Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1377–1381, February 1989
Medical Sciences

Mutants of human colon adenocarcinoma, selected for thymidylate synthase deficiency

(tumorigenicity/5-fluorouracil/thymidine salvage)

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Communicated by George H. Hitchings, November 3, 1988

ABSTRACT **GC_3/c1 human colon adenocarcinoma cells were treated with the mutagen ethyl methanesulfonate, and three clones deficient in thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) activity were selected and characterized. Growth in medium deficient in thymidine caused cell death in two clones (TS^-c_1 and TS^-c_3), whereas one clone (TS^-c_2) showed limited growth. Growth correlated with thymidine synthase activity and 5-fluoro-2′-deoxyuridine 5′-monophosphate-binding capacity and with incorporation of 2′-deoxy[6-^{3}H]uridine into DNA. In the presence of optimal thymidine, growth rates were only 5–18% that of the parental clone (GC_3/c1), which grew equally well in thymidine-deficient or -replete medium. Analysis of poly(A)$^+$ RNA showed normal levels of a 1.6-kilobase transcript in TS^-c_1 and TS^-c_2 but decreased levels (≈6% control) in TS^-c_3. Clone TS^-c_3 was 32-, 750-, and >100,000-fold more resistant than the parental clone to 5-fluorouracil, 5-fluoro-2′-deoxyuridine, and methotrexate, respectively. When inoculated into athymic nude mice, each TS^- clone produced tumors, demonstrating continued ability to proliferate *in vivo*.**

Thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45; dTMP synthase) catalyzes the conversion of dUMP to dTMP and in mammalian cells functions as the only *de novo* source of dTMP. This enzyme has thus been of considerable interest as a target for cancer chemotherapeutic agents (1–3). dTMP synthase is considered to be the primary site of action for 5-fluorouracil (FUra), 5-fluoro-2′-deoxyuridine (FdUrd), and the folate analogues 5,8-dideazaisofolic acid and N^{10}-propargyl-5,8-dideazafolic acid (3–5). Further, polyglutamyl derivatives of methotrexate are inhibitors of this enzyme (6). Despite the availability of potent inhibitors of dTMP synthase, most human cancers are intrinsically resistant to agents acting at this locus. For FUra many mechanisms for intrinsic and acquired resistance have been proposed (reviewed in ref. 3). In studies using human colon adenocarcinomas maintained in mice as xenografts, intrinsic resistance to FUra appeared to be a consequence of relatively transient inhibition of dTMP synthase (7). This appeared to be due to low levels of 5,10-methylenetetrahydrofolate (CH_2-H_4PteGlu), or its polyglutamyl derivatives, required to stabilize the covalent ternary complex of the FUra metabolite FdUMP, enzyme, and CH_2-H_4PteGlu (8). An alternative mechanism of intrinsic resistance, namely salvage of thymidine (dThd), appeared of little significance, as cloned lines deficient in dThd kinase (9), growing as xenografts, were no more sensitive to FUra than the parental line. These data also indicated that colon adenocarcinoma cells, obligated to dTMP synthesis *de novo*, were tumorigenic in mice. However, the significance of dThd salvage *in vivo* under conditions of complete and prolonged inhibition of dTMP synthase remained unanswered. In experiments reported here we selected and characterized three clones of GC_3/c1, a human colon adenocarcinoma (9), each deficient in dTMP synthase (TS^-). Each TS^- clone retained tumorigenic potential in athymic mice and retained histologic characteristics of the parental cloned line.

MATERIALS AND METHODS

Materials. Sephadex G-25 columns (PD10) were obtained from Pharmacia. [5-^{3}H]dUMP, [6-^{3}H]dUrd, [5-^{3}H]dUrd, and [6-^{3}H]FdUMP (specific activities, 20–22 Ci/mmol; 1 Ci = 37 GBq) were purchased from Moravek Biochemicals (Brea, CA). Methotrexate (MTX) and aminopterin were obtained from the Drug Development Branch, National Cancer Institute, and FUra and FdUrd were obtained through the pharmacy at Saint Jude Children's Research Hospital. All other chemicals were purchased from Sigma.

Cell Culture. The GC_3/M cell line, derived from a human colon adenocarcinoma grown initially as a xenograft in mice, has been described (9). From this line, clones were obtained by conventional methods (10). GC_3/c1 was maintained in RPMI 1640 (Hazleton Research Products, Reston, VA) containing 10% fetal bovine serum (FBS; Hyclone) without added antibiotics.

Selection of TS^- Cells. Clones of GC_3/c1 deficient in dTMP synthase were selected (on passage 56 for TS^-c_2 and TS^-c_3 and on passage 57 for TS^-c_1) by a procedure similar to that of Li and Chu (11). Cells (2×10^6 per T75 flask) were exposed for 18 hr to ethyl methanesulfonate (600 μg/ml), washed, and refed with RPMI 1640 containing 10% FBS and 10 μM dThd. Cells were refed after 3 days with supplemented medium. After an additional 2 days, cells from 10 culture flasks were pooled and plated at 7×10^5 cells per 100-mm dish. Cells were grown in selection medium (RPMI 1640 containing 5% dialyzed FBS, 1.0 μM aminopterin, 10 μM dThd, 0.1 μM folinic acid, and 100 units of penicillin and 100 μg of streptomycin per ml) and subsequently were maintained in this medium for 19 days, at which time 17 colonies were isolated by using cloning cylinders. Selection frequency was 8.5×10^{-7}. Cells from individual colonies were trypsinized and replated in one well of a 24-well plate. Cells were maintained in selection medium for a further 8 days, at which time cells from 9 of the original colonies were viable. Four of

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Abbreviations: FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FBS, fetal bovine serum; MTX, methotrexate; CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate; TS^-, thymidylate synthase-deficient.

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these were grown in RPMI 1640 containing 10% FBS, 50 μM dThd, 0.1 μM folinic acid, and antibiotics.

Growth Inhibition. Cells were plated at 1.5×10^5 per cm^2 and incubated for 24 hr in drug-free medium (RPMI 1640 containing 10% FBS and dThd). Cells were harvested from representative plates, enumerated, and incubated in drug-containing medium for 72 hr, at which time cell number was again determined. For each concentration of drug, triplicate cultures were examined.

Assay of dTMP Synthase. Cells were seeded at a density of 5.7×10^3 per cm^2 and harvested by mild trypsinization on day 4. Cells were washed twice in ice-cold phosphate-buffered saline and once in disruption buffer (20 mM Tris·HCl/70 mM 2-mercaptoethanol/100 mM NaF/5 mM AMP). After centrifugation (200 × *g*, 5 min, 4°C), cells were resuspended at 10^8 per ml in disruption buffer and homogenized [Polytron (Brinkmann) setting 10; twice for 15 sec, 4°C]. The homogenate was centrifuged (15,000 × *g*, 15 min, 2°C) and the supernatants were used for assay of dTMP synthase activity as described (7). To remove endogenous dUMP, 0.5-ml supernatants were centrifuged (200 × *g*, 4 min, 4°C) on a Sephadex G-25 column (PD10), preequilibrated with disruption buffer as described by Meyer *et al.* (12). In preliminary experiments this removed >90% of [5-^{3}H]dUMP added to supernatants. For assay the final reaction mixture contained 10 μM [5-^{3}H]dUMP (specific activity, 632 mCi/mmol), 100 μM (6*RS*)CH_2-H_4PteGlu [racemic mixture of the natural (6*R*) and unnatural (6*S*) diastereoisomers of CH_2-H_4PteGlu], 70 mM 2-mercaptoethanol, 100 mM NaF, 5 mM AMP, and 300 μl of cell supernatant in a volume of 600 μl. Protein concentrations were determined by the method of Bradford (13); cell number was determined by counting nuclei (Coulter ZM) after cell lysis, according to the procedure of Butler (14).

FdUMP Binding Assay. Cell supernatants were prepared as above, and [6-^{3}H]FdUMP (87.7 μM) was substituted for [5-^{3}H]dUMP. Formation of covalent ternary complex was assayed by adsorption of unbound FdUMP to charcoal–albumin–dextran (7).

Incorporation of [6-^{3}H]dUrd into DNA. Cells were plated (5×10^6 per dish) in 35-mm dishes (Falcon 3001) and allowed to attach for 14–16 hr. Medium was aspirated and replaced with 1 ml of RPMI 1640 containing 10% FBS and 2 μCi of [6-^{3}H]dUrd. Cells were incubated at 37°C for up to 30 min. At the appropriate time, radiolabeled medium was aspirated and the monolayer was washed twice with ice-cold phosphate-buffered saline. Cells were solubilized in 1 M KOH (37°C, 60 min), and DNA was extracted by a modified Schmidt–Thannhauser procedure (15). Radiolabel incorporated into alkali-stable, acid-precipitable material was determined after hydrolysis in 1 M perchloric acid (65°C, 1 hr). In some experiments [5-^{3}H]dUrd was substituted for [6-^{3}H]dUrd.

For autoradiographic studies, 10^5 cells were plated per plastic microscope slide (9) and, after 24 hr, exposed to [6-^{3}H]dUrd (1 μCi/ml) for 1 hr. Cells were fixed in 95% ethanol and autoradiographs were produced. For analysis of [6-^{3}H]dUrd labeling index, at least 5000 cells were scored.

Growth of Heterografts. Cells selected for dTMP synthase deficiency were resuspended at 10^8 per ml and inoculated subcutaneously into both flanks of three BALB/c athymic nude mice (The Jackson Laboratory). Mice were housed in filter-top cages in a humidity- and temperature-controlled room. Upon reaching a diameter >1.5 cm tumors were excised, and fragments were transplanted subcutaneously in both dorsal flanks of female CBA/CaJ mice that had been immune-deprived by thymectomy at age 4 weeks, followed in 3 weeks by 925-cGy whole-body irradiation and rescue with 5×10^6 nucleated marrow cells (16).

Hybridization Studies. RNA was extracted from 10^8 cells by a modified method of Feramisco *et al.* (17). Poly(A)$^+$ RNA was purified by chromatography over oligo(dT)-cellulose and quantitated by determining optical density (OD_{260}/OD_{280} > 1.8; 1 OD_{260} unit = 40 μg of RNA per ml). For Northern blot analysis, poly(A)$^+$ RNA was separated according to size by electrophoresis in 1% agarose containing 6 M formaldehyde and transferred to nitrocellulose by conventional techniques. For dot blot quantitation, poly(A)$^+$ RNA denatured in 10× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) containing 4.68 M formaldehyde was applied to nitrocellulose. Filters were incubated for at least 2 hr at 42°C in prehybridization buffer containing 50% formamide, 5× SSC, 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.1% SDS, 20 mM sodium phosphate (pH 6.5), and 250 μg of denatured salmon sperm DNA per ml. Hybridization was allowed to proceed for at least 15 hr at 42°C in a minimal amount (50 μl/cm^2) of the same buffer with added ^{32}P-labeled probe (10^6 cpm/ml). Filters were washed four times for 5 min at room temperature in 2× SSC/0.1% SDS and four times for 15 min at 50°C in 0.1× SSC/0.1% SDS before they were processed for autoradiography.

pcHTS-1, a functional cDNA for dTMP synthase isolated from a human expression library (ref. 18; a gift from T. Seno), was labeled by nick-translation with [α-^{32}P]dCTP and [α-^{32}P]dTTP. To normalize for mRNA, filters were dehybridized and rehybridized with pA_1, a β-actin probe provided by D. Cleveland (19). Hybridization signals were quantitated by densitometry (EC densitometer) of autoradiographs; a Shimadzu C-R3A Chromatopak was used to integrate peak areas.

RESULTS

Primary TS$^-$ Clones. Four clones were derived from the selection procedure described in *Materials and Methods*. To determine whether these were dThd auxotrophs, duplicate cultures were grown in RPMI 1640 containing 10% dialyzed FBS and supplemented with 50 μM dThd or no dThd. Three clones degenerated rapidly, whereas the fourth was capable of growth without dThd. This clone was discarded. For TS$^-c_1$ and TS$^-c_3$ there was, respectively, a 58% and a 30% decrease in cells recovered after 3 days in the absence of dThd. TS$^-c_2$ demonstrated slight growth in the absence of dThd. Optimal growth required >5 μM dThd. In contrast, maximal growth for the parent line GC_3/c1 was determined in the absence of exogenous dThd. Even under conditions for

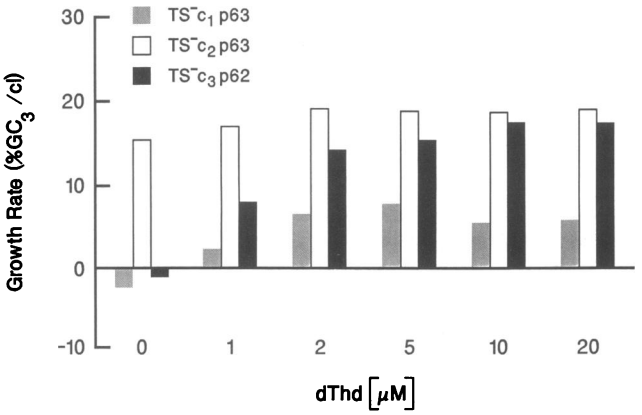

FIG. 1. Growth of TS$^-$ clones relative to GC_3/c1 at different dThd concentrations. Increase in cell number over 6 days was determined for TS$^-c_1$ at passage 63 (TS$^-c_1$p63; passage 6 after selection), TS$^-c_2$p63 (passage 7 after selection), and TS$^-c_3$p62 (passage 6 after selection) in the presence of various dThd concentrations. At optimal dThd, growth rates for TS$^-$ clones were 5%, 18%, and 16% that of GC_3/c1 grown in the absence of dThd. Each result is the mean of three determinations.

Table 1. dTMP synthase activity in GC_3/c1 and primary TS^- clones

Clone and passage (p) no.	Activity	
	pmol per min per 10^8 cells	%
GC_3/c1p134	17.55 ± 1.25	100 ± 7.1
TS^-c_1p64	0.16 ± 0.01	0.92 ± 0.1
TS^-c_2p64	1.60 ± 0.16	9.07 ± 0.24
TS^-c_3p63	0.93 ± 0.25	5.25 ± 1.03

maximal growth TS^-c_1, TS^-c_2, and TS^-c_3 grew at 5%, 18%, and 16%, respectively, of the rate for GC_3/c1 (Fig. 1).

For each clone, dTMP synthase activity was determined 4 days after plating, during which time cultures were in mid-logarithmic growth. For TS^-c_1p64 (passage 7 after selection), no activity was detected (<1.0% control) even when the assay period was extended to 45 min or when endogenous dUMP was removed by Sephadex G-25 filtration (data not shown). For GC_3/c1, dTMP synthase activity was 17.55 ± 1.25 pmol per min per 10^8 cells (Table 1). Some activity was detected in TS^-c_3p63 (5.2%), whereas TS^-c_2p64 had ≈9% of the activity determined in GC_3/c1. Enzyme activity paralleled incorporation of [6-^{3}H]dUrd into DNA (Fig. 2), where for TS^-c_1 and TS^-c_3 incorporation was 1.1 ± 0.6% and 9.7 ± 1.5% of the rate for GC_3/c1. Duplicate experiments using [5-^{3}H]dUrd showed no incorporation of label into DNA (data not shown). Similarly, [6-^{3}H]FdUMP binding activity in TS^-c_1 was undetectable, and in TS^-c_3 it was 13.6% of that in GC_3/c1 (1.69 pmol per 10^8 cells). For TS^-c_2p73 binding activity was 39.5% of control. An autoradiograph of reaction mixtures analyzed by SDS/PAGE under reducing conditions is shown in Fig. 3. For GC_3/c1 the ternary complex migrated at 36 kDa; a similar band, but with reduced intensity, was observed for TS^-c_2 and TS^-c_3. No ternary complex was observed in TS^-c_1 extracts. Thus each assay gave qualitatively similar data for these clones.

To determine whether low levels of dTMP synthase activity were a consequence of altered transcription, Northern blots of mRNA were probed with pcHTS-1 radiolabeled by nick-translation. mRNA complementary to pcHTS-1 was detected in each of the TS^- clones, although the level was lower in TS^-c_3 (Fig. 4). In each case mRNA migrated with

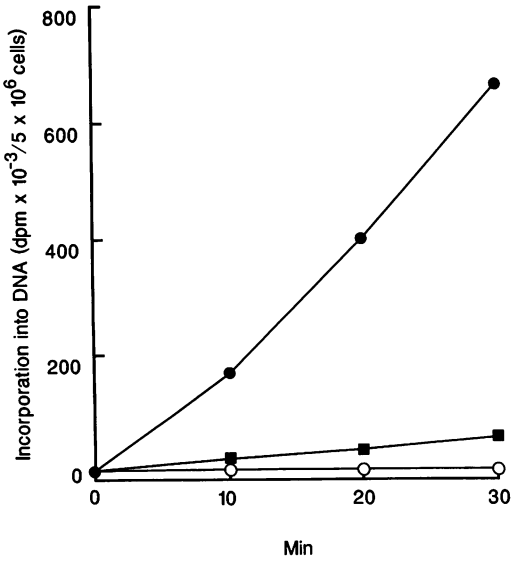

FIG. 2. Incorporation of [6-^{3}H]dUrd into DNA of GC_3/c1 and TS^- clones. Cells (5×10^6) were plated and allowed to attach overnight. [6-^{3}H]dUrd-containing medium was applied and incorporation into alkali-stable, acid-precipitable material was determined at the times indicated. Each result is the mean of duplicate experiments. ●, GC_3/c1; ○, TS^-c_1; ■, TS^-c_3.

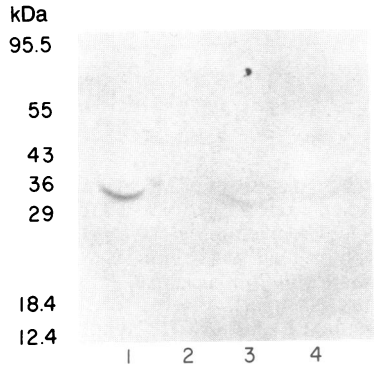

FIG. 3. Formation of [6-^{3}H]FdUMP–dTMP synthase–CH_2-H_4PteGlu complex in GC_3/c1 and TS^- clones. Reactions were terminated at 30 min by adding 25 μl of SDS sample buffer (4% SDS/2% 2-mercaptoethanol/40% glycerol) to 100 μl of reaction mixture and placing the sample at 100°C for 2 min; 100 μl was analyzed by SDS/9% PAGE. Lanes: 1, GC_3/c1; 2, TS^-c_1; 3, TS^-c_2; 4, TS^-c_3.

that from the parent line (1.6 kilobases). Dot blot analysis (Fig. 5), and rehybridization of filters with a probe for actin mRNA (19), allowed relative quantitation of the dTMP synthase mRNA. For clones TS^-c_1 and TS^-c_3, levels similar to the parent GC_3/c1 were determined (93% and 121%), whereas in TS^-c_3 the level was ≈6% that of the parent line.

Growth studies were conducted to determine the sensitivity of TS^-c_3 to chemotherapeutic agents that exert cytotoxic activity through a direct or indirect effect on dTMP synthase. Growth-inhibitory effects of FUra, FdUrd, and MTX were examined at 6.3 or 17.3 μM dThd to allow optimal growth of TS^-c_3 (Table 2). For parental GC_3/c1 cells, increasing the concentration of dThd (from 6.3 to 17.3 μM) caused protection from both FUra and FdUrd (15- and 140-fold, respectively) but not from MTX. These data indicated that the primary site of cytotoxicity for FdUrd and FUra in this human line was dTMP synthase. For TS^-c_3 the IC_{50} values for FUra and FdUrd (at 6.3 μM dThd) were 32-fold and 750-fold greater than for GC_3/c1. For MTX, the IC_{50} was in excess of 1 mM, indicating that TS^-c_3 was $>10^5$-fold more resistant than GC_3/c1 to this agent.

Secondary TS^- Clones. The phenotype of TS^-c_1 and TS^-c_3 clones remained relatively constant, whereas TS^-c_2 cultures regained dTMP synthase activity over nine serial transfers (passages 64–73). To examine the stability and growth characteristics of TS^-c_1 and TS^-c_3 clones, subclones were selected after exposure to 500 μM MTX in the presence of 20 μM dThd. Independent clones from TS^-c_1 (designated TS^-c_1/c_{1-3}) and TS^-c_3 (TS^-c_3/c_{1-4}) were expanded to $>10^{10}$ cells and incorporation of [6-^{3}H]dUrd was examined on 10^5 cells by autoradiography. This technique was used to distinguish between low incorporation in a high proportion of cells and high incorporation in very few cells (true revertants). For each clone the population doubling time was between 47.6 and 52.6 hr during logarithmic growth. Data are presented in

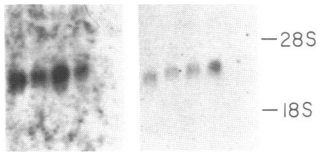

FIG. 4. Northern blot analysis of poly(A)$^+$ RNA from GC_3/c1 and TS^- clones. Poly(A)$^+$ RNA (5 μg per lane) was electrophoresed in formaldehyde/agarose gel and transferred to nitrocellulose. (*Left*) Hybridization with nick-translated pcHTS-1 to detect dTMP synthase mRNA. (*Right*) Same filter rehybridized with pA1 actin probe. Lanes left to right: GC_3/c1, TS^-c_1, TS^-c_2, and TS^-c_3. Note that TS^-c_3 was overloaded to demonstrate pcHTS-1 hybridization.

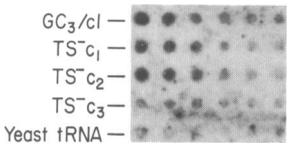

FIG. 5. Dot blot analysis of dTMP synthase mRNA. Poly(A)$^+$ RNA was extracted from GC_3/c1p89, TS^-c_1p66, TS^-c_2p66, and TS^-c_3p74. Samples (from left to right: 4 μg and serial 1:2 dilutions) were bound to nitrocellulose and hybridized to ^{32}P-labeled pcHTS-1. Results demonstrate very low signal in TS^-c_3 extracts.

Table 3 and indicate that TS^-c_1 subclones are generally stable, whereas TS^-c_3/c_2 had reverted, was able to grow in dThd-free medium, and had 15% of control dTMP synthase activity.

Growth as Heterografts. Previous work demonstrated that mutants of GC_3/c1 deficient in dThd kinase were tumorigenic in immune-deprived mice (ref. 9 and unpublished data). It was thus of interest to determine whether clones deficient in dTMP synthase (dThd auxotrophs) were also capable of forming tumors *in vivo*. Approximately 10^7 cells from each primary TS^- clone (passage 64 or 65) were inoculated bilaterally into the subcutaneous space of BALB/c athymic nude mice. Each line was tumorigenic, producing tumors in two of three mice, and the tumors maintained the poorly differentiated histological characteristics of the parental clone. Subsequently, each line was transplanted into immune-deprived CBA/CaJ mice after growth in nude mice for 122 and 173 days, respectively. Progressively growing TS^-c_1 and TS^-c_3 second-passage tumors were examined 250 and 294 days after initial inoculation into mice. Immune-deprived mice received [6-^{3}H]dUrd (1 μCi/g of body weight), tumors were excised after 1 hr, and autoradiographs were produced. The labeling index for TS^-c_1 and TS^-c_3 was 42% and 44%, respectively, of that for GC_3/c1.

DISCUSSION

Although TS^- mutants of rodent cells have been reported (11, 20) and extensively characterized (21), no mutants of human cells have been reported. We were specifically interested in deriving TS^- mutants from a human colon adenocarcinoma as this locus is a major focus for therapy of these cancers and the consequences of thymidine restriction remain to be fully characterized in human cells. Three clones were selected, two of which were auxotrophic for dThd. Assay for dTMP synthase activity using [5-^{3}H]dUMP revealed low or undetectable activity in TS^-c_3 and TS^-c_1, respectively. Growth of TS^-c_1 and TS^-c_3 was optimal at >5 μM dThd, whereas its parental clone GC_3/c1 grew optimally without dThd. However, in comparison to the parental clone, apparent growth rates were 5%, 18%, and 16% for TS^-c_1, TS^-c_2, and TS^-c_3, respectively. The low growth rate in TS^-c_1 is a consequence of an increased population doubling time of 52 hr compared to 24 hr for GC_3/c1. Incorporation of [6-^{3}H]dUrd into DNA paralleled enzyme activity in TS^-c_1 and TS^-c_3 being 1.1% and 9.7% of the rate for the parental clone. To determine whether TS^-c_1 could incorporate dUTP into DNA, similar experiments using [5-^{3}H]dUrd were performed. There was no incorporation of radiolabel into DNA. FdUMP binding assays gave results qualitatively similar to those of the catalytic assay, with no detectable binding in TS^-c_1. Further, in TS^-c_1, no ternary complex could be detected after SDS/PAGE.

Table 2. Sensitivity of GC_3/c1 and TS^-c_3 to antimetabolites

Clone	dThd, μM	IC_{50},* M		
		FUra	FdUrd	MTX
GC_3/c1	6.3	2.1×10^{-7}	3.2×10^{-8}	1.3×10^{-8}
	17.3	3.1×10^{-6}	4.5×10^{-6}	2.1×10^{-8}
TS^-c_3	6.3	6.8×10^{-6}	2.4×10^{-5}	$>1.0 \times 10^{-3}$
	17.3	1.0×10^{-5}	1.8×10^{-5}	$>1.0 \times 10^{-3}$

*Concentration of agent required to inhibit growth by 50% over a 72-hr exposure.

Both TS^-c_1 and TS^-c_2 appeared to have normal levels of mRNA detected by hybridization with a human cDNA dTMP synthase probe. Analysis revealed a major band at ≈1.6 kilobases, with a minor band at 3.8 kilobases, similar to that reported for TIG-1 normal human fibroblasts (22). For TS^-c_3 the level of dTMP synthase mRNA (relative to the signal for an actin probe) was lower, ≈6% of the ratio determined in GC_3/c1 cells. Whether this is a consequence of reduced transcription or of altered stability of this mRNA remains to be determined.

We further characterized TS^-c_3 with respect to its sensitivity to FUra, FdUrd, and MTX (Table 2). For both fluoropyrimidines, dThd reversed growth inhibition in the parent clone (GC_3/c1), indicating that dTMP synthase was the primary target for cytotoxicity. In the presence of dThd to allow growth (6.3 μM), the IC_{50} values for FUra and FdUrd were increased 32-fold and 750-fold in TS^-c_3 relative to GC_3/c1. These results are in contrast to those of Akazawa *et al.* (23), who reported increasing cytotoxicity of FUra in the presence of dThd and no difference in sensitivity between mouse mammary tumor line FM3A and its TS^- derivative. The TS^-c_3 line was also highly resistant to MTX (>100,000-fold) as would be anticipated in a cell line in which dTMP synthase activity was negligible, since the loss of reduced folates through the dTMP synthase reaction under conditions of dihydrofolate reductase inhibition would be reduced. Data also suggested that in this line, MTX or its polyglutamyl metabolites *per se* did not exert growth inhibition due to secondary or tertiary sites of action [e.g., phosphoribosylaminoimidazolecarboxamide formyltransferase (24)].

Table 3. Characterization of secondary TS^- clones

Clone	[6-^{3}H]dUrd labeling index, % (passage)	dTMP synthase activity, % of GC_3/c1 (passage)	Growth in dThd-free medium,* % (passage)
GC_3/c1	26.0 (187)	100 (182)	ND
TS^-c_1/c_1	<0.00 (74)	<0.5 (70)	−78 (70)
TS^-c_1/c_2	<0.00 (74)	<0.5 (70)	−87 (69)
TS^-c_1/c_3	0.06 (74)	<0.5 (70)	−80 (70)
TS^-c_3/c_1	<0.00 (72)	<0.5 (69)	−85 (69)
TS^-c_3/c_2	30.8 (72)	15.5 (69)	+111 (69)
TS^-c_3/c_3	0.02(?)† (72)	<0.5 (69)	−85 (69)
TS^-c_3/c_4	0.35 (72)	<0.5 (69)	−91 (69)

ND, not determined.
*Percent change in cell number over 5 days.
†One cell with 6 grains in 6009 cells scored.

Data indicated that clones TS^-c_1 and TS^-c_3 were deficient in dTMP synthase. Clone TS^-c_2 appeared to have some enzyme activity and by passage 67 was capable of limited growth in the absence of dThd, whereas TS^-c_1 and TS^-c_3 were more stable. To examine stability of TS^-c_1 and TS^-c_3, secondary cloning was undertaken, and individual subclones were expanded to $>10^{10}$ cells over a period of 10 weeks. At this time only one clone, TS^-c_3/c_2, demonstrated growth in dThd-free medium; however, TS^-c_3/c_4 demonstrated few (0.35%) heavily radiolabeled cells by autoradiography after exposure to [6-^{3}H]dUrd. In contrast, clones derived from TS^-c_1 appeared relatively stable, and radiolabeled cells were detected in only one clone (TS^-c_1/c_3), at very low frequency. The growth rate of both primary and secondary TS^- clones suggests that salvage of dThd was inadequate for optimal growth in these cells.

It was thus of interest to determine whether these TS^- clones were tumorigenic in mice, where plasma concentrations of dThd have been reported to be <1 μM (25, 26). Tumor cells were inoculated into BALB/c athymic mice, and each clone produced tumors in two of three mice. Thus, at a time when primary clones were dThd auxotrophs (passages 63 and 64), they retained tumorigenic potential. However, analysis of second-passage transplants demonstrated incorporation of [6-^{3}H]dUrd into $\approx$5% of tumor cells (40% of that in GC_3/c1). Thus, within 250 days after initial inoculation, TS^+ cells comprised at least 40% of the tumor population. Whether tumor growth was initiated by inoculating very few TS^+ cells remains to be examined in detail, by using secondary TS^- clones. However, if the differential growth rate *in vitro* between TS^- and TS^+ clones of GC_3 were maintained in mice, the overgrowth of TS^- cells by revertants would be rapid. That the labeling index was only 40% of the control level suggests that at this time TS^- cells are present in the tumor population. Studies to examine early stages of tumor growth are necessary to resolve this. However, other data confirm that relatively low concentrations of dThd may protect GC_3/c1 cells when dTMP synthase is inhibited. At a concentration of FUra that reduced clonogenicity to <3% of control, addition of 0.1 or 1.0 μM dThd increased colony formation to 25% and 66%, respectively (S. Radparvar, P.J.H., and J.A.H., unpublished data). Hence, dThd concentrations found in humans and mice may provide adequate protection for cells in which dTMP synthase has been inhibited, and may support growth of TS^- mutants.

The current data indicate that *in situ* salvage of dThd may limit cytotoxicity due to complete and prolonged inhibition of dTMP synthase induced by chemotherapeutic agents. These mutants will be of value in determining the consequences of dTTP depletion and events that may cause death (27) of human colon cancer cells.

Note Added in Proof. TS^-c_1/c_1p69 cells injected into athymic mice formed tumors within 100 days of inoculation.

We are grateful to Dr. T. Seno and Dr. D. W. Cleveland for providing the cDNA probes used in this study. We thank Ruby Cook-Tharp and Pamela Cheshire for excellent technical assistance with animal studies. This work was supported by Public Health Service Grant CA32613 from the National Cancer Institute and by the American Lebanese Syrian Associated Charities.

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