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In Vivo Kinetics of Thymidylate Synthetase Inhibition in 5-Fluorouracil-sensitive and -resistant Murine Colon Adenocarcinomas[1]

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ABSTRACT

The predictive utility of several biochemical parameters of 5-fluorouracil (5-FUra) action was evaluated in four murine colonic adenocarcinomas: 5-FUra-sensitive Tumor 38 and 5-FUra-resistant Tumors 07/A, 51, and 06/A. Thymidylate synthetase (TS) was determined by a tritiated 5-fluoro-2′-deoxyuridylate (FdUMP)-binding assay. Bolus 5-FUra (80 mg/kg, i.p.) administration caused in all tumors a rapid decrease in free TS levels. Only Tumor 38, however, showed inhibition of TS to undetectable (<0.05 pmol/g) levels, which lasted up to 6 hr after treatment; correction for dissociation of endogenous TS:FdUMP:folate ternary complex during the TS assay was required. Total TS (free enzyme plus ternary complex) was determined with experimental conditions that achieved quantitative recovery of free TS from ternary complex. By 48 hr after 5-FUra, Tumor 38 showed a decrease in total TS proportional to the estimated log kill/dose of 5-FUra; in contrast, the resistant tumors showed no such decrease from pretreatment levels. Assay of FdUMP showed that the free nucleotide was formed rapidly in all tumors in excess over available TS-binding sites. However, tumor sensitivity did not correlate with peak or residual FdUMP levels or with deoxyuridylate levels, which were low and remained so in all tumors. Tumor sensitivity to 5-FUra also could not be explained by the small differences among the tumors in total perchloric acid-soluble metabolites of 5-FUra or drug incorporation into RNA. We conclude from these data that levels of free TS in the tumor after 5-FUra treatment are predictive of chemotherapeutic response in these murine models of human colonic adenocarcinoma.

INTRODUCTION

The antitumor drug 5-FUra[3] is widely used as a palliative treatment for disseminated internal cancers and as curative therapy for several kinds of epithelial neoplasms (1, 9). The principal mechanism of cytotoxicity of 5-FUra has long been thought to result from the inhibition of TS, which provides the only *de novo* source of thymidylate for DNA synthesis (17). TS is inactivated rapidly in the presence of the 5-FUra metabolite, FdUMP, and CH_2FH_4, by the formation of an enzyme:FdUMP:CH_2FH_4 covalently bonded ternary complex (10, 25, 43), from which native TS is slowly released (11, 29, 48).

Evidence that TS inactivation is the critical therapeutic effect of 5-FUra has been largely indirect, because of difficulties in the assay of low, growth-limiting levels of TS. Recent studies using a sensitive tritium release TS assay (42), however, have indicated correlations between 5-FUra cytotoxicity and decreases in tumor TS activity (3, 12). *In vivo,* P388 mouse leukemia ascites cells sensitive to 5-FUra show a lower rate of recovery of TS activity after 5-FUra treatment than do cells that have acquired resistance to 5-FUra (3). Mouse cells sensitive to 5-FUra (Sarcoma 180, MMT, and L-cells) show lower TS activities following continuous *in vitro* 5-FUra exposure (10 μM for 3 hr) than do human cells (HeP-2, HeLa, and KB) that have some innate resistance to 5-FUra (12).

Resistance to 5-FUra, both innate and acquired, has often been explained by metabolic events that could contribute to diminished TS inactivation. These events include decreases in enzymes that anabolize 5-FUra (3, 17, 22, 26, 40) and increases in 5-FUra catabolism (17), resulting in inadequate FdUMP formation (2) or persistence (24, 30, 36). TS inhibition can lead to massive dUMP accumulation (2, 33, 36, 37); this effect might limit enzyme inhibition because of competition between dUMP and FdUMP for binding to TS. With correction for dUMP pool expansion, the rate of DNA synthesis measured by the incorporation of tritiated deoxyuridine into DNA has also been used as an indication of the degree of TS inhibition (36). The innate resistance (15) of murine colon Tumor 51 to 5-FUra treatment has, thus, been related to rapid recovery of tritiated deoxyuridine uptake into DNA (2). Several examples have been described of increased TS activity accompanying acquired resistance to 5-fluorodeoxyuridine (4, 39, 50). Another factor that contributes to incomplete TS inhibition by FdUMP and concomitant acquired resistance includes an alteration in the affinity of TS for FdUMP (18). Insufficient concentrations of intracellular folate cofactor for optimal binding of FdUMP to TS have also been suggested to mediate the innate 5-FUra resistance of several human colon carcinoma xenografts (19).

Thymidine administration should bypass the *de novo* block to synthesis of thymidylate but does not always relieve the cytotoxic effects of 5-FUra (12, 31). This phenomenon and other observations (7, 30, 45, 49) have suggested the chemotherapeutic importance of a second site of action of 5-FUra, namely, its effects on RNA metabolism. The most important of these effects is probably drug incorporation into RNA and consequent inhibition of rRNA maturation (17). Several investigators have observed decreased drug incorporation into RNA in 5-FUra-resistant cells, together with decreased total acid-soluble 5-FUra metabolites (predominantly ribonucleotides) (3,

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[3] The abbreviations used are: 5-FUra, 5-fluorouracil; TS, thymidylate synthetase; FdUMP, 5-fluoro-2′-deoxyuridylate; dUMP, 2′-deoxyuridylate; CH_2FH_4, L-(+)-5,10-methylenetetrahydrofolate; TS_{tot}, total thymidylate synthetase; TS_f, free non-5-fluoro-2′-deoxyuridylate-bound thymidylate synthetase; TS_b, ternary complex 5-fluoro-2′-deoxyuridylate-bound thymidylate synthetase; FH_4, L-(+)-tetrahydrofolate; TS_f^{app}, experimentally determined [^{3}H]5-fluoro-2′-deoxyuridylate-binding sites.

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14). Enhancement of 5-FUra ribonucleotide formation by the administration of ribose donors, unfortunately, does not increase the therapeutic efficacy of 5-FUra in murine leukemias (21). The relationship between the effects of 5-FUra on TS and RNA is poorly understood. It appears, however, that these 2 effects have maximal expression in S and G_1 phases, respectively, thus requiring cell cycle traverse for both toxicities to occur (31). TS activity is not significantly inhibited by the ribonucleotide, 5′-fluorouridylate (16, 33).

We recently reported highly specific methods for the assay of TS, FdUMP, and dUMP that are considerably more sensitive than previous methods (33). The enzyme assay was modified in the current study in order to determine both TS_{tot} and TS_f, and by the difference, TS_b. Application of these methods is now reported for 4 chemical carcinogen-induced transplantable murine colonic adenocarcinomas, including the 5-FUra-sensitive colon Tumor 38. Our study, which also includes measurement of total acid-soluble 5-FUra metabolites and incorporation of drug into RNA, suggests that chemotherapeutic sensitivity to bolus, parenteral 5-FUra is closely related to reduction of TS_f to undetectable levels after 5-FUra treatment in these tumors.

MATERIALS AND METHODS

Materials. [6-^{3}H]FdUMP and 5-[6-^{3}H]FUra were purchased from Moravek Biochemicals, Brea, Calif. [^{14}C]Formaldehyde was purchased from New England Nuclear, Boston, Mass. Purified *Lactobacillus casei* TS and FH_4 were prepared as reported (33). Purified TS from human CCRF-CEM lymphoblasts (29) was a gift of Dr. Arnold Lockshin. FdUMP was a gift of Dr. Peter V. Danenberg. DEAE-cellulose was obtained from Eastman Organic Chemicals, Rochester, N. Y. 5-FUra was from Roche, Inc., Nutley, N. J. All other reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Buffer A consisted of 0.6 M NH_4HCO_3, pH 8.0, containing 100 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM cytidylate. Buffer B consisted of 50 mM potassium phosphate buffer, pH 7.4, with 20 mM 2-mercaptoethanol, 100 mM NaF, 15 mM cytidylate, and 2% bovine serum albumin. Cofactor solution consisted of Buffer B which also contained 2 mM FH_4, 16 mM sodium ascorbate, and 9 mM formaldehyde. All buffers were sterilized before use by 0.22-μm membrane filtration (Millipore Corp., Bedford, Mass.).

Tumor Models. The 4 colon adenocarcinomas of this study originated in BALB/c mice (Tumors 51, 06/A, and 07/A) or C57Bl/6 mice (Tumor 38) following s.c. 1,2-dimethylhydrazine hydrochloride (15). Histologically, they all showed intermediate grades of differentiation, although Tumor 51 was mucin producing. The tumors were passaged in the host of origin by axillary transplantation and were studied at transplant generations 18 to 23 (Tumor 38), 17 to 23 (Tumors 07/A), 75 to 80 (Tumor 51), and 21 to 27 (Tumor 06/A).

For chemotherapeutic sensitivity experiments, Tumor 38 was transplanted into C57Bl/6 × DBA/2 (hereafter called $BD2F_1$) mice and the other 3 tumors were transplanted into BALB/c × DBA/2 (hereafter called $CD2F_1$) mice. Tumor size was calculated using length and width caliper measurements by the formula for an ellipse in revolution about its long axis and was determined twice weekly. Chemotherapy with 5-FUra was begun when the tumors grew to the 100- to 300-mg size range (about 1 to 3 × 10^8 cells); mice with tumors not in the desired range were excluded. Techniques of chemotherapy and data analysis, including determinations of percentage increase in host life span, tumor growth delay, and $\log_{10}$ kill/dose, have been detailed elsewhere (8, 15) and are summarized in the footnotes to Table 1. Patterns of tumor metastasis and drug sensitivities have been reported for colon Tumors 38 and 51 (8).

For the biochemical studies, a single dose of 5-FUra, 80 mg/kg, was given i.p. to mice bearing 21-day-old tumors (average size, 1.0 ± 0.10 g). In each experiment, 2 pretreatment mice and one mouse/time point after 5-FUra were sacrificed by cervical dislocation, and s.c. tumor freed of epidermal attachments was immediately frozen in dry ice or liquid N_2 in polyethylene vials. Data shown are the mean values of replicate experiments, as indicated in the legends.

General Methodology. Tumors were thawed at 4° and placed in a 4-fold excess of 0.2 M Tris-HCl buffer, pH 7.4, containing 20 mM 2-mercaptoethanol and the phosphatase inhibitors cytidylate (15 mM) and NaF (100 mM). The tissues were disrupted by use of a ground-glass hand homogenizer followed by sonication with the medium probe of a Braunsonic 1510 at 100 watts, using 20-sec bursts 3 to 5 times with ice:salt cooling. Aliquots of the crude sonicates were removed for 105,000 × *g* centrifugation and TS assay of the cytosol supernatant (175 μl), for 1 M acetic acid extraction of nucleotides (300 μl), and for DNA and RNA assays (100 μl). The acetic acid extracts were lyophilized to dryness and dissolved in 2 ml of 5 mM potassium phosphate buffer, pH 7.2, and the 2400 × *g* supernatants were applied to DEAE-cellulose minicolumns (1.0 x 3.0 cm) for NH_4HCO_3 (pH 8.0) separation prior to assay of FdUMP and dUMP as described previously (33) and briefly outlined in the legends to Charts 4 and 5.

Nucleic acids in the crude sonicates were extracted by a modified Schmidt-Tannhauser procedure (35), following cold 0.5 M perchloric acid precipitation and extraction of phospholipids in the pellet with 95% ethanol (with 2% sodium acetate, pH 8.0) and ethanol:ether (3:1). RNA was digested overnight by 0.3 N KOH at 37° and determined in the perchloric acid-soluble fraction by the orcinol reaction (20). DNA in the pellet was extracted 3 times by heating at 70° for 15 min with 1.5 N perchloric acid and assayed by a modified Burton procedure (41).

TS Assay. The TS assay as reported (33) was modified in order to quantitate total cytosolic [^{3}H]FdUMP-binding sites present either as TS_f or TS_b.

TS_{tot} was determined experimentally by causing the TS_b present in the cytosol to fully dissociate to TS_f at pH 8.0 in a preincubation period prior to the addition of [^{3}H]FdUMP. Dissociation of TS_b was achieved by addition of 50 μl of Buffer A to 50 μl of the 105,000 × *g* cytosol and incubating the mixture for 3 hr at 30°. TS_{tot} levels were then assayed (33) by the addition of 6 pmol of [6-^{3}H]FdUMP (18 Ci/mmol) in 50 μl of 5 mM potassium phosphate buffer, pH 7.4, plus 25 μl of cofactor solution. The concentration of TS_f^{app} was determined in parallel, by omission of the preincubation dissociation period and addition of Buffer A together with the [^{3}H]FdUMP and cofactor solutions. TS_f was calculated from the results of TS_{tot} and TS_f^{app} determinations (see below). The tubes were incubated in triplicate at 30° for 20 min. Then a cold 1.0-ml slurry of albumin- and T-70 dextran-treated 3% (w/v) activated charcoal (0.1 N in HCl) was added; this permitted isolation of protein-bound [^{3}H]FdUMP in the supernatant by 400 × *g* centrifugation for 20 min. Results were calculated using Buffer B as a blank in place of the 105,000 × *g* supernatant and, as a standard, either purified TS obtained from human CCRF-CEM cells (29) or purified *L. casei* TS (33) at pH 7.4. These conditions gave a standard curve with a slope of 68 dpm [^{3}H]FdUMP bound per fmol TS_f. The results were corrected for [^{3}H]FdUMP isotope dilution by free FdUMP present in the cytosols.

Assay results were unaffected by repeated freeze-thawing of enzyme in the crude sonicates. TS_f was completely stable over the 3-hr preincubation period in the presence of 50 mM 2-mercaptoethanol. Variation in assay pH from 7.4 to 8.4 did not significantly affect [^{3}H]FdUMP binding to TS_f partially purified from CCRF-CEM cells.

Precise quantitation of TS_f in the presence of TS_b required correction for the rate of exchange of [^{3}H]FdUMP into cytosolic nonlabeled ternary complex during the 20-min assay. This rate of exchange (k_e) was experimentally determined as follows. To 0.5 ml of the high-speed supernatant obtained from untreated tumor were added 0.1 ml of Buffer B containing 0.5 μM FdUMP and 0.233 ml of cofactor solution. After 30° incubation for 20 min, an equal volume of cold, neutral 10% (w/v) charcoal was added, and the mixture was vortexed and centrifuged to

separate ternary complex from charcoal-bound FdUMP and cofactor. A 50-μl aliquot of the 4000 × *g* supernatant was added to 50 μl of Buffer A, and the TS_f^{app} activity was then determined as above by addition of [^{3}H]FdUMP and cofactor solution, as a function of time at 30° before addition of charcoal.

Incorporation of 5-FUra into RNA. In separate experiments, mice were given 5-[6-^{3}H]FUra (7 mCi/mmol) at 80 mg/kg. The perchloric acid-, lipid-extracted pellets were digested by KOH and assayed for RNA and DNA as above. Although lipid solvent-extractable radioactivity was negligible, omission of the organic solvent step markedly reduced RNA recovery. Radioactivity associated with DNA was regularly less than 1% of that present in tissue sonicates. The low level of radioactivity used in this study permitted the routine performance of TS, FdUMP, and dUMP assays, without correction for increased background.

RESULTS

Tumor Response to 5-FUra Treatment. Colon Tumor 38 showed moderate sensitivity to 5-FUra given i.p. at maximally tolerated dosage to mice bearing relatively advanced (about 200-mg) s.c. tumors. The indices of therapeutic response, the percentage increase in life span and the tumor growth delay shown in Table 1, are only somewhat lower than values resulting from 5-FUra treatment of early-stage (about 60-mg) disease and are also similar to results obtained with 5-fluorodeoxyuridine (8). The more relevant parameter may be the tumor growth delay time, which is analogous to the period of clinical remission that describes response duration in patients. In the other 3 tumors, 5-FUra did not show significant evidence of chemotherapeutic activity, although colon Tumor 07/A had been modestly responsive at earlier transplant generations (data not shown).

Sensitivity to 5-FUra did not correlate with tumor growth rate or metastatic potential. Tumor volume-doubling times were 3.4, 2.7, 4.2, and 3.2 days for colon Tumors 38, 07/A, 51, and 06/A, respectively, and were similar to growth rates observed in the mice used for passage. In the latter animals, this corresponded to an average radial growth of 0.35 ± 0.13 (S.E.) mm/day for the 4 tumor types. In separate studies of untreated mice with far-advanced disease, the incidence of gross pulmonary metastasis was found to decrease in the order Tumor 51 (100%), Tumor 06/A (>75%), Tumor 38 (<50%), and Tumor 07/A (<20%).

Labeling of TS_b by [^{3}H]FdUMP. Chart 1 shows the results of measuring the rate of exchange of [^{3}H]FdUMP into preformed unlabeled ternary complex in the TS assay, using the 105,000 × *g* cytosolic enzyme obtained from untreated CCRF-CEM cells (33), colon Tumors 38 and 06/A, or a human breast adenocarcinoma. Control values (*i.e.*, total [^{3}H]FdUMP-binding sites) in these experiments were obtained from the results of tubes carried in parallel but without the addition of nonlabeled FdUMP. At high CH_2FH_4 concentration, the rate of appearance of radioactivity in ternary complex, k_e, is governed by the rate of ternary complex dissociation, which is slow compared to the rate of ternary complex formation (11, 29, 43). Linear regression by least-squares analysis of the combined data up to 90 min ($N = 23$; $r = 0.9633$) showed a k_e value of 6.37×10^{-3} min^{-1}. Thus, regardless of the tumor source, about 13% of TS_b dissociated and rapidly reformed ternary complex containing [^{3}H]FdUMP during the 20-min TS assay incubation period and appeared as TS_f. TS_f was therefore calculated from the following relationship:

$$TS_f = TS_{tot} - TS_b = (TS_f^{app} - 0.13\ TS_{tot})/0.87$$

where TS_f^{app} is the experimentally determined concentration of [^{3}H]FdUMP-binding sites present at the end of 20 min in the standard TS assay. The k_e value for CCRF-CEM TS at pH 7.4 was 4.14×10^{-3} min^{-1}, or 65% of the rate at pH 8.0.

The determination of TS_{tot} is therefore highly useful for increased accuracy in the assay of TS_f. This is particularly true if the enzyme is predominantly in the form of the ternary complex. Assay of TS_{tot} depends on the full recovery of native FdUMP-binding sites from the ternary complex by complete

Table 1

Advanced-stage[a] colon adenocarcinomas: therapeutic effects of 5-FUra treatment[b] and parameters of TS target enzyme response[c]

	5-FUra response			TS (pmol/g)		
				TS_f		
Tumor	%ILS[d]	T − C[e]	log_{10} kill[f]/dose	Pretreatment	Nadir	TS_{tot} at 48 hr
06/A	0	0.6	0.01	150.2	11.1	163.5
51	−17	1.5	0.03	28.0	1.9	38.9
07/A	−25	2.0	0.06	21.3	7.5	30.1
38	58	11.1	0.25	32.1	≤0.05[g]	16.9

[a] s.c. tumors (20- to 30-mg transplantation size) were allowed to grow to 100- to 300-mg size range prior to 5-FUra treatment.

[b] 5-FUra given at the maximally tolerated dosage of 50 mg/kg/day i.p. a total of 4 times at 4-day intervals beginning Day 11 (Tumor 06/A), Day 15 (Tumor 51), and Day 13 (Tumors 07/A and 38) after transplantation.

[c] TS responses based on single-dose 5-FUra given 80 mg/kg i.p. on Day 21 after transplantation.

[d] Percentage increase in life span, based on median survival after transplantation compared to control mice ($N = 10$ in each group); control mice lived 34 days (Tumor 38), 57 days (Tumor 07/A), 99 days (Tumor 51), and 34 days (tumor 06/A).

[e] Tumor growth delay, treated minus control ($T - C$): the time lag, in days, for treated tumors to reach a predetermined size (1250 mg) compared to controls; the median time to reach 1250 mg in control mice was 25 days (Tumor 38), 22 days (Tumor 07/A), 39 days (Tumor 51), and 21 days (Tumor 06/A).

[f] Log_{10} kill/dose = $[(T - C) \times \log 2/Td \times$ number of doses], where Td = the tumor volume-doubling time, in days, at the 100- to 400-mg size range.

[g] Represents the lower limit of TS_f detection.

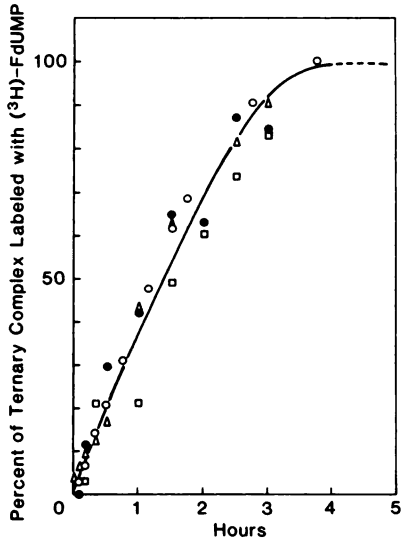

Chart 1. Rate of exchange of [^{3}H]FdUMP into ternary complex (k_e) in the TS assay. The rate of [6-^{3}H]FdUMP exchange into preformed, charcoal-isolated enzyme:FdUMP:CH_2FH_4 ternary complex is shown for standard pH 8.0 TS assay conditions at 30° using 286 μM FH_4 and enzyme obtained from colon Tumor 38 (●), colon Tumor 06/A (○), CCRF-CEM cells (△), and a human breast adenocarcinoma (□). Experimental details are given in "Materials and Methods." The results indicate that 13% of TS_b, regardless of the source, dissociates during the 20-min TS assay and appears as TS_f^{app}.

dissociation of the complex during the 3-hr preincubation period. For the determination of TS_{tot}, no added cofactor is present in the preincubation mixtures, in which cytosolic folates present are lowered 90% by dilution. Low preincubation reduced folate concentrations were expected to decrease the rate of ternary complex formation so that equilibrium at 3 hr favored the TS_f state. To demonstrate the absence of appreciable ternary complex formation at low preincubation FH_4 concentrations, 0.38 pmol of TS from untreated CCRF-CEM cells was labeled with 6 pmol of [^{3}H]FdUMP in a final volume of 250 μl using standard preincubation conditions, except that added FH_4 was varied from 0 to 1 mM final concentration and 1.0 mM formaldehyde and 1.6 mM ascorbate were present. At the end of 3 hr, protein-bound radioactivity (determined by charcoal absorption) obeyed the relationship, $TS_b/TS_f = [FH_4]/51.3\ \mu M$, which was linear ($r = 0.9995$; $N = 10$) down to 0.5 μM FH_4. Thus, for example, at a FH_4 concentration of 0.5 μM in the preincubation mixture using colon tumor cytosols, only 1.0% of the available enzyme sites would have been bound by cytosolic FdUMP (assuming [FdUMP] $\geq$ [TS]).

Several lines of experimental evidence indicate that full recovery of enzyme in the TS_f state occurs during the 3-hr preincubation. (*a*) Treatment of tumor cytosols with 10% neutral charcoal before enzyme assay permitted separation of ternary complex(es) and TS_f from endogenous folates and nucleotides; assay of charcoal-treated 4000 × *g* supernatant fractions for TS_f and TS_{tot} at all time points after 5-FUra treatment revealed no significant differences from results obtained without treatment of these cytosols with charcoal. (*b*) Examination of neutral charcoal-treated cytosols of Tumors 38 and 51, 1 hr after 5-FUra administration, showed that the rates of increase in [^{3}H]FdUMP-binding sites during the preincubation period, after the period of rapid labeling of free enzyme, were 6.86×10^{-3} min^{-1} and 6.22×10^{-3} min^{-1}, respectively. These rates are indistinguishable from k_e values obtained using ternary complex formed with FH_4 prior to [^{3}H]FdUMP addition. Thus, under our TS assay conditions, the contribution of cytosolic folylpolyglutamates to k_e appears to be negligible. (*c*) Extension of the preincubation period to 4 hr caused less than a 3% increase in TS_{tot} in these tumors. (*d*) There was no significant difference between TS_f and TS_{tot} analyzed in cytosols from untreated tumors, and the differences between pretreatment TS_f values and TS_{tot} at short times after 5-FUra were quite small (see below). Therefore, we conclude that TS_{tot} determinations at short times after 5-FUra exposure yield excellent approximations of pretreatment TS_f values.

***In Vivo* Kinetics of TS Inhibition.** The results of TS_f and TS_{tot} determination for the 4 colon tumors are shown in Charts 2 and 3 and are summarized in Table 1. Pretreatment values of TS_f showed wide variation among the tumor lines but did not correlate with the response to 5-FUra or with the biological behavior of the tumors. However, Tumor 06/A, possibly the most 5-FUra resistant (Table 1), possessed 4 to 6 times higher pretreatment TS_f than the other tumors.

Within the first hr after 5-FUra administration, all tumors showed a profound decrease in TS_f (Chart 2). Most striking was the TS_f reduction found for 5-FUra-sensitive colon Tumor 38, in which TS_f was undetectable during the period 0.5 to 6 hr after 5-FUra administration. Colon tumor 38 also showed the lowest TS_f of the 4 tumors at 12 and 24 hr following treatment. The time course of the increase in TS_f subsequent to initial TS_f

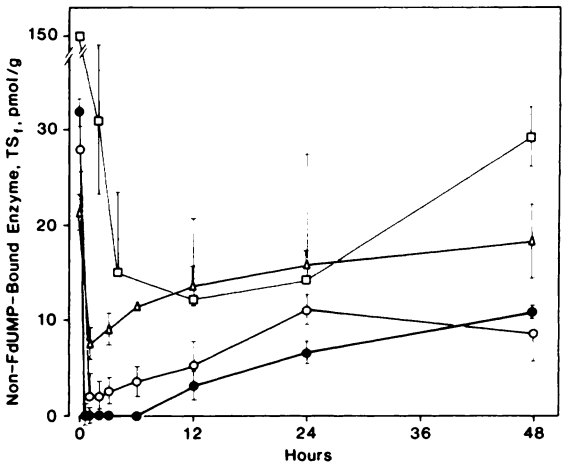

Chart 2. *In vivo* kinetics of inhibition of TS_f in colon tumors following 80 mg 5-FUra per kg i.p. The results are corrected for labeling of TS_b as described in "Results." *Point*, average of 2 separate experiments for colon Tumors 38 (●) 51 (○), 07/A (△), and 06/A (□); *bars*, S.D.; S.D.s of the 2-, 3-, and 6-hr time points of Tumor 38 were less than 0.3 pmol/g.

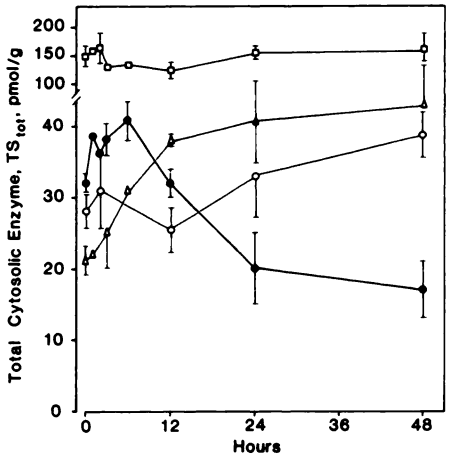

Chart 3. *In vivo* kinetics of change in TS_{tot} in colon tumors following 80 mg 5-FUra per kg i.p. *Point*, average of 2 separate experiments for colon Tumors 38 (●), 51 (○), 07/A (△), and 06/A (□); *bars*, S.D.

inhibition was similar for the tumors with similar pretreatment TS_f levels, namely, Tumors 38, 07/A, and 51. The increase in TS_f at 48 hr was greatest for Tumor 06/A, which had the highest pretreatment TS_f. From the pretreatment growth rates and TS_f values of these tumors, it can be calculated that up to one-half of the regeneration of TS_f seen in Chart 2 is attributable to new tumor growth.

The 24- and 48-hr TS_{tot} levels in 5-FUra-sensitive colon Tumor 38 were about one-half of its pretreatment level (Chart 3). In marked contrast, tumor TS_{tot} either increased or showed no significant change from pretreatment values through 48 hr following 5-FUra administration to mice bearing the resistant colon tumors 06/A, 51, and 07/A. The decrease in TS_{tot} of Tumor 38 was due to a decrease in TS_b, since the TS_f steadily increased after initial inhibition. The TS_b decrease was not offset by an equivalent increase in TS_f and therefore could not be solely attributable to intracellular ternary complex dissociation. A likely cause of this decrease in TS_b in Tumor 38 was cell death, since the estimated $\log_{10}$ kill/dose of 0.25 (Table 1) predicted a 44% decrease in viable cell number and hence, presumably, a corresponding decrease in TS. The early, tran-

sient increases in TS_{tot} observed in Tumors 07/A and 38 were suggestive of a cell synchrony effect (38). These data shown in Charts 2 and 3 were not appreciably changed when normalized by tissue DNA content.

Nucleotide Assays. No correlation was found between the sensitivity to 5-FUra (Table 1) and either peak or persistent levels of FdUMP (Chart 4). Intracellular FdUMP formation in the 4 murine colon tumors was rapid. The highest levels of the free nucleotide were found at the earliest time point evaluated, 0.5 hr. All tumors showed a sharp decrease in free FdUMP concentration at 1 hr, synchronous with the inhibition of TS_f and formation of ternary complex. In Tumors 38 and 51, however, ternary complex formation could not account for most of the free FdUMP losses. The ratio of peak FdUMP to pretreatment TS_f generally correlated with the nadir of TS_f inhibition. However, this ratio was highest for Tumor 51, which showed incomplete TS_f inhibition. Free FdUMP persisted at low levels (<60 pmol/g) throughout the period of study; in Tumors 06/A and 07/A, free FdUMP after 6 hr was consistently lower than available TS_f-binding sites.

Studies with [^{3}H]FdUMP-labeled ternary complex showed that, although ternary complex was extracted into the acetic acid supernatant, 0.0% and <0.2% of the radioactivity cochromatographed with the dUMP and FdUMP fractions on DEAE-cellulose, respectively. Hence, our method of estimation of free FdUMP excludes FdUMP bound to enzyme.

Pretreatment dUMP levels in the 4 murine tumors were similarly low (3 to 8 nmol/g) and did not increase following 5-FUra treatment (Chart 5).

Nucleic Acid Content. DNA contents of colon Tumors 38, 07/A, 51, and 06/A were respectively 19.9, 21.3, 9.8, and 12.5 mg/g, wet weight. RNA contents were 8.1, 7.5, 4.2, and 6.6 mg/g, respectively. These values are similar to those of normal rodent intestine (27).

Incorporation of 5-FUra into RNA. Colon Tumor 38 showed a slightly higher concentration of total perchloric acid-soluble 5-[^{3}H]FUra metabolites than the other tumors, at 3, 12, and 24 hr after drug administration (Table 2). However, this small difference was not apparent when the results were normalized on the basis of tumor RNA content. Peak acid-soluble metabolite levels were found at the earliest post-5-FUra time point studied, 3 hr, for all tumors except 06/A, which showed fairly constant metabolite levels through 48 hr. The perchloric acid-soluble metabolites accounted for approximately 90% of the total radioactivity found in the crude sonicates.

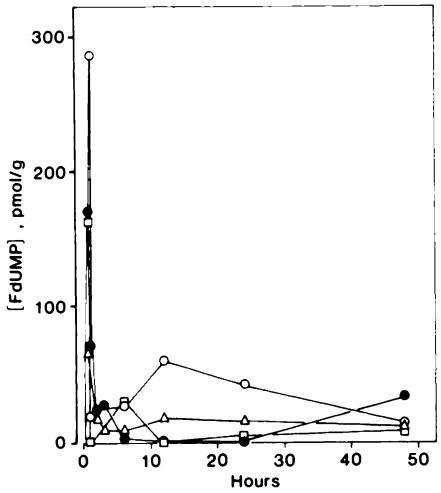

Chart 4. Colon tumor free FdUMP concentration as a function of time after 5-FUra administration. DEAE column-separated FdUMP fractions, obtained from lyophilized acetic acid extracts of tissue sonicates, were assayed in quadruplicate by measuring the competitive, isotope dilution effects of FdUMP on [^{3}H]FdUMP binding to *L. casei* TS_f in the presence of excess CH_2FH_4 (33). These results plus concurrent [(TS_{tot} − TS_f) × 1.7] data give estimates of total tumor FdUMP levels. *Point*, average of 3 separate experiments. Tumors studied and symbols used are those of Chart 2.

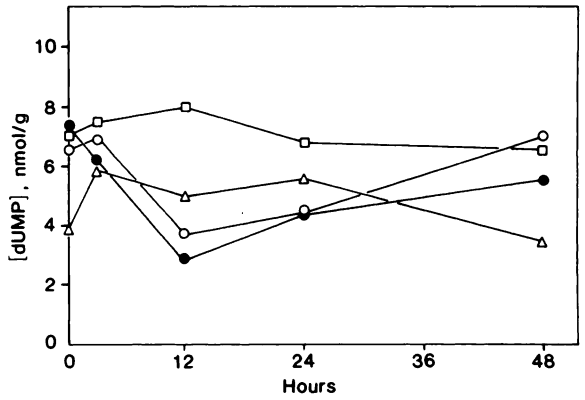

Chart 5. Colon tumor dUMP concentration at time points after 5-FUra administration. Samples derived from DEAE column-isolated dUMP fractions were obtained from acetic acid extracts and were incubated with [^{14}C]CH_2FH_4 and *L. casei* TS_f. Quantitative conversion of dUMP to [^{14}C]thymidylate was measured after ammonium formate:DEAE column removal of [^{14}C]formaldehyde as described (33). *Point*, average of 3 separate experiments. Tumors studied and symbols used are those of Chart 2.

The specific activities of RNA isolated from Tumors 38, 07/A, and 51 were not significantly different. The highest levels of 5-FUra incorporation into RNA of these tumors were at the earlier time points and represented about 1 fluororibonucleotide base/600 ribonucleotides. Colon Tumor 06/A showed slower drug incorporation into RNA,which paralleled the slower rate of TS_f inhibition and slower acid-soluble metabolite formation in this tumor. 5-FUra incorporation into RNA was a higher proportion of total acid-soluble metabolites (25%) in Tumor 07/A than in Tumor 38 (14%), Tumor 51 (9%), or Tumor 06/A (12%). There was no obvious relationship between 5-FUra incorporation into RNA and FdUMP levels, although differences among the tumors in any case were not great.

DISCUSSION

5-FUra-sensitive colon Tumor 38 showed quantitative and qualitative differences from the 3 resistant tumors in the time course of inhibition of TS (Charts 2 and 3). TS_f was undetectable up to 6 hr after chemotherapeutic 5-FUra administration in Tumor 38; standard deviations showed no overlap with TS_f values of the 5-FUra-resistant tumors during this time period. The resistant tumors never had less than 1.9 pmol of TS_f per g, which apparently was sufficient to allow pretreatment rates of tumor growth. The rate of return of TS_f levels after initial inhibition was similar in the 4 tumors and was probably twice the rate attributable to renewed or continued tumor growth alone. Intracellular enzyme in Tumor 38 was entirely in the inactive TS_b state up to 6 hr after 5-FUra treatment. By 48 hr, TS_{tot} in Tumor 38 declined 47% from its pretreatment level, in agreement with the estimated $\log_{10}$ kill/dose of 0.25, or 44% cell kill/dose (Table 1). These results are compatible with our hypothesis that reduction in TS_f to growth rate-limiting levels

Table 2

Studies with [6-^{3}H]5-FUra: tumor uptake into total perchloric acid-soluble metabolites and incorporation into tumor RNA

	Total acid-soluble 5-FUra metabolites (nmol/g)				Incorporation of 5-FUra into RNA (nmol/mg RNA)			
	3[a]	12	24	48	3	12	24	48
Tumor 38	176 ± 25[b]	116 ± 30	129 ± 9	71 ± 40	3.2 ± 1.6	4.9 ± 1.2	1.2 ± 0.4	2.1 ± 1.1
Tumor 51	138 ± 3	102 ± 4	80 ± 15	79 ± 9	2.8 ± 1.4	5.0 ± 1.5	1.2 ± 0.8	2.2 ± 0.3
Tumor 07/A	131 ± 3	75 ± 1	73 ± 3	54 ± 9	4.0 ± 1.1	3.1 ± 0.4	3.1 ± 0.5	1.6 ± 0.2
Tumor 06/A	82 ± 31	109 ± 10	100 ± 20	88 ± 3	0.5 ± 0.02	3.1 ± 1.6	3.9 ± 0.6	1.0 ± 0.7

[a] Time of sacrifice (hr) after 5-[6-^{3}H]FUra.
[b] Average ±S.E. of 5-[6-^{3}H]FUra (7 mCi/mmol) given i.p. at 80 mg/kg to 3 tumor-bearing mice/time point.

ultimately resulted in death of cells containing high amounts of TS_b relative to TS_f levels. High amounts of TS_b *per se* would not be expected to correlate with cytotoxicity, as shown by the results in Tumor 06/A.

Although the ternary complex among TS, FdUMP, and folate cofactor is covalently linked, the reaction that results in complex formation is reversible (11). This implies that dissociation of the complex in the cell would allow a recovery of enzyme activity in the presence of excess dUMP as has been discussed previously (2, 33, 36, 37). Moreover, this reversibility has major practical importance for the measurement of TS_f using any technique that employs either V_{max} determination in the presence of excess dUMP or labeling of active sites with [^{3}H]-FdUMP. Thus, spectrophotometric (47) or tritium release (3, 12, 42) assays of enzyme activity in the presence of substantial levels of TS_b will overestimate TS_f, unless the results are extrapolated to true initial rates. Likewise, titration of TS_f with [^{3}H]FdUMP (19, 33, 43) must be corrected for the rate of TS_b dissociation that occurs during the period of exposure to [^{3}H]-FdUMP, since exchange of [^{3}H]FdUMP with FdUMP in TS_b occurs at a modest rate (Chart 1). Hence, the technique discussed in this report for the determinations of TS_{tot} and TS_f on a single sample allows an accurate estimate to be made of how much enzyme was, in fact, free for *de novo* synthesis of thymidylate in the tissue. Our previous estimates of TS_f in 5-FUra-inhibited CCRF-CEM cells had suggested that approximately 20% of the TS_{tot} content of these cells was present as TS_f in spite of the presence of excess FdUMP; in view of the data of Chart 1, this clearly was an overestimate. The potential for error in TS_f determinations is illustrated by our observation that the storage of colon tumor cytosols at 4° for several days in the absence of added CH_2FH_4 can lead to nearly complete dissociation of TS_b (data not shown).

The increased efficiency of TS_f inhibition in Tumor 38 was not a result of particularly high peak formation or persistence of FdUMP (Chart 4), whose levels were highest at the earliest time studied (0.5 hr) and reflected the rapidly changing balance among events of 5-FUra transport and activation and loss of free FdUMP by catabolism and binding to TS_f. The rapid FdUMP losses in all tumors within an hr of treatment were similar to the kinetics described recently in P388 cells *in vivo* (3) and human carcinoma cells *in vitro* (12). At most time points, the highest free FdUMP contents were found in Tumor 51, although at 48 hr Tumor 38 showed the highest FdUMP, 34 pmol/g. The late rise of free FdUMP in Tumor 38 may have been caused by ternary complex destruction during cell death *in vivo*, resulting in release of extracellular FdUMP. In the resistant tumors, the coexistence of free FdUMP and TS_f at early time points suggests that intracellular conditions did not allow complete ternary complex formation or stabilization.

Tissue dUMP contents of these tumors corresponded to cytosol dUMP concentrations in the 5 to 10 μM range and, therefore, were probably too low to have slowed rates of ternary complex formation (29, 36). In addition, differences among the dUMP levels of these tumors were too small to explain the greater TS_f inhibition in the 5-FUra-sensitive Tumor 38. In contrast to our previous results in human CCRF-CEM cells continuously exposed to 5-FUra (33), these murine colon adenocarcinomas did not show appreciable dUMP accumulation after 5-FUra treatment.

In comparison with normal colon tissue, colon Tumor 38 may have somewhat higher fluorouracil phosphoribosyltransferase activity (6), which has provided an explanation (44) for the apparently enhanced therapeutic index when 5-FUra is preceded by allopurinol administration in this tumor. However, our study of 5-FUra metabolism to total acid-soluble metabolites (which presumably are predominantly fluororibonucleotides) and incorporation into RNA revealed no differences significant enough to explain the 5-FUra sensitivity of colon Tumor 38, although the slow rates of these events in Tumor 06/A may have been an additional factor in the resistance of this tumor.

Adequate 5-FUra transport, activation to nucleotide(s), and incorporation into RNA may still be insufficient events *per se* to cause therapeutically useful sensitivity to 5-FUra at maximally tolerated bolus i.p. doses. Lowering of TS_f to levels approaching zero may be necessary for growth inhibition by 5-FUra. Marked TS_f inhibition requires tight FdUMP binding to enzyme. At FdUMP concentrations equivalent to or exceeding TS_f, a critical variable that determines ternary complex stability is the concentration of reduced folates. Although nearly all TS studies have used CH_2FH_4, a monoglutamyl form, this folate probably is not the active cofactor for this enzyme intracellularly (32). Intracellular folates exist as polyglutamates (5, 34, 46), which may differ from CH_2FH_4 in their kinetics of interaction with TS_f (10, 13, 23, 28). Washtien and Santi (48) have shown that considerably slower ternary complex dissociation occurs in intact cells in culture than in high CH_2FH_4-containing cell-free cytosols. It thus seems likely that differences in the concentration of the reduced folates in these colon tumors could be a determining factor in causing inhibition of TS_f to tumoricidal levels. This hypothesis is supported by the recent suggestion of Houghton *et al.* (19) that cytosols of 5-FUra-resistant human colorectal xenografts required the addition of exogenous CH_2FH_4 in order for increased FdUMP binding to occur.

In conclusion, our study has provided evidence that tumor sensitivity to 5-FUra correlates with immediate and complete inhibition of TS_f. Dissociation of ternary complex is sufficiently facile that active enzyme may be overestimated (3, 12, 33). We have found that correction for such dissociation is essential for the accurate determination of active TS_f *in vivo* after exposure to 5-FUra. Other indices of 5-FUra action that we studied (incorporation into RNA, dUMP levels, FdUMP levels, total

acid-soluble metabolites) did not correlate with 5-FUra sensitivity in these tumors.

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