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RELATIONSHIP BETWEEN 5-FLUORO-2′-DEOXYURIDYLATE, 2′-DEOXYURIDYLATE, AND THYMIDYLATE SYNTHASE ACTIVITY SUBSEQUENT TO 5-FLUOROURACIL ADMINISTRATION, IN XENOGRAFTS OF HUMAN COLON ADENOCARCINOMAS

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Abstract—5-Fluorouracil (FUra) has been administered to mice bearing xenografts of human colon adenocarcinomas. In two tumor lines, $HxGC_3$ and $HxVRC_5$, intrinsically resistant to FUra, 2′-deoxyuridylate (dUMP) accumulated 13.4- and 23.9-fold above basal levels. In $HxELC_2$ xenografts, which demonstrated some sensitivity to FUra, there was a decrease in dUMP concentration after drug administration. Maximal intratumor levels of 5-fluoro-2′-deoxyuridylate (FdUMP) were found at 1 hr, but decreased in all tumor lines by 4 hr after administration of FUra. Data derived in tumor cytosols suggested that FdUMP levels *in situ* were not rate-limiting for formation of covalent ternary complex, but that accumulation of dUMP would retard the rate of complex formation. Subsequent to administration of FUra, thymidylate synthase activity was reduced >75% in all tumors, but it recovered rapidly in tumors resistant to FUra. In addition, the pretreatment level of activity of thymidylate synthase was 12.7-fold greater in $HxVRC_5$ tumors than in $HxELC_2$ tumors. This elevated activity in $HxVRC_5$ tumors appears not to be a consequence of gene amplification. Formation of FdUMP or the accumulation of dUMP did not correlate with the activity of phosphatases measured at pH 5.8 or pH 9.2 in each tumor line. Further, inhibition of phosphatase activity did not alter, significantly, the net rate of dissociation of the FdUMP–thymidylate synthase–[6*R*]-CH_2-H_4PteGlu complex.

The cellular pharmacology of the antineoplastic agent 5-fluorouracil (FUra‡) is complex. Cytotoxicity may be effected by several known mechanisms including incorporation into RNA with subsequent alteration in posttranscriptional processing or translation [1–3], incorporation into DNA and excision which may cause DNA fragmentation [4], and depletion of dTTP formed *de novo* through inhibition of thymidylate synthase by the proximal metabolite FdUMP [5, 6]. The exact mechanism may depend upon metabolic characteristics of the cell line, experimental conditions, the concentration of FUra, and exposure time. Consequently, we have attempted to study these complex interactions under conditions that may more readily approximate to human colon adenocarcinoma *in situ*, by using human adenocarcinomas growing subcutaneously as xenografts in mice. There are now considerable data which support the fidelity of such tumors (reviewed in Ref. 7), but the importance to this study is that metabolism of pyrimidines *de novo* appears similar to that of human colon adenocarcinomas. Thus, in xenografts that anabolize FUra by the sequential activities of uridine phosphorylase and uridine kinase [8, 9], the activity of the kinase has been determined to be considerably lower than that of uridine phosphorylase [8, 9]. A similar situation with respect to FdUrd kinase and phosphorylase was found in human colon adenocarcinoma [10]. Further, in xenografts of colon tumors cell proliferation kinetics are consistent with the limited data obtained in patients [11]. Using this model system, the formation and maintenance of FdUMP, accumulation of dUMP, and thymidylate synthase activity have been examined subsequent to administration of FUra to tumor-bearing mice. Levels of these nucleotides, and the formation and stability of ternary complexes formed between FdUMP, thymidylate synthase and [6R]-CH_2-H_4PteGlu, have been examined with respect to the activities of acid and alkaline phosphatases.

MATERIALS AND METHODS

Tumor lines. Human colon adenocarcinomas $HxVRC_5$, $HxGC_3$ and $HxELC_2$ have been described

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‡ Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; AMP, adenosine-5′-monophosphate; [6*R*,*S*]-CH_2-H_4PteGlu, racemic mixture of the natural [6*R*] and unnatural [6*S*] diastereoisomers of 5,10-methylenetetrahydropteroylmonoglutamate; dUMP, 2′deoxyuridine-5′-monophosphate; thymidylate synthase, 5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45; dTTP, thymidine-5′-triphosphate; SDS, sodium dodecyl sulfate; and HPLC, high performance liquid chromatography.

previously [11]. Briefly, $HxELC_2$ shows some sensitivity to 5-fluoropyrimidines, whereas $HxVRC_5$ and $HxGC_3$ are intrinsically resistant [12]. Tumors were passaged bilaterally in the subcutaneous space of mice immune-deprived by thymectomy, cytosine arabinoside and whole body irradiation as described [13].

Determination of FdUMP and dUMP in vivo. Mice bearing bilateral xenografts received FUra (100 mg/kg) as a single i.p. administration. Tumors were excised at the appropriate times, and dUMP levels were determined using the method of Moran *et al.* [14]. Levels of FdUMP were determined by TLC as previously described [12], or by HPLC using the method of Garrett and Santi [15]. Samples were eluted from a Partisil 10/25 SAX column (Whatman, NJ) using 2.5 mM ammonium phosphate, pH 3.5, for 15 min followed by a linear gradient from 2.5 to 500 mM ammonium phosphate at pH 3.5. Flow rate was 1 ml/min, and the retention time for FdUMP was 25 min. Radioactivity was determined in 0.5-ml fractions.

Determination of thymidylate synthase activity. To determine thymidylate synthase activity in xenografts before and subsequent to FUra administration, mice bearing bilateral subcutaneous tumors were injected with 0.9% NaCl or FUra (100 mg/kg). At the appropriate time mice were killed, and tumors were excised, frozen in liquid N_2 and stored at −70° for up to 1 week prior to assay. The assay will be described in detail elsewhere. Briefly, four tumors (each 0.3 to 0.6 g) from each time point were pooled, thawed on ice, and homogenized three times for 15 sec (Polytron, Brinkmann Instruments) in 2 vol. of ice-cold buffer (20 mM Tris–HCl, pH 7.5, containing 70 mM 2-mercaptoethanol, 100 mM NaF, 5 mM AMP and 1.5 mM NaN_3). Homogenates were centrifuged (105,000 *g*, 60 min, 4°), and the supernatant fractions were retained on ice. Endogenous nucleotide was removed by addition of an equal volume of ice-cold 10% charcoal suspension (containing 1% bovine serum albumin and 0.1% dextran) [16]. In preliminary experiments it was found that subsequent to this treatment, isolation of [6-^{3}H]-FdUMP covalent ternary complex using 5% charcoal suspension [16] (with albumin and dextran) was greatly reduced compared to that formed in untreated cytosols. This led to substantial error in determining enzyme levels. Metabolism of [5-^{3}H]-dUMP was not altered by treatment with 10% charcoal suspension, consequently this procedure was used to determine thymidylate synthase activity. Reaction mixtures (150 μl) contained 10 μM [5-^{3}H]-dUMP (sp. act. 1.9 Ci/mmole), 100 μM [6*R*,*S*]-CH_2H_4PteGlu, and 110 μl charcoal-treated cytosol. Reactions were terminated (10 min, 37°) by pipetting 40 μl into an Eppendorf centrifuge tube containing 10 μl of 0.75 M perchloric acid (ice-cold), with rapid mixing. To this, 1.2 ml of ice-cold 5% charcoal (containing 0.5% BSA, and 0.05% dextran) was added, and the mixture maintained on ice for 15 min. After centrifuging (12,000 *g*, 5 min, 4°) to precipitate the charcoal, the supernatant fraction was passed through a glass fiber filter (Gelman GF/A) and 800 μl filtrate was used to determine radioactivity. Reactions were linear for at least 15 min.

DNA blot analysis. Deoxyribonucleic acid was extracted from xenografts in the following manner. Fresh tissues were minced finely using crossed scalpels, and incubated (50°, 12 hr) in Tris–HCl (pH 8.0) containing 10 mM NaCl, 1 mM EDTA, 100 μg/ml proteinase K, and 0.5% SDS. Subsequently procedures were similar to those of Blin and Stafford [17]. DNA concentration and purity was determined from 280/260 nm absorbance ratio. Equal quantities of DNA were bound to nitrocellulose using the procedures of Kafatos *et al.* [18], using a Hybridot apparatus (Bethesda Research Laboratories). The cDNA probe used was a PstI fragment of pMTS-3 (obtained from L. F. Johnson, Ohio State University). This plasmid contains cDNA for mouse thymidylate synthase [19]. Plasmid MTS-3 was restricted using PstI, and a fragment (≈760 base pairs) was isolated after electrophoresis through low melting point agarose (0.8% Seaplaque; FMC Corp.). This fragment was purified and nick translated using standard procedures. Hybridization conditions were as described by Kafatos *et al.* [18].

Measurement of the catabolism of [^{3}*H*]*FdUMP*. Tumors were rapidly excised and homogenized (Polytron, Brinkmann Instruments, 3 × 15 sec) in 4 vol. of ice-cold 10 mM Tris–HCl, pH 7.4, containing 4 mM dithiothreitol (DTT). Homogenates were centrifuged (12,000 *g*, 4°, 10 min), and supernatant fractions were used immediately. The reaction mixture for acid phosphatase contained, in a volume of 60 μl, cytosol (40 μl), [^{3}H]FdUMP 1 mM (sp. act. 2.42 mCi/mmole), and 25 mM sodium acetate (pH 5.8). Prewarmed cytosol (2 min, 37°) was added to prewarmed reagents to initiate the reaction. To terminate the reaction (10 min), an aliquot (10 μl) was removed and mixed with 3 μl of 1 M perchloric acid (ice-cold) and retained on ice for 5 min. Potassium hydroxide (1 M, 5.3 μl) was added, and after a further 5 min 30 μg FdUrd was added, and the mixture centrifuged (12,000 *g*, 4°, 4 min). Ten microliters of the supernatant fraction was analyzed by HPLC. Samples were eluted from a Partisil 10/25 SAX column (Whatman, NJ) using a linear gradient from 2.5 to 125 mM ammonium phosphate at pH 3.5. Both FdUrd and FUra eluted with the void volume (≈3.5 min), whereas the retention time for FdUMP was 17.6 min. Radiolabel was determined in 0.5-min fractions. Protein concentration was determined using the BioRad assay (BioRad, Richmond, CA). For alkaline phosphatase activity measurements, 25 mM ammonium bicarbonate, pH 9.2, was substituted for pH 5.8 buffer. Reaction rates were linear over 10 min.

Competition between dUMP and FdUMP. Excised tumors were homogenized for 30 sec (Polytron, Brinkmann Instruments) in ice-cold buffer A (60 mM sodium phosphate, pH 7.4, containing 0.12% (w/v) bovine serum albumin and 1 mM β-mercaptoethanol). Homogenates were centrifuged at 100,000 *g* (60 min, 4°), and supernatant fractions were separated from endogenous nucleotide by gel filtration at 4° on a G-25 Sephadex column (14 × 2.5 cm) equilibrated in buffer A.

Competition between dUMP and FdUMP was carried out using inhibitor and substrate concentrations far in excess of enzyme-ligand binding

concentrations. The final concentration of [^{3}H]-FdUMP binding sites was between 0.4 and 7.52 nM, and [^{3}H]FdUMP was 56.8 to 79.2 nM, dependent upon the tumor preparation, [6*R*,*S*]-CH_2-H_4PteGlu was 100 μM, and dUMP concentrations were varied between 0 and 28 μM. Alternatively, FdUMP was 1.09 μM, and dUMP concentrations were varied between 0 and 500 μM. Reactions were initiated by adding preincubated cytosol to the other reactants, and ternary complex formation was measured over the initial 4 min during which time the reaction was linear. Reactions were terminated by pipetting aliquots of reaction mixture (300 μl) into 1.2 ml of ice-cold quench solution (2% charcoal, 0.5% albumin and 0.05% dextran) as described [20].

The substrate binding constant (K_s) was calculated from the equation [21]

$$\ln \frac{[E_0] - [X]}{[E_0]} = - \frac{k_a [I_o] t}{1 + \frac{[S]}{K_s}}$$

where $[E_0]$ is the initial concentration of ligand binding sites, $[I_0]$ is the initial concentration of inhibitor, $[X]$ is the concentration of [^{3}H]FdUMP–enzyme–CH_2-H_4PteGlu complex at time t (min), and $[S]$ is the initial substrate concentration (μM).

For determination of the rate of formation of complex with respect to the concentration of FdUMP (10–200 nM), the initial rate was determined over 2 min.

Measurement of net dissociation of [^{3}H]FdUMP-labeled ternary complex. Ternary complex was formed using 28 nM [^{3}H]FdUMP, 100 μM [6*R*,*S*]-CH_2-H_4PteGlu and $HxVRC_5$ tumor cytosol, incubated at 37° for 120 min, and separated from unbound [6*R*,*S*]-CH_2-H_4PteGlu and nucleotide by gel filtration on G-25 Sephadex at 4° as described above. In the eluted protein fractions greater than 95% of radiolabel could be precipitated using perchloric acid, indicating only slight contamination with unbound nucleotide. Complex, 2.44 nM, was incubated in the presence of folate at 37°, with or without 1 mM AMP. At appropriate times aliquots of reaction mixture were pipetted into ice-cold quench solution. In all experiments, quenched reactions were allowed to stand on ice for 20 min prior to centrifugation (12,000 *g*, 4°, 10 min). The supernatant fraction was then filtered through a glass fiber filter (Gelman GF/A) packed into a disposable 1-ml syringe, and radioactivity was determined in 0.9 ml of the filtrate.

RESULTS

Concentrations of FdUMP achieved in each tumor line after administration of FUra (100 mg/kg) are presented in Table 1. In each line, maximal levels were found 1 hr after injection and decreased by 4 hr. Similar levels of FdUMP were achieved in $HxELC_2$ and $HxGC_3$ tumors, although FdUMP concentrations decreased more rapidly in the responsive tumor ($HxELC_2$); FdUMP levels were higher in $HxVRC_5$ extracts. Analysis of FdUMP by the method of Garrett and Santi [15] yielded similar data for $HxGC_3$ tumors, being at 1 hr and 4 hr 2.41 ± 0.85 and 0.41 ± 0.15 nmoles/g respectively. Basal levels of dUMP were 8.24, 2.12 and 5.50 nmoles/g in $HxELC_2$, $HxGC_3$ and $HxVRC_5$ tumors respectively. Subsequent to FUra administration, there was a rapid increase in dUMP levels within the first hr in both $HxGC_3$ and $HxVRC_5$ tumors, this being 7- and 4.4-fold respectively. Maximal levels were achieved at 24 hr in $HxGC_3$ tumors and at 48 hr in $HxVRC_5$ tumors (Fig. 1). However, in the tumor line that shows some sensitivity to FUra, $HxELC_2$, there was no accumulation of dUMP. In contrast to other lines, in $HxELC_2$ xenografts dUMP levels decreased by 61% by 8 hr after administration of FUra.

Table 1. Concentration of FdUMP in human colon tumor xenografts after administration of FUra

	FdUMP (pmoles/g)				
	Time (hr)				
Tumor line	1	4	24	48	96
$HxELC_2$	3,436	316	265	209	145
$HxGC_3$	2,345	450	730	754	488
$HxVRC_5$	10,641	4,512	1,917	1,656	842

Each value is the mean of two to six determinations.

For each tumor line thymidylate synthase activity was determined before and subsequent to administration of FUra (Fig. 2). The greatest pretreatment activity was measured in $HxVRC_5$ tumors, this being 13-fold greater than that measured in $HxELC_2$ xenografts. $HxGC_3$ had intermediate activity. Four hours after administration of FUra, thymidylate synthase activity had decreased by >75% in each tumor. However, even at its nadir, activity in $HxVRC_5$ xenografts exceeded by 2.5-fold the pretreatment activity in $HxELC_2$ tumors. Recovery of enzyme activity in both FUra resistant tumors was relatively rapid. By

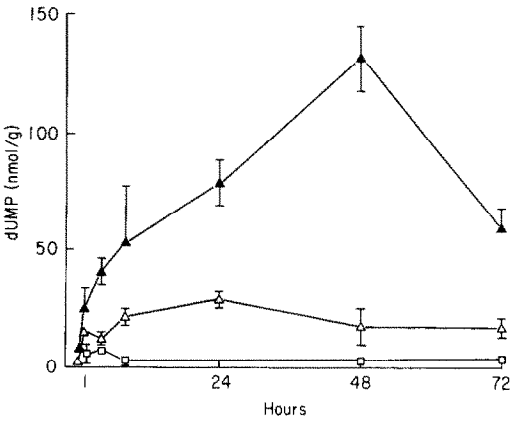

Fig. 1. Accumulation of dUMP in human colon adenocarcinomas growing as xenografts. At various times between 1 and 72 hr after FUra (100 mg/kg) injection, tumors were excised and up to eight tumors were pooled for each time point. Levels of dUMP were determined as described in Materials and Methods. Results represent the mean ± S.D. of three to ten determinations at each time point. Key: $HxVRC_5$ (▲); $HxGC_3$ (△); and $HxELC_2$ (□).

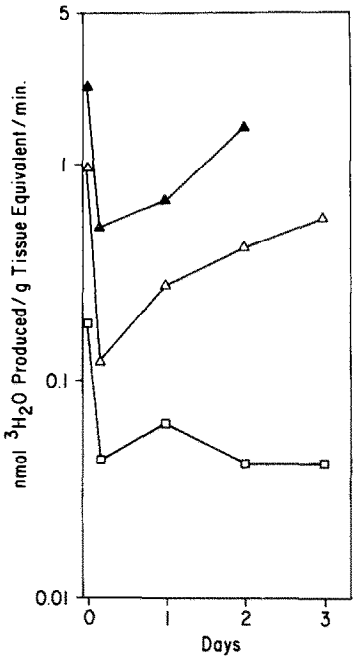

Fig. 2. Activities of thymidylate synthase in human colon adenocarcinoma xenografts. At various times between 4 and 72 hr after injection of FUra (100 mg/kg), tumors were excised, and four tumors were pooled for each time point. Thymidylate synthase activity in untreated and FUra-treated tumors was determined as described in Materials and Methods. Results represent the mean of three determinations at each time point (S.D. ≤ 5.5% of mean). Key: $HxVRC_5$ (▲); $HxGC_3$ (△); and $HxELC_2$ (□).

48 hr after administration of FUra, enzyme activity had recovered to 65 and 43% of the pretreatment values in $HxVRC_5$ and $HxGC_3$ tumors respectively. In contrast, even at 72 hr thymidylate synthase activity in $HxELC_2$ xenografts was only 25% of control values.

The differences in pretreatment activity of thymidylate synthase correlated well with binding of [6-^{3}H]FdUMP (data not shown). These differences could have been due to spontaneous amplification of thymidylate synthase genes which could occur during tumor progression [22]. Deoxyribonucleic acid was extracted from $HxVRC_5$, $HxGC_3$ and $HxELC_2$ tumors and analyzed by dot-blot hybridization (Fig. 3). For equal quantities of DNA bound to the nitrocellulose filter (determined by absorbance at 260 nm), there was an equal signal for DNA derived from different tumors. This result suggests that elevated thymidylate synthase activities were not a consequence of gene amplification that could be detected.

Recently, it has been reported that resistance to FdUrd is related to increased activities of phosphatases (or 5′-nucleotidase) that degrade FdUMP [23]. It was therefore of interest to determine whether FdUMP levels or dUMP accumulation in colon tumor xenografts correlated with phosphatase activities. These are presented in Table 2. The activity of acid (pH 5.8) phosphatase exceeded that of alkaline (pH 9.2) phosphatase in each tumor examined. Greatest activity was measured in $HxVRC_5$ tumors and lowest activity was determined in $HxELC_2$ tumors. At physiologic pH (7.4), phosphatase activities were reduced considerably. Thus, in these tumors, there was little correlation between FdUMP concentrations achieved, or accumulation of dUMP, and phosphatase activity with FdUMP as substrate.

Formation of ternary complex. The rate at which ternary complex (FdUMP–thymidylate synthase–[6*R*]-CH_2-H_4PteGlu) forms is dependent upon the concentration of nucleotide and folate. Further, competition between dUMP and FdUMP will slow formation of ternary complex. For $HxVRC_5$ tumors, the rate of ternary complex formation, with respect to FdUMP concentration, is shown in Fig. 4. Maximal reaction velocity was obtained at approximately 200 nM FdUMP. Thus, from data presented in Table 1, FdUMP achieved in tumors *in situ* should not be limiting for complex formation. Similar results were obtained in $HxGC_3$ and $HxELC_2$ xenografts (data not shown). However, under suboptimal conditions for formation of ternary complex (79.2 nM FdUMP; 100 μM [6*R*,*S*]-CH_2-H_4PteGlu), dUMP at relatively low concentrations decreased the rate of formation (Fig. 5). Thus, in the presence of 28 μM dUMP, the rate at which ternary complex formed was 40% of control (no dUMP), determined in cytosols of $HxVRC_5$ xenografts. Similar data were obtained when 1.09 μM FdUMP was used, and dUMP was varied between 0 and 500 μM (data not shown). At

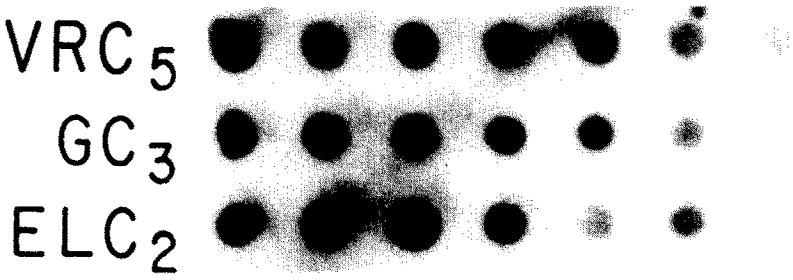

Fig. 3. Comparison of thymidylate synthase DNA in total genomic DNA of colon adenocarcinoma xenografts. Total DNA was isolated and bound to nitrocellulose as described in Materials and Methods. The dot-blot shows a series of 2-fold dilutions of DNA (2.5 to 0.02 μg) and the hybridization signal using nick translated thymidylate synthase cDNA. The result indicates similar thymidylate synthase gene copy number in DNA from each tumor.

Table 2. Activities of phosphatase and/or nucleotidase for FdUMP in the supernatant fraction of colon tumor xenografts

Tumor line	Activity (units)*		
	pH 5.8	pH 7.4	pH 9.2
$HxELC_2$	1.51 ± 0.2†	0.01‡	0.55 ± 0.06
$HxGC_3$	8.58 ± 0.8	0.2	2.17 ± 0.2
$HxVRC_5$	13.9 ± 2.2	1.2	3.98 ± 0.54

* One unit is defined as the conversion of 1 nmole FdUMP/min/mg protein at 37°.
† Mean ± S.D. (N = 3).
‡ Mean for duplicate determinations.

48 hr after FUra administration, the dUMP concentration in $HxVRC_5$ tumors *in situ* was calculated to be approximately 260 μM in cell water.

The effect of dUMP on the rate of binding of [^{3}H]FdUMP to thymidylate synthase was therefore examined in each tumor line. Inhibition was a linear function of the substrate concentration over the range examined (0–20 μM) in each tumor, but only slight differences in substrate binding constant values (K_s) were determined. The lowest value (0.7 ± 0.09 μM) was found in cytosols derived from $HxVRC_5$. Values determined in $HxELC_2$ and $HxGC_3$ tumors were 3.0 ± 0.8 and 2.1 ± 0.3 μM respectively.

Stability of ternary complex. It has been shown previously [21] that the apparent rate of dissociation of ternary complex is first order and is dependent upon the concentration of unbound [6*R*]-CH_2-H_4PteGlu, but not dependent upon the concentration of unbound FdUMP. Similar results were obtained using ternary complexes formed in cytosols of $HxELC_2$, $HxGC_3$ and $HxVRC_5$ tumors, where the $t_{\frac{1}{2}}$ for net dissociation of complex in the absence of CH_2-H_4PteGlu was between 31 and 33 min (data not shown). Consequently, it was of interest to determine whether phosphatase or nucleotidase activities would alter the apparent stability of ternary complex, by reducing the rate at which FdUMP reassociated with enzyme. Complex was formed, and separated from unbound [6*R*,*S*]-CH_2-H_4PteGlu and nucleotide

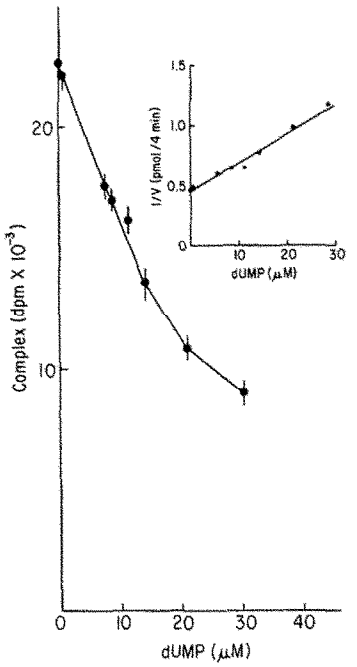

Fig. 5. Effect of dUMP on ternary complex formation by [^{3}H]FdUMP. Ternary complex was determined at 4 min after addition of nucleotide to $HxVRC_5$ cytosols. FdUMP was 79.2 nM and [6*R*,*S*]-CH_2-H_4PteGlu was 100 μM. Each point represents the mean of three determinations ± S.D. Inset: semi-reciprocal plot of ternary complex at 4 min with respect to dUMP concentration.

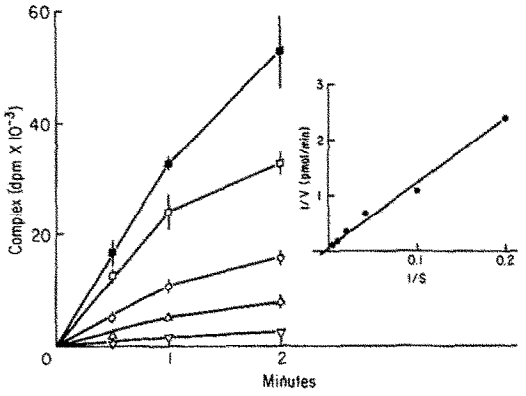

Fig. 4. Rate of formation of ternary complex in cytosols from $HxVRC_5$ xenografts. Cytosols were incubated at 37° with 100 μM [6*R*,*S*]-CH_2-H_4PteGlu, and complex formation was determined over 2 min after addition of [^{3}H]-FdUMP to a concentration of (■) 200; (□) 100; (○) 50; (△) 25; and (▽) 10 nM. Each point represents the mean of three determinations ± S.D. Inset: replot of data showing reciprocal plot of rate of formation of complex against FdUMP concentration.

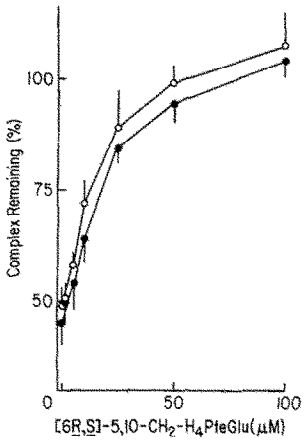

Fig. 6. Stability of covalent ternary complex with respect to the concentration of [6*R*,*S*]-CH_2-H_4PteGlu. Complex was formed and separated from free nucleotide and folate by gel filtration as described in Materials and Methods. Complex was then incubated at 37° for 40 min with various concentrations of [6*R*,*S*]-CH_2-H_4PteGlu in the presence (○) or absence (●) of 1 mM AMP.

as described in Materials and Methods. Complex ($HxVRC_5$) was then incubated at 37° in the presence or absence of 1 mM AMP, with various concentrations of [6*R*,*S*]-CH_2-H_4PteGlu. In preliminary experiments, it was determined that in $HxVRC_5$ cytosols addition of AMP to a final concentration of 1 mM inhibited nucleotidase action on [^{3}H]FdUMP (data not shown). As shown in Fig. 6, the proportion of complex remaining at 40 min (37°) was similar over the range of [6*R*,*S*]-CH_2-H_4PteGlu concentration (0–100 μM) examined whether or not AMP was included.

DISCUSSION

The cellular pharmacology of FUra is complex, and the mechanism(s) causing cytotoxicity is quite controversial. In this study, factors influencing the interaction of FdUMP with thymidylate synthase under conditions of growth *in situ* have been addressed. Specifically, the time courses for formation of FdUMP and accumulation of dUMP have been examined in xenografts of human colon adenocarcinomas. Maximal levels of FdUMP were found 1 hr after administration of FUra in each of the colorectal xenografts. The highest level was found in $HxVRC_5$ tumors (intrinsically resistant), which supports the observation of Berger and Hakala [24] that the capacity to accumulate free FdUMP does not alone guarantee that inhibition of thymidylate synthase will be growth limiting. By 4 hr after FUra administration FdUMP levels had fallen considerably in each line, but between 24 and 168 hr they remained relatively constant. Similar results, i.e. prolonged periods during which FdUMP levels remain relatively high, have been reported in rodent tumor systems [25, 26]. Levels of FdUMP measured in this study are higher than those determined in biopsy specimens of human adenocarcinoma [27]. This may be due to differences in methodology used to determine FdUMP, differences in tumor size and degree of necrosis (xenografts were 0.5 to 0.8 g), differences in drug pharmacokinetics, or possibly to the rapidity with which the tumor was removed after the vascular supply had been occluded and, hence, to levels of phosphatase or nucleotidase activity. In this study tumors were frozen within 15 sec. Analysis by HPLC of FdUMP in periodate-treated samples [15] gave results very similar to those obtained using TLC methods. Kinetic studies in which the rate of ternary complex formation was examined in tumor cytosols showed that velocities achieved at 100–200 nM were close to V_{max} under conditions of saturating [6*R*,*S*]-CH_2-H_4PteGlu. Consequently, *in situ* FdUMP concentration (145–10,641 nM) would appear not to be limiting in the rate of complex formation.

Although dUMP does not displace FdUMP, or alter the rate of dissociation of FdUMP from the ternary complex [28], it can protect newly synthesized or regenerated enzyme from inactivation, by competing with FdUMP for binding (Fig. 5). Deoxyuridylate accumulated rapidly in $HxGC_3$ and $HxVRC_5$ tumors after administration of FUra. Maximal levels of dUMP were determined between 24 and 48 hr after treatment and were elevated 23.9- and 13.4-fold in $HxVRC_5$ and $HxGC_3$ tumors respectively. In $HxELC_2$ tumors there was a decrease in dUMP pools subsequent to FUra administration. The degree to which dUMP accumulates after FUra administration *in vivo* appears to depend upon the model system used. For rodent colon adenocarcinomas, dissimilar results have been reported using the same putative tumor line. Thus, in one study dUMP was elevated 13-fold in colon tumor #38 [25], whereas in the more extensive studies of Spears *et al.* [29], only slight increases in dUMP pools were observed in four lines of colon tumors. In the latter study [29], colon tumor #38 (moderately sensitive to FUra) behaved in a manner similar to $HxELC_2$ human colon adenocarcinomas, presented in the current study, i.e. dUMP levels decreased. In cytosols from $HxVRC_5$ tumors, the rate at which ternary complex was formed at 79 nM or 1.09 μM FdUMP was a function of the dUMP:FdUMP ratio. The rate at which complex was formed (y) could be described best by the equation $\ln y = e^{(4.59 - 0.0026X)}$, where X is the molar ratio of dUMP to FdUMP. In $HxVRC_5$ tumors, the maximum dUMP:FdUMP ratio determined was approximately 80. Consequently, even under ideal conditions where the concentrations of CH_2-H_4PteGlu were high, reassociation of FdUMP or binding of FdUMP to newly synthesized enzyme would be slowed by approximately 20% (Fig. 5), and this would be accentuated under conditions of low [6*R*]-CH_2-H_4PteGlu. Determination of the substrate binding constant (K_s) gave the lowest value (i.e. greatest affinity for dUMP) in $HxVRC_5$ tumors, although differences between these lines were not great. Under the experimental conditions used, dUMP competed with FdUMP more effectively in cytosols from $HxVRC_5$ tumors compared to $HxGC_3$ or $HxELC_2$ preparations. This observation, together with data demonstrating greater accumulation of dUMP, may relate to the intrinsic resistance to 5-fluoropyrimidines in $HxVRC_5$ tumors. In biopsies of human colon tumors, no accumulation of dUMP was found subsequent to administration of FUra, although inhibition of thymidylate synthase was determined [27]. In xenografts, accumulation occurred within 1 hr and was maximal between 24 and 48 hr. Both studies used similar methodology to determine dUMP, thus differences in data obtained may relate to tissue procurement and processing. In view of the relatively high activity of phosphatases in tumors, it may be of importance to determine the relationship between the time interval between vascular occlusion and tissue freezing with respect to nucleotide levels.

Determination of thymidylate synthase activity before and subsequent to FUra administration showed that tumors intrinsically resistant to FUra had (a) higher endogenous activity and (b) more rapid recovery of thymidylate synthase activity. Thus, these data are in agreement with reports that the sensitivity of mouse or human gastrointestinal tumor cell lines to 5-fluoropyrimidines varies inversely with the intracellular concentration of the free target enzyme [29, 30]. However, two additional human colorectal adenocarcinomas (HxBR and $HxAC_4$) have low thymidylate synthase levels (determined by [^{3}H]FdUMP binding), and neither respond

to FUra (data not presented). Consequently, such a simple relationship cannot be generally established *in vivo*. Thymidylate synthase activity recovered more rapidly in both FUra insensitive lines ($HxVRC_5$, $HxGC_3$), which also accumulated dUMP. Whether accumulation of endogenous substrates protects newly synthesized enzyme, or enzyme that is regenerated subsequent to dissociation of ternary complex, requires further study.

The difference between activities of thymidylate synthase in each tumor appears not to be a consequence of spontaneous gene amplification. Such a phenomenon has been reported for the N-myc oncogene during progression of neuroblastomas [22]. Data derived using dot-blot procedures of total genomic DNA show similar results for DNA isolated from these tumors (Fig. 3). Thus, there appears to be no obvious correlation between enzyme activity (or level using [6-^{3}H]FdUMP binding) and gene copy number detected. These differences in basal levels of thymidylate synthase activity require further examination.

The activity of acid phosphatase exceeded that of alkaline phosphatase in each tumor, in contrast to data derived in human CCRF-CEM leukemic cells in culture [23]. Further, the additive activities in each line of xenograft were considerably lower than those in CCRF-CEM cells sensitive to FdUrd. Whether this activity is indeed phosphatase or 5'-nucleotidase will require further characterization. The effect of phosphatase activity may either reduce FdUMP concentration or reduce dUMP accumulation. In the three tumor lines examined, there was no obvious relationship between nucleotide levels and phosphatase activity or recovery of thymidylate synthase activity. In $HxELC_2$ tumors, which had the lowest ability to degrade FdUMP, no dUMP accumulation was measured. Conversely, in $HxVRC_5$ tumors, demonstrating the greatest phosphatase activities, the greatest nucleotide levels were determined. At pH 7.4, phosphatase activities were reduced in each tumor examined.

It is now established that the covalent ternary complex between [6*R*]-CH_2-H_4PteGlu, thymidylate synthase, and FdUMP dissociates at a rate proportional to the concentration of free [6*R*]-CH_2-H_4PteGlu. Previous studies using these xenografts have indicated that the concentration of [6*R*]-CH_2-H_4PteGlu may be low [31]. Consequently, it was of interest to determine whether the activity of phosphatases would reduce the reassociation of FdUMP liberated from ternary complex at different concentrations of free [6*R*,*S*]-CH_2-H_4PteGlu. Data presented in Fig. 5 show that complexes were stabilized by increasing folate concentration, but (at pH 7.4) addition of 1 mM AMP to inhibit FdUMP catabolism did not significantly stabilize the complex (i.e. increase the ability of FdUMP to reassociate).

With respect to cytotoxicity mediated by the inhibition of thymidylate synthase in xenografts of human colon tumors, three parameters appear important. These are (1) the stability of the ternary complex, (2) accumulation of dUMP, and possibly (3) the endogenous level or activity of thymidylate synthase. In tumors, such as $HxVRC_5$, which have elevated thymidylate synthase activities, it remains to be determined whether the degree of inhibition obtained at acceptable dose levels of FUra is sufficient to limit DNA synthesis. Stability will be determined by the free concentration of [6*R*]-CH_2-H_4PteGlu, or its polyglutamate forms which may have greater affinity for thymidylate synthase [32] and possibly by characteristics of the enzyme [33]. Increasing the stability of this complex by increasing concentration of folylpolyglutamates may negate the effect of dUMP accumulation, and provides rationale for combining a fluoropyrimidine with a folate such as leucovorin.

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