxycytidine is incorporated into the DNA of murine cells as 5-bromo- or 5-iododeoxyuridylate (4, 5). Deamination of 5-halogenated deoxycytidine analogues could occur either at the nucleoside level, catalyzed by cytidine deaminase, or at the nucleotide level, catalyzed by deoxycytidylate deaminase. In the first case phosphorylation would occur after deamination and be catalyzed by thymidine kinase. In the second case the deoxycytidine analogue would be phosphorylated by deoxycytidine kinase prior to deamination. In the current study the relative activities of these two possible pathways for the incorporation of 5-bromodeoxycytidine into DNA are investigated by inhibition of cytidine deaminase with tetrahydrouridine.

METHODS

Chemicals. Tetrahydrouridine was a generous gift from Dr. Gary Neil of the Upjohn Company and from Dr. Harry Wood of the National Cancer Institute. 5-Fluorodeoxycytidine was purchased from Calbiochem. [5-^{3}H]Deoxycytidine and 5-bromo[6-^{3}H]deoxycytidine were purchased from New England Nuclear Corporation. More than 99.9% of the ^{3}H label in [5-^{3}H]deoxycytidine was shown to be in position 5 by halogenation (data of the manufacturer). [5-^{3}H]Deoxyuridine was purchased from Amersham/Searle and had a radiochemical purity greater than 95%.

Growth of tumors. Lymphoma M3445 (6), Lymphoma M5183, and mammary Adenocarcinoma M3659 were maintained in the laboratory of Dr. W. F. Dunning (Papanicolaou Cancer Research Institute) by unilateral subcutaneous implantation in the flanks of adult male C3H, male BALB/c, and female C3H mice, respectively. Mice were bred by Dr. W. F. Dunning. Purina laboratory chow and water were provided ad libitum during all experiments.

Incorporation of precursors into DNA. Normal or tumor-bearing mice received intraperitoneal injections of tetrahydrouridine and radioactive nucleosides dissolved in 0.9% NaCl. Mice were killed 24 hr after injection, and tissues to be studied were removed and stored at −30°.

DNA was extracted by a modification of the method of Schmidt and Thannhauser (7). Tissues were homogenized at 0° in a Sorvall OmniMixer in 15 ml of 5% perchloric acid. Precipitates were collected by centrifugation, washed twice with 30 ml of 5% perchloric acid, and extracted three times with 20 ml of ethanol–ether (1:1). When indicated in particular experiments, RNA was hydrolyzed in 10 ml of 0.5 N NaOH at 37°. DNA was reprecipitated at 0° by addition of 2 ml of 70% perchloric acid, collected by centrifugation, and washed with 20 ml of 5% perchloric acid. Nucleic acids were hydrolyzed by heating at 90° for 45 min in 5% perchloric acid. Insoluble material was removed by centrifugation.

The radioactivity of hydrolysates was determined by liquid scintillation counting. Quenching was corrected by the method of channel ratios. The deoxyribonucleotide content of hydrolysates was determined by the diphenylamine reaction (8).

RESULTS

Effect of tetrahydrouridine on incorporation of deoxycytidine into DNA. [5-^{3}H]Deoxycytidine is a specific precursor of ^{3}H-labeled DNA deoxycytidylate. It cannot be incorporated into ^{3}H-labeled DNA thymidylate, since synthesis of thymidylate will result in the loss of ^{3}H label from position 5. Control experiments, in which the incorporation of [5-^{3}H]deoxycytidine into RNA was determined by alkaline hydrolysis, demonstrated that the specific activity of RNA (disintegrations per minute per milligram) was less than 2% of that of DNA isolated from the M3445 lymphoma. Furthermore, the incorporation of [5-^{3}H]deoxyuridine into nucleic acids of the M3445 lymphoma, mouse spleen, and small intestine was less than 10% of that obtained with [5-^{3}H]deoxycytidine (Table 1). The limited incorporation of [5-^{3}H]-deoxyuridine may have resulted from the incorporation of [5-^{3}H]uracil into DNA deoxycytidylate or from radiochemical impurity (less than 5%) in the [5-^{3}H]deoxyuridine preparation. It should be noted that the radiochemical purity of the [5-^{3}H]deoxycyti-

TABLE 1

Utilization of [5-³H]deoxycytidine and [5-³H]deoxyuridine for DNA synthesis

Mice received injections 11 days after implantation of the M3445 lymphoma and were killed 24 hr after injection. The specific activity of DNA isolated from tumor and normal tissues was determined after alkaline hydrolysis of RNA for 18 hr at 37°. [5-³H]Deoxycytidine and [5-³H]deoxyuridine were administered at a dose of 10 μmoles/kg (60 μCi/μmole). Data are presented as picomoles of administered precursor incorporated per milligram of DNA, and as means ± standard deviations of the numbers of animals indicated in parentheses.

DNA precursor	Incorporated precursor		
	M3445	Small intestine	Spleen
		pmoles/mg DNA	
[5-³H]Deoxycytidine	300 ± 100 (5)	1200 ± 100 (5)	1000 ± 100 (5)
[5-³H]Deoxyuridine	20 ± 10 (5)	100 ± 30 (5)	60 ± 10 (5)

TABLE 2

Effect of tetrahydrouridine on incorporation of [5-³H]deoxycytidine into DNA of mouse neoplasms

Mice received injections 11 days after implantation of M3445 or M5183 and 12 days after M3659 implantation. Tetrahydrouridine (400 μmoles/kg) was injected 30 min before [5-³H]deoxycytidine (10 μmoles/kg, 60 μCi/μmole). Mice were killed 24 hr after injection, and the specific activity of isolated DNA was determined. Data are presented as in Table 1.

Neoplasm	[5-³H]Deoxycytidine incorporated	
	−Tetrahydrouridine	+Tetrahydrouridine
	pmoles/mg DNA	
M3445 lymphoma	310 ± 110 (16)	800 ± 170 (12)[a]
M5183 lymphoma	880 ± 200 (10)	1800 ± 340 (10)[a]
M3659 mammary adenocarcinoma	600 ± 160 (9)	1700 ± 460 (7)[a]

[a] Significantly different from mice which did not receive tetrahydrouridine ($P < 0.05$).

dine preparation was greater than 99.9% (see METHODS). These results demonstrate that the deamination of [5-³H]deoxycytidine *in vivo* would prevent the incorporation of ³H label into DNA. Therefore, if deamination is a limiting factor in deoxycytidine utilization, the incorporation of [5-³H]deoxycytidine could be increased by tetrahydrouridine administration.

The incorporation of [5-³H]deoxycytidine into the DNA of the three mouse neoplasms studied was increased 2–3-fold by tetrahydrouridine (Table 2). The optimal dose of tetrahydrouridine was 200–400 μmoles/kg, administered 30 min prior to [5-³H]deoxycytidine (Table 3). Similar dose responses were obtained when tetrahydrouridine was administered orally instead of by intraperitoneal injection. A similar effect of tetrahydrouridine on the incorporation of [5-³H]-deoxycytidine into DNA of the M3445 lymphoma was observed when [5-³H]deoxycytidine was administered at doses of 30 or 100 μmoles/kg rather than 10 μmoles/kg. Inhibition of cytidine deaminase with tetrahydrouridine also increased the incorporation of [5-³H]deoxycytidine into the DNA of mouse bone marrow and testes, but not of the intestine or spleen (Table 4). It is possible that the incorporation of exogenous deoxycytidine into DNA of the spleen or intestine is limited by the activity of anabolic pathways, and therefore is not significantly affected by inhibition of catabolism.

Effect of tetrahydrouridine on incorporation of 5-bromodeoxycytidine into DNA. Administration of tetrahydrouridine resulted in approximately 70% inhibition of the incorporation of 5-bromo[6-³H]deoxycytidine into DNA of the M3445 lymphoma, small intes-

tine, and spleen (Table 5), rather than an increase in incorporation as was observed in the case of deoxycytidine. The incorporation of higher doses of 5-bromo[6-^{3}H]deoxycytidine (50, 100, and 200 μmoles/kg) into DNA of the M3445 lymphoma and small intestine was also inhibited 50–75% by tetrahydrouridine at a dose of 400 μmoles/kg.

Effect of tetrahydrouridine on toxicity of 5-fluorodeoxycytidine. Male C3H mice received various doses of 5-fluorodeoxycytidine (0, 35, 70, 180, or 350 μmoles/kg) either with or without combined administration of tetrahydrouridine (400 μmoles/kg). Compounds were administered daily for a 4-day period by intraperitoneal injection. Tetrahydrouridine was administered 30 min prior to 5-fluorodeoxycytidine. Groups of 5–10 mice were used for each combination of drugs. Toxic deaths were observed between 1 and 2 weeks after the initial injection of chemicals. In those groups of mice which did not receive tetrahydrouridine, 4 out of 10 mice which received 350 μmoles/kg of 5-fluorodeoxycytidine died of toxicity; no deaths were observed among the mice which received lower 5-fluorodeoxycytidine doses. When tetrahydrouridine was administered together with 5-fluorodeoxycytidine, toxic deaths occurred in the groups of mice which received 70 μmoles/kg (two of five mice), 180 μmoles/kg (all of 10 mice), and 350 μmoles/kg (all of 10 mice) of 5-fluorodeoxycytidine. No toxicity (diarrhea or weight loss) was observed in mice which received tetrahydrouridine alone. These results suggest that the toxicity of 5-fluorodeoxycytidine was significantly increased by inhibition of cytidine deaminase with tetrahydrouridine.

TABLE 3

Effect of varied doses of tetrahydrouridine on [5-^{3}H]deoxycytidine incorporation into DNA of M3445 lymphoma

Mice received injections 11 days after implantation of the M3445 lymphoma. Tetrahydrouridine was administered 30 min prior to [5-^{3}H]deoxycytidine (10 μmoles/kg, 60 μCi/μmole). The specific activity of isolated tumor DNA was determined as described in Table 2.

Tetrahydrouridine	[5-^{3}H]Deoxycytidine incorporated
μmoles/kg	*pmoles/mg DNA*
0	310 ± 110 (16)
80	350 ± 100 (8)
200	840 ± 220 (15)[a]
400	800 ± 170 (11)[a]

[a] Significantly different from mice which did not receive tetrahydrouridine ($p < 0.05$).

DISCUSSION

Administration of tetrahydrouridine, a potent inhibitor of cytidine deaminase *in vitro* and *in vivo*, increased the incorporation of deoxycytidine into the DNA of mouse neoplastic tissues, bone marrow, and testes, suggesting that deamination of deoxycytidine limits its anabolic utilization in these tissues. Similarly, the toxicity of 5-fluorodeoxycytidine was increased approximately 5-fold by tetrahydrouridine administration.

TABLE 4

Effect of tetrahydrouridine on incorporation of [5-^{3}H]deoxycytidine into DNA of normal mouse tissues

Tetrahydrouridine (400 μmoles/kg) was administered 30 min prior to [5-^{3}H]deoxycytidine (10 μmoles/kg, 60 μCi/μmole) to normal C3H male mice. The specific activity of isolated DNA was determined as described in Table 2.

Tissue	[5-^{3}H]Deoxycytidine incorporated	
	−Tetrahydrouridine	+Tetrahydrouridine
	pmoles/mg DNA	
Bone marrow	780 ± 220 (9)	2000 ± 500 (9)[a]
Testes	180 ± 35 (9)	360 ± 90 (10)
Spleen	450 ± 200 (5)	360 ± 50 (5)
Small intestine	1000 ± 290 (10)	1200 ± 370 (8)

[a] Significantly different from mice which did not receive tetrahydrouridine ($P < 0.05$).

TABLE 5

Effect of tetrahydrouridine on incorporation of 5-bromo[6-^{3}H]deoxycytidine into DNA

Chemicals were administered 11 days after implantation of the M3445 lymphoma. Tetrahydrouridine (400 μmoles/kg) was administered 30 min prior to 5-bromo[6-^{3}H]deoxycytidine (10 μmoles/kg, 5 μCi/μmole). The specific activity of isolated DNA was determined as described in Table 2.

Tissue	5-Bromo[6-^{3}H]deoxycytidine incorporated	
	−Tetrahydrouridine	+Tetrahydrouridine
	pmoles/mg DNA	
M3445 lymphoma	180 ± 40 (5)	60 ± 10 (5)[a]
Small intestine	1200 ± 300 (5)	360 ± 100 (5)[a]
Spleen	720 ± 90 (5)	160 ± 40 (5)[b]

[a] Significantly different from animals which did not receive tetrahydrouridine ($p < 0.05$).

[b] Significantly different from animals which did not receive tetrahydrouridine ($p < 0.001$).

In related studies it has been shown that inhibition of cytidine deaminase by administration of tetrahydrouridine increased the antileukemic activity of cytosine arabinoside (1). Previous work in our laboratory (9) has demonstrated that the incorporation of pyrimidine bases into nucleic acids *in vivo* is also limited by the catabolic activity of dihydrouracil dehydrogenase. Catabolism of purine bases has similarly been shown to limit their utilization for nucleic acid synthesis *in vivo* (10, 11).

Maley and Maley (12) have shown that tetrahydrodeoxyuridylate inhibits deoxycytidylate deaminase, and that tetrahydrodeoxyuridylate increases the incorporation of deoxycytidylate into DNA deoxycytidylate in cell-free chick embryo extracts. These results are similar to those obtained in the present investigation; however, in the work of Maley and Maley (12) deamination was inhibited at the level of deoxycytidylate deaminase rather than cytidine deaminase. It is unlikely that our results with tetrahydrouridine reflect inhibition of deoxycytidylate deaminase, since neither tetrahydrouridine nor tetrahydrouridylate is an effective inhibitor of this enzyme (12).

In contrast to the increased utilization of deoxycytidine for DNA synthesis which resulted from inhibition of cytidine deaminase, the incorporation of 5-bromodeoxycytidine into DNA was inhibited by tetrahydrouridine. This suggests that the incorporation of 5-bromodeoxycytidine into DNA proceeds by deamination of the nucleoside to 5-bromodeoxyuridine, followed by phosphorylation by thymidine kinase. The alternative pathway, involving the sequential activities of deoxycytidine kinase and deoxycytidylate deaminase, appears to be relatively inactive. This conclusion is consistent with the observation that P815Y murine neoplastic mast cells lacking thymidine kinase are resistant to the cytotoxic effects of 5-bromodeoxycytidine (4). In the accompanying paper (13) we show that the incorporation of 5-bromodeoxycytidine into DNA by the deoxycytidine kinase–deoxycytidylate deaminase pathway is limited by the restricted substrate specificity of deoxycytidine kinase. In contrast to 5-bromodeoxycytidine, 5-fluorodeoxycytidine is shown to be a good substrate for deoxycytidine kinase (13). This is consistent with the increased toxicity of 5-fluorodeoxycytidine which resulted from tetrahydrouridine administration and with the observation (4) that P815Y cells lacking thymidine kinase retain their sensitivity to the cytotoxic effects of 5-fluorodeoxycytidine.

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