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Modulation of 5-Fluoro-2′-deoxyuridine Response by Folinic Acid in Human Colonic Tumor Cell Lines: The Role of Thymidylate Synthase

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SUMMARY

Recent investigations have revealed a significant increase in cytotoxic response to (5-fluoropyrimidine, FP) agents in the presence of the folate folinic acid (CF). It has been suggested that CF provides a source of intracellular reduced folates which, in turn, enhances the inhibition of the cellular target thymidylate synthase (TS) by the FP metabolite 5-fluoro-2′-deoxyuridylate (FdUMP). The extent of variation in the response to FP-CF combinations is unknown but it is an important consideration in view of the utilization of these combinations for the therapy of colorectal carcinoma. In the present study, variation in the response to 5-fluoro-2′-deoxyuridine (FdUrd)-CF combinations was observed between two human colorectal tumor cell lines, RCA and C. The response of both cell lines to FdUrd increased with increasing CF, but the effect was more pronounced in cell line RCA. RCA was 4-fold less responsive than cell line C to FdUrd at low CF concentrations, whereas both cell lines exhibited similar sensitivity at high CF concentrations. RCA accumulated lower levels of TS folate cosubstrates after CF than did C; however, this was not the sole mechanism accounting for the differential response to FdUrd-CF. The two cell lines responded differently to equivalent intracellular levels of 5,10-methylenetetrahydrofolate ($CH_2H_4PteGlu$) derivatives, the folate ligands involved in tight-binding inhibition of TS by FdUMP. The differential response to $CH_2H_4PteGlu$ was not due to lack of folate polyglutamation; the predominant $CH_2H_4PteGlu$ derivative in both cells was the hexaglutamate form. The difference in response to $CH_2H_4PteGlu$ was associated with a reduction in the affinity of the RCA TS for $CH_2H_4PteGlu$, relative to the C enzyme. Thus, a cell line has been identified that responds poorly to FdUrd at physiological levels of CF and that contains a variant TS enzyme.

The uracil antimetabolite FUra has been utilized in the therapy of solid tumors for 25 years. The drug exerts its cytotoxic effects by at least two mechanisms of action (1). FUra is converted to FUTP, which is subsequently incorporated into RNA; the resulting fraudulent RNA molecules have altered biological activity. FUra is also anabolized to FdUMP, which is a tight-binding inhibitor of the enzyme TS in the presence of the folate cosubstrate $CH_2H_4PteGlu$; inhibition of TS results in thymidylate deprivation and, hence, inhibition of DNA biosynthesis. Which mechanism underlies cytotoxicity is dependent upon cell phenotype and nutrient conditions (1).

As a single agent, FUra has produced responses of 15–20% in patients bearing tumors of the gastrointestinal tract, pancreas, breast, and ovary; in combination with the folate CF, clinical responses of 45% have been reported (2). The utilization of CF and FUra in combination is an extension of studies with mammalian tumor cells in culture, which demonstrated that CF increases the cytotoxicity of FUra and directs the action of FUra at TS (3). CF is thought to enhance the action of FUra at TS by serving as a precursor of intracellular reduced folates, including $CH_2H_4PteGlu$ and its polyglutamated derivatives. Intracellular folates play a critical role in the interaction of FdUMP with TS as shown by *in vitro* studies revealing that the stability of FdUMP-TS complexes is enhanced by 5–6 orders of magnitude by $CH_2H_4PteGlu$ monoglutamate (4, 5). The resulting ternary complex composed of FdUMP, TS, and $CH_2H_4PteGlu$ contains two covalent bonds, one between FdUMP and TS and the other between FdUMP and $CH_2H_4PteGlu$ (1). Folates other than $CH_2H_4PteGlu$ may stimulate ternary complex formation; however, these complexes are less stable because they contain only one covalent bond, between FdUMP and TS (6, 7). Ternary complexes formed from polyglutamate derivatives of $CH_2H_4PteGlu$ and other folates are more stable than those derived from monoglutamate forms

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ABBREVIATIONS: FUra, 5-fluorouracil; FUTP, 5-fluorouridine-5′-triphosphate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; TS, thymidylate synthase; $CH_2H_4PteGlu$, 5,10-methylenetetrahydrofolic acid; CF, citrovorum factor, calcium leucovorin, 5-formyltetrahydrofolic acid; FdUrd, 5-fluoro-2′-deoxyuridine; PteGlu, folic acid; $5\text{-}CH_3H_4PteGlu$, 5-methyltetrahydrofolic acid; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum; $10\text{-}CHO\text{-}H_4PteGlu$, 10-formyltetrahydrofolic acid; $H_4PteGlu$, tetrahydrofolic acid; ID_{50}, does required for 50% inhibition of cell growth.

(6, 8, 9). Folylpolyglutamates, which are folate derivatives that contain greater than one glutamate residue, are the major intracellular forms of folate (10). Thus, the capacity of CF to serve as a source of intracellular reduced folates, particularly polyglutamated derivatives, may be an important determinant of the cellular response to FUra and other fluoropyrimidine agents.

In order to examine the role of CF in the TS-directed action of fluoropyrimidine agents, FdUrd, rather than FUra, has been utilized. Because TS is the growth-limiting target of FdUrd in the cells used in this study (data not shown), the relationship between growth response and TS-directed action is more distinctly defined with FdUrd than with FUra. In this study, human colorectal tumor cell lines were utilized to assess the role of CF in FdUrd response, because the CF-FUra combination is currently an experimental protocol for advanced colorectal carcinoma. A major objective of this study was to determine whether cell lines vary in the response to FdUrd-CF combinations. For this purpose, two cell lines of differing phenotype were chosen as a model system. The cell lines, RCA and C, differ in growth rate, differentiation status, tumorigenicity, and sensitivity to FdUrd and FUra (11, 12). In the present study, we have shown that the two cell lines differ in the response to FdUrd-CF combinations and we have identified a mechanism that may underlie the response variation.

Experimental Procedures

Materials. [6-^{3}H]FrdUMP (18 Ci/mmol) and [3′,5′,7,9-^{3}H]PteGlu were purchased from Moravek Biochemicals (Brea, CA). FdUrd, (6*RS*)-CF, PteGlu, (6*RS*)5-CH_3H_4PteGlu, hypoxanthine, thymidine, glycine, sodium ascorbate, SDS, acid-washed charcoal, Sephadex G-50-80, and DEAE-cellulose microgranular anion exchanger were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 medium, folate-free RPMI 1640 medium, and FBS were purchased from GIBCO (Grand Island, NY). 10-CHO-H_4PteGlu was prepared from (6*RS*)-CF as described by Rabinowitz (13). (6*RS*)-CH_2H_4PteGlu was prepared from (6*RS*)-H_4PteGlu as described by Dunlap *et al.* (14); (6*RS*)-H_4PteGlu was prepared from PteGlu by the method of Zakrzewski and Sansone (15). $PteGlu_n$ (n = 2–6 glutamate residues) forms were purchased from C. Krumdieck (University of Alabama, Birmingham, AL). $PteGlu_n$ forms were reduced to (6*S*)-$H_4PteGlu_n$ derivatives with *Lactobacillus casei* dihydrofolate reductase (16), then converted to (6*R*)-$CH_2H_4PteGlu_n$ derivatives by the method of Dunlap *et al.* (14). (6*S*)-H_4PteGlu and (6*R*)-CH_2H_4PteGlu are the biologically active forms of these reduced folates. [^{32}P]FdUMP was prepared by incubating [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear, Boston, MA) with FdUrd in the presence of partially purified *Escherichia coli* thymidine kinase (17); the labeled nucleotide (620 Ci/mmol) was purified by DEAE-cellulose chromatography (18). Charcoal-stripped serum was prepared by exposing FBS to acid-washed charcoal at pH 4.2 (19). This procedure removed all free serum folate and 77% of folate bound to serum proteins, as determined with [3′,5′,7,9-^{3}H]PteGlu.

Cell culture and growth conditions. Human colorectal tumor cell lines RCA and C (obtained from M. Brattain, Baylor College of Medicine, Houston, TX) were maintained as monolayers in RPMI 1640 medium supplemented with 5% FBS. The cells were routinely monitored for the absence of mycoplasma by the Mycotrim detection system (New England Nuclear). Before growth inhibition studies, cells were depleted of folates by growth for 12 days in folate-free RPMI 1640 medium supplemented with 100 μM hypoxanthine, 30 μM thymidine, 30 μM glycine, and 5% charcoal-stripped FBS (20). All growth inhibition studies were carried out in folate-free RPMI 1640 medium supplemented with varying concentrations of CF and 5% charcoal-stripped FBS. T-25 flasks were inoculated with 100,000 cells and the cells were incubated for 24 hr before 3-hr exposure to FdUrd (3). Growth was continued for 5–6 cell generations, after which relative growth was determined by protein analysis using the method of Lowry *et al.* (21).

Extraction of cellular folates. Cells were depleted of folates for 12 days, then exposed to varying concentrations of CF for 24 hr. Cells were harvested in phosphate-buffered saline (10 mM sodium phosphate, pH 7.5, 153 mM NaCl) and folates were extracted from the cell pellets (250 mg) at 90° with 50 mM Tris·HCl, pH 7.4/1% sodium ascorbate. Two 10-min extractions removed 93% of intracellular radioactivity from cells incubated for 24 hr in 10 μM [3′,5′,7,9-^{3}H]PteGlu (0.1 mCi/mmol). Under the conditions of the extraction, no conversion of CH_2H_4PteGlu to H_4PteGlu or H_4PteGlu to CH_2H_4PteGlu was detected, as determined by the addition of folate standards to sonicates of folate-depleted and folate-containing cells.

Folate-binding assays. A modification of the TS ligand-binding assay of Moran *et al.* (18) was developed to determine the level of intracellular folates that stimulate ternary complex formation with TS *in vitro*. The reaction (0.1 ml) contained 0.1 μM *L. casei* TS (provided by R. B. Dunlap, University of South Carolina, Columbia, SC), 0.3 μM [6-^{3}H]FdUMP, 50 mM Tris·HCl, pH 7.4, 38 mM sodium ascorbate, 19 mM 2-mercaptoethanol, 25 mM $MgCl_2$, 37.5 μg of bovine serum albumin, and either 1.0–30 nM (6*RS*)-CH_2H_4PteGlu or ascorbate cell extract. After 1 hr at 30° in the dark, the levels of ternary complexes stimulated either by total folates or by CH_2H_4PteGlu derivatives were analyzed. To determine total folates, cold albumin-treated charcoal suspension was added to the reaction mixture as described by Moran *et al.* (18). CH_2H_4PteGlu levels were determined after heating reaction mixtures at 90° in the presence of 1% SDS and isolating the surviving ternary complexes by Sephadex G-50 centrifugal elutriation (16).

Glutamyl chain-length analysis of cellular CH_2H_4PteGlu. Ternary complexes were formed *in vitro* under the conditions described for the folate-binding assay among [^{32}P]FdUMP (20 Ci/mmol), TS from human HEp-2/500 cells (22) partially purified by ammonium sulfate fractionation (23), and either (6*R*)-$CH_2H_4PteGlu_n$ standards or ascorbate cell extracts. In these studies, TS from human cells was utilized rather than that from *L. casei* because anomalous gel bands were observed with ternary complex reactions utilizing the bacterial enzyme, [^{32}P]FdUMP, and CH_2H_4PteGlu monoglutamate; no such bands were present in complex reactions prepared with human enzymes nor were these bands present in complex reactions utilizing [^{3}H]FdUMP and TS from either source. The complexes were denatured in 6 M urea and separated by isoelectric focusing as described previously (12). After electrophoresis, the gels were fixed, dried, and subjected to autoradiography at −70°.

Affinity of TS for ligands. RCA and C were grown in folate-free medium (see above) to prevent interference from folylpolyglutamates in the folate-binding assay. TS enzymes from folate-depleted cells were partially purified by ammonium sulfate fractionation (23). The affinity of the enzymes for FdUMP was determined by a modification of the folate-binding assay utilizing 0.2 μM TS enzyme, 0.02–3.5 μM [6-^{3}H]FdUMP, and 135 μM (6*R,S*)-CH_2H_4PteGlu; the affinity for CH_2H_4PteGlu was determined in this assay utilizing 0.3 μM [6-^{3}H]FdUMP and 19–380 nM (6*R,S*)-CH_2H_4PteGlu. The extent of ternary complex formation was determined by the SDS-centrifugal elutriation procedure (see above).

TS level determination. To quantitate the level of TS enzyme utilized in these studies, the amount of [^{3}H]FdUMP bound to enzyme in the presence of CH_2H_4PteGlu was determined by a modification of the procedure of Moran *et al.* (18) as described previously (12).

Determination of radioactivity. Aqueous samples were counted in 10 ml of Liquiscint scintillation solution (National Diagnostics, Manville, NJ) using a Packard Tri-Carb model 4660 liquid scintillation counter.

Results

Growth response to FdUrd in the presence of CF. As shown in Fig. 1, the response of both cell lines to FdUrd was

modulated by CF. Above 10^{-8} M, increasing the CF concentration enhanced the sensitivity to FdUrd (as demonstrated by a reduction in the ID_{50} value). Thus, the sensitivity of RCA to FdUrd was increased by 10-fold over this CF concentration range, whereas that of C was increased by 4-fold. It is apparent that the two cell lines differed in the extent of modulation by CF, with RCA being considerably more responsive. Below 10^{-8} M, decreasing the CF concentration paradoxically increased the sensitivity of RCA to FdUrd. At these CF concentrations, the growth of RCA is suboptimal, with 80% and 30% of maximal cell growth occurring at 3×10^{-9} M and 10^{-9} M, respectively; in C, CF concentrations below 10^{-8} M support less than 10% of optimal cell growth (data not shown). The mechanism underlying the inverse modulation in RCA below 10^{-8} M CF is unknown; however, this phenomenon has been observed recently in mouse leukemia L1210 cells exposed to either FUra or FdUrd and CF concentrations limiting for maximal cell growth (24).

Analysis of intracellular TS folate co-substrates. A folate-binding assay was developed to determine the level of intracellular folates that stimulate ternary complex formation with *L. casei* TS and [^{3}H]FdUMP. A linear relationship was observed between the extent of ^{3}H-labeled ternary complex formation and the concentration of $CH_2H_4PteGlu$ in the assay. Standard curves relating ^{3}H-labeled ternary complex formation and either (6*R,S*)-$CH_2H_4PteGlu$ or (6*R*)-$CH_2H_4PteGlu$ concentration were similar in nature, indicating that the inactive isomer is inert in this assay. Because dUMP, the substrate for TS, is expected to interfere with the assay by virtue of direct competition with FdUMP for binding to TS as well as by stimulating the conversion of $CH_2H_4PteGlu$ to $H_2PteGlu$ during enzyme catalysis, the effect of dUMP was determined on the assay. The formation of radiolabeled ternary complexes was dependent on dUMP concentration; in fact, the assay is a sensitive radioligand-binding method for quantitation of dUMP. Because cellular dUMP may interfere with determination of TS folate co-substrates by this assay, $CH_2H_4PteGlu$ standards were added to folate-depleted cell extracts; no reduction in ternary complex formation was observed, suggesting that the intracellular levels of dUMP are <1 μM in these cells.

Folates other than $CH_2H_4PteGlu$ may stimulate ternary complex formation between TS and FdUMP in the charcoal-binding method of the assay (see Experimental Procedures). The capacity of $H_4PteGlu$, 5-$CH_3H_4PteGlu$, CF, and 10-CHO-$H_4PteGlu$ to stimulate ternary complex formation was determined by utilizing 15 nM of each folate in the assay. Relative to an equivalent amount of $CH_2H_4PteGlu$, $H_4PteGlu$, 5-$CH_3H_4PteGlu$, CF, and 10-CHO-$H_4PteGlu$ stimulated 7.2%, <1%, <1%, and <1%, respectively, of ternary complexes (data not shown).

The levels of cellular folates formed from CF that stimulate ternary complex formation *in vitro* are shown in Fig. 2. At all concentrations of CF, the levels of TS folate cosubstrates in C were higher than in RCA. In both cell lines, $CH_2H_4PteGlu_n$ represented a relatively small proportion of the total TS folate cosubstrates; 11–29% in C and 14–24% in RCA. Thus, C, which is more sensitive to FdUrd at all concentrations of CF, accumulated higher levels of TS folate cosubstrates than did RCA. When the relationship between sensitivity to FdUrd and intracellular $CH_2H_4PteGlu_n$ levels was examined, it became apparent that the two cell lines require different levels of folate to promote equivalent TS-directed cytotoxicity (Fig. 3). The $CH_2H_4PteGlu_n$ levels were normalized to TS levels to facilitate comparison between the cell lines; TS levels remained unaltered in these cells under folate-depleted and folate-repleted conditions.[1] Thus, RCA required higher levels of $CH_2H_4PteGlu_n$ than did C to achieve a similar FdUrd sensitivity. The differential requirement of the cell lines for $CH_2H_4PteGlu_n$ could derive from a differential capacity to form $CH_2H_4PteGlu$ polyglutamates or from the presence of TS enzymes with differential affinity for $CH_2H_4PteGlu_n$.

Glutamyl chain-length distribution of $CH_2H_4PteGlu$. The affinity of most TS enzymes for $CH_2H_4PteGlu$ increases

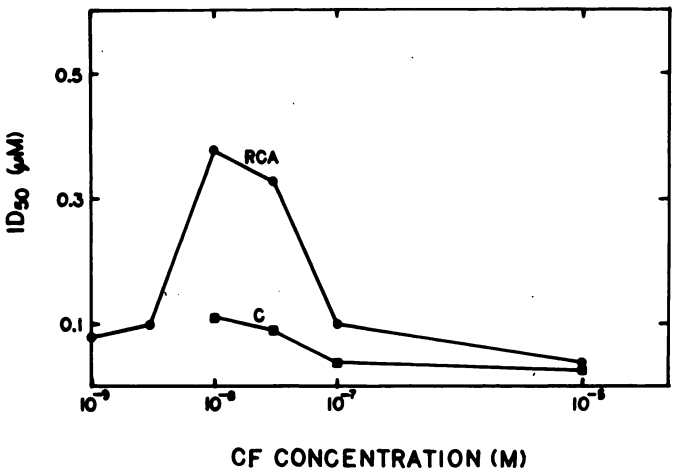

Fig. 1. Effect of CF on FdUrd sensitivity. Folate-depleted cells were exposed to CF-supplemented medium for 24 hr and then exposed to FdUrd for 3 hr in CF-supplemented medium. After drug removal, the cells were grown in CF-supplemented medium for 5–6 days before growth was measured by protein determination. The ID_{50} is the concentration of FdUrd required to inhibit cell growth by 50%, relative to untreated cells. The data are the mean of two separate determinations, each carried out in triplicate.

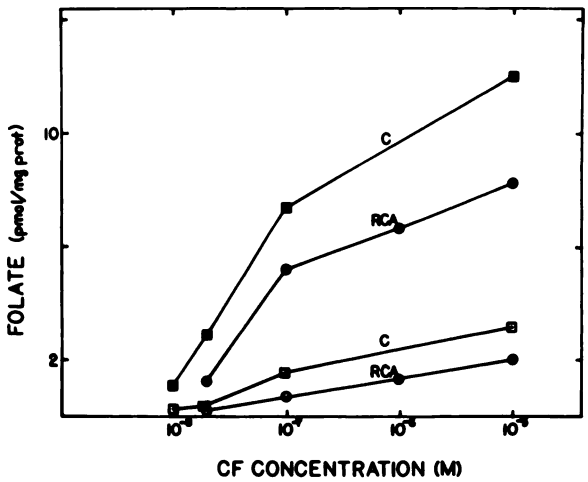

Fig. 2. Intracellular TS folate cosubstrates formed from CF. Folates from folate-depleted cells exposed to CF for 24 hr were extracted with hot Tris-buffered ascorbate and quantitated by the folate-binding assay (see Experimental Procedures). Total folate cosubstrates (*closed symbols*) were determined by the charcoal adsorption procedure; the data are the mean values of at least three separate determinations, each carried out in triplicate. The levels of $CH_2H_4PteGlu$ (*open symbols*) were determined by the gel filtration method; the data are the mean values of at least four separate determinations.

[1] C. Stevens and S. Berger, unpublished results.

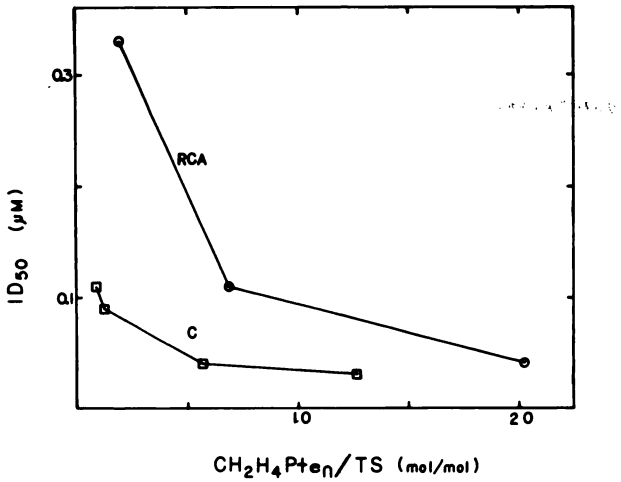

Fig. 3. Relationship between FdUrd sensitivity and $CH_2H_4PteGlu$ levels. FdUrd sensitivity as a function of CF concentration, expressed as the ID_{50} value, was determined as described in the legend to Fig. 1. The $CH_2H_4PteGlu$ levels were determined as described in the legend to Fig. 2. The $CH_2H_4PteGlu$ levels are expressed per mole of TS to normalize the data.

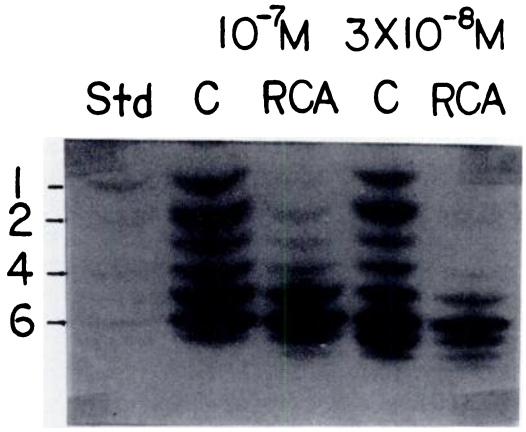

Fig. 4. Glutamyl chain length distribution of TS complexes formed from endogenous folates. Cellular folates were extracted from cells exposed to CF for 24 hr, as described in the legend to Fig. 2. Ternary complexes were formed among partially purified human HEp-2 TS, [^{32}P]FdUMP, and extracted folates under conditions in which folate is limiting; the complexes were denatured, separated by isoelectric focusing, and detected by autoradiography. Complexes prepared with $CH_2H_4PteGlu$ derivatives containing 1–6 glutamyl residues were utilized as mobility standards. *Std*, standard.

with increasing glutamyl chain length (8, 25). Furthermore, ternary complexes containing polyglutamate derivatives of $CH_2H_4PteGlu$ are more stable than complexes with the monoglutamate derivative (8, 9). Thus, the level and distribution of $CH_2H_4PteGlu$ polyglutamates formed from CF were analyzed. Ternary complexes prepared with cellular folates were denatured, allowing the survival of only complexes stimulated by $CH_2H_4PteGlu_n$. The complexes were separated by electrophoresis and the mobility was compared with that of complexes prepared with $CH_2H_4PteGlu_n$ standards. As shown in Fig. 4, both cell lines formed higher chain-length polyglutamates; in fact, the proportion of $CH_2H_4PteGlu$ pools represented by higher chain-length derivatives is greater in RCA than in C. The hexaglutamyl derivative was the predominant intracellular $CH_2H_4PteGlu$ form in both cell lines at these CF concentrations. At higher CF concentrations, the pentaglutamate derivative was the major form (data not shown). The data are qualitative, in that equivalent extract volumes, not extract protein, were utilized; quantitation by the *in vitro* binding assay revealed that the pools of $CH_2H_4PteGlu_n$ at both concentrations of CF are 2-fold higher in C. These data indicate that the RCA requirement for higher $CH_2H_4PteGlu_n$ levels to achieve TS-directed cytotoxicity equivalent to that of C is not a result of inability to polyglutamate $CH_2H_4PteGlu$.

Affinity of TS for ligands. An alternative explanation for the differential response to $CH_2H_4PteGlu_n$ is a difference between the TS enzymes in the affinity for ligands involved in ternary complex formation. Thus, the affinity of the enzymes from RCA and C for both FdUMP and $CH_2H_4PteGlu$ was determined. No difference was observed between the two enzymes in the affinity for FdUMP (data not shown); however, as shown in Fig. 5, the enzyme from RCA required 3-fold higher $CH_2H_4PteGlu$ levels than that from C to achieve 50% of maximal ternary complexes. That the *in vitro* data reflect TS-specific radiolabeling was verified by isoelectric focusing gel electrophoresis of the ternary complexes; a single radiolabeled band was observed which comigrated with ternary complexes formed with human TS and $CH_2H_4PteGlu$ monoglutamate. Densitometric scans of the autoradiograms revealed that the TS enzyme from RCA required 3-fold higher $CH_2H_4PteGlu$ for maximal ternary complex formation, relative to the C enzyme (data not shown).

Discussion

These studies are the first to demonstrate that variation in the response to fluoropyrimidine-CF combinations is associated with TS. Cell line C is sensitive to FdUrd, regardless of the presence of CF, whereas RCA is highly responsive to FdUrd in the presence of pharmacological concentrations of CF but poorly responsive when CF concentrations are more physiological. The term physiological refers to concentrations of CF that are minimal for optimal cell growth, namely 10^{-8} M in both cell

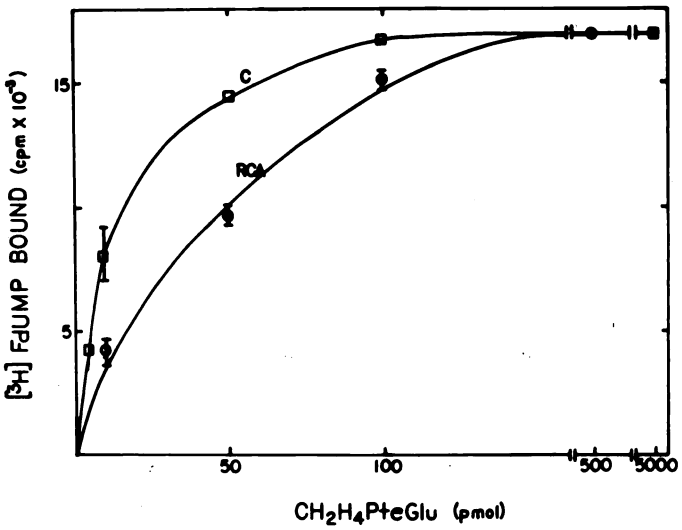

Fig. 5. $CH_2H_4PteGlu$ binding to TS enzymes from RCA and C. TS, partially purified from cell lines RCA or C, was incubated with excess [^{3}H]FdUMP and varying concentrations of $CH_2H_4PteGlu$, as described in Experimental Procedures; the resulting complexes were analyzed by gel filtration. The data are the mean of three separate determinations; the *error bars* indicate the standard deviation.

lines.[2] The identification of the RCA response phenotype provides support for the clinical utilization of combinations containing high-dose CF and fluoropyrimidines in the therapy of colorectal carcinoma. The fact that the inclusion of CF significantly increases clinical response to FUra suggests that the RCA phenotype may be representative of colon tumor phenotypes encountered clinically. An additional response phenotype associated with FdUrd-CF combinations has been observed in this laboratory. Two human colorectal tumor cell lines have been identified that are resistant to FdUrd, regardless of CF concentration.[3] Elucidation of the mechanism(s) underlying the response differences among human colorectal tumor cell lines may have diagnostic and therapeutic application for the effective clinical utilization of fluoropyrimidine-CF combinations.

An additional important outcome of these studies is the identification of a naturally occurring variant of human TS, with altered affinity for $CH_2H_4PteGlu$. This is the second report of a human TS enzyme with functional variation that is expressed in cells not previously exposed to fluoropyrimidine agents (26). Although these TS variants may have arisen in the tumor during progression or during cultivation *in vitro*, the possibility exists that the TS enzyme is naturally variant in the human population. If so, these variants and others, yet unidentified, may be utilized as markers of functional alteration and, hence, of reduced response to fluoropyrimidine chemotherapy. The association of enzyme variation with reduced response to fluoropyrimidine agents may contribute significantly to the more rational clinical utilization of these agents.

Several, more specific comments, concerning interpretation of the data are appropriate. The observation of inverse modulation, that is, increased sensitivity to FdUrd with decreasing CF, has been reported previously (24). In both studies, the phenomenon occurred in cells exposed to concentrations of CF that are suboptimal for cell growth. It is possible that, in the presence of FdUMP, intracellular reduced folates, which are already limiting for maximal cell growth, are depleted further by binding to TS in ternary complexes. Thus, cytotoxicity could result from thymidine and/or purine deprivation, depending on which reduced folates become limiting for metabolite biosynthesis.

The studies aimed at analyzing the intracellular levels of TS folate cosubstrates indicate that $CH_2H_4PteGlu_n$ represents a small proportion of the total TS folate co-substrates (Fig. 2); hence, the major proportion of complexes formed after CF are composed of folates noncovalently bound to FdUMP in the ternary complex. In folate-depleted mouse leukemia L1210 cells, the predominant intracellular folates present after exposure to CF include 10-CHO-H_4PteGlu, CF, 5-CH_3H_4PteGlu, and H_4PteGlu (27). Due to the limited capacity of these monoglutamates to stimulate ternary complex formation *in vitro*, it is likely that the major proportion of TS folate cosubstrates formed from CF *in situ* are polyglutamate derivatives. In fact, it has been shown that polyglutamate forms comprise the major proportion of folate pools after CF (27). The contribution of reduced folates other than $CH_2H_4PteGlu$ to the cytotoxicity of FdUrd has not been investigated, although it has been demonstrated that several of these folates promote covalent interactions between FdUrd and TS (7); moreover, it has been shown that polyglutamate derivatives of folates other than $CH_2H_4PteGlu$ participate in the formation of stable ternary complexes with FdUMP and TS (6).

With regard to the mechanism(s) underlying the differential response to FdUrd-CF, these studies reveal that the two cell lines have a differential capacity to accumulate TS folate cosubstrates. Clearly, this difference may contribute to the variation in response to FdUrd-CF; however, the cells respond differently to equivalent levels of $CH_2H_4PteGlu_n$. This indicates that additional mechanisms are contributing to the response variation. Because it has been demonstrated that increasing the glutamyl chain length increases the rate of ternary complex formation and decreases ternary complex dissociation (8), an impaired capacity to form higher chain-length glutamyl derivatives could underlie the differential response to equivalent $CH_2H_4PteGlu_n$ pools. No significant difference was observed between the cells in the capacity to generate higher chain-length glutamyl derivatives of $CH_2H_4PteGlu$ at concentrations of CF associated with considerable differences in response to FdUrd; in both cells, the predominant $CH_2H_4PteGlu$ derivative is the hexaglutamate form. An alternative explanation for the differential response to $CH_2H_4PteGlu_n$ is a variation in the affinity of TS for these folate derivatives. The binding data revealed that the RCA enzyme has a reduced affinity for $CH_2H_4PteGlu$, relative to the C enzyme. This is consistent with the differential response to FdUrd-CF and to equivalent intracellular $CH_2H_4PteGlu_n$. That the folate-binding variation observed for the partially purified RCA TS is intrinsic to the enzyme is suggested by recent attempts to purify the enzyme to homogeneity. In contrast to other human TS enzymes, the RCA TS does not bind to a 10-formylfolate-Sepharose affinity column. Studies in progress are aimed at purifying the TS enzymes from both cell lines and at analyzing the kinetic and binding characteristics of the two enzymes.

The variation in the RCA enzyme may be due to variation in the TS gene or in posttranscriptional processing. Previous studies revealed that the TS gene in RCA is variant with respect to the gene in six other human colorectal tumor cell lines, including C (12). The DNA variation in RCA could underlie or be tightly linked with functional alternation in the TS enzyme; thus, it would serve as a marker for TS enzyme variation. Alternatively, the DNA variation could be unlinked to the enzyme alteration. With regard to differences in posttranscriptional processing, the TS mRNA molecules are identical in size (12) and the TS polypeptides are identical in structure (11) and in size (data not shown), within the limits of detection of the analyses. In order to determine whether the functional alteration derives from the TS gene, studies in progress are aimed at isolating cDNA molecules corresponding to each TS mRNA. These studies will provide resources for correlating enzyme function with gene structure in heterologous systems that are TS-negative.

Acknowledgments

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