Isolation and Functional Effects of Monoclonal Antibodies Binding to Thymidylate Synthase†

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ABSTRACT: Monoclonal antibodies against electrophoretically pure thymidylate synthase from HeLa cells have been produced. Antibodies (M-TS-4 and M-TS-9) from hybridoma clones were shown by enzyme-linked immunoassay to recognize thymidylate synthase from a variety of human cell lines, but they did not bind to thymidylate synthase from mouse cell lines. The strongest binding of antibodies was observed to enzyme from HeLa cells. These two monoclonal antibodies bind simultaneously to different antigenic sites on thymidylate synthase purified from HeLa cells, as reflected by a high additivity index and results of cross-linked radioimmunoassay. Both monoclonal antibodies inhibit the activity of thymidylate synthase from human cell lines. The strongest inhibition was observed with thymidylate synthase from HeLa cells. Monoclonal antibody M-TS-9 (IgM subclass) decreased the rate of binding of [^{3}H]FdUMP to thymidylate synthase in the presence of 5,10-methylenetetrahydrofolate while M-TS-4 (IgG1) did not change the rate of ternary complex formation. These data indicate that the antibodies recognize different epitopes on the enzyme molecule.

Thymidylate synthase (EC 2.1.1.45) is the terminal enzyme in thymidylate biosynthesis which catalyzes the reaction between deoxyuridylate and the cofactor 5,10-methylenetetrahydrofolate (Blakley, 1969; Walsh, 1979). This enzyme has been the object of considerable interest because of its important function in mammalian DNA synthesis as the only de novo source of thymidylate (Kornberg, 1980). For the same reason thymidylate synthase has been a target for the design of inhibitors with potential chemotherapeutic applications (Danenberg, 1977; Maley & Maley, 1981).

Limited information is available concerning 5-fluoro-2′-deoxyuridine (FdUrd)[1]-resistant cell lines with an altered thymidylate synthase (Heidelberger et al., 1960; Bapat et al., 1983; Jastreboff et al., 1983a), the intracellular localization of this enzyme (Baril et al., 1973; Reddy & Pardee, 1980; Reddy, 1982), and the regulation of thymidylate synthase gene expression (Navalgund et al., 1980). The unique properties of monoclonal antibodies, which recognize single antigenic determinants, allow their use as sensitive probes to study enzyme structure, function, and intracellular localization.

This paper reports the production of monoclonal antibodies to HeLa cell thymidylate synthase. Two antibodies are described that bind to human thymidylate synthase at different antigenic sites and that have different functional effects upon this enzyme.

Experimental Procedures

Materials

dl-Tetrahydrofolate was prepared by hydrogenation of folic acid over platinum oxide in neutral solution (Blakley, 1957) and purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). [5-^{3}H]dUMP (22 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) and [6-^{3}H]FdUMP[1] (4 Ci/mmol) from Moraveck Biochemicals (City of Industry, CA). Tetrahydropteroylpolyglutamates (synthesized from oxidized pteroylpolyglutamates provided by Dr. J. K. Coward, Rensselaer Polytechnic Institute, Troy, NY) were kindly provided by Dr. J. J. McGuire, Department of Pharmacology, Yale University School of Medicine. Sodium [^{125}I]iodide was obtained from Amersham and Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) from Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade. Antisera to the mouse immunoglobulin subclasses were obtained from Litton Bionetics. A Hybridoma Screening Kit from Bethesda Research Laboratories was used for the ELISA procedure. Media and sera for tissue culture were obtained from Grand Island Biological Co.

Cell Lines. Human cell lines HeLa (Gey et al., 1952), HCT-8 (Tompkins et al., 1974), HCT-8^R (the subline resistant to 2.5 × 10^{-6} M FdUrd; Jastreboff et al., 1984), CCRF-CEM (Foley et al., 1965), and K-562 (Lozzio & Lozzio, 1973) were routinely maintained in culture in RPMI 1640 medium with 15% heat-inactivated fetal calf serum. Mouse cell lines L1210 (Moore et al., 1966) and L5178Y (Fischer, 1957) were grown in Fischer's medium with 10% horse serum and 3T6 (Todaro & Green, 1963) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum. All media contained antibiotics in a final concentration of 100 units/mL penicillin and 100 μg/mL streptomycin, except medium for HL-60 (Collins et al., 1977) cells which was supplemented with gentamicin in a concentration of 50 μg/mL. The cells were grown at 37 °C in a 5% CO_2 atmosphere. The cells from these lines were used for purification of thymidylate synthase.

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[1] Abbreviations: FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolic acid; 5,10-CH_2-H_4Pte(Glu)$_n$, 5,10-methylenetetrahydropteroylpolyglutamates, where n is the total number of glutamates; ELISA, enzyme-linked immunoassay; AI, additivity index; PBS, phosphate-buffered saline; BSA, bovine serum albumin; M-TS-4 and M-TS-9, monoclonal antibodies to thymidylate synthase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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The nonsecreting 8-azaguanine-resistant myeloma cell line P3-X63-Ag8.653 (Kearny et al., 1979) was grown in RPMI 1640 with 10% HyClone fetal bovine serum. Monoclonal antibody producing hybridomas were maintained in DMEM supplemented according to a previously published procedure (Lerner, 1981). The cells were grown at 37 °C in a humidified 5% CO_2 atmosphere.

Methods

Collection of Cells. The plates of monolayer growing cells in the logarithmic phase of culture growth were placed on ice and scraped in 0.01 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol. Cells growing in suspension were harvested by centrifuging at 600*g* for 5 min and washed with PBS containing 0.01 M 2-mercaptoethanol. The cells were stored at −20 °C until use.

Purification of Thymidylate Synthase. Enzyme was purified according to a method previously described (Rode et al., 1979). An affinity chromatography procedure based on reversible deoxyuridylate-dependent binding of the enzyme to methotrexate, instead of 10-formyl-5,8-dideazafolate, immobilized on aminoethyl-Sepharose, was used (Dolnick & Cheng, 1977). The thymidylate synthase preparations were tested for homogeneity by polyacrylamide gel electrophoresis at 4 °C as described by Rode et al. (1980).

Enzyme Assay. Thymidylate synthase activity was measured as previously described (Jastreboff et al., 1982). The enzyme activity is expressed in units defined as the amount required to release 1 μg equivalent of tritium from [5-^{3}H]-dUMP per min under conditions of the assay.

FdUMP Binding Assay. Binding of [^{3}H]FdUMP to thymidylate synthase in the presence of cofactor was determined by nitrocellulose disc filtration according to a method described previously (Lockshin et al., 1979; Santi et al., 1974).

Protein Determination. The procedure of Sedmak & Grossberg (1977) was used with bovine serum albumin as standard.

Production of Hybridoma Monoclonal Antibodies. The hybridomas were produced according to a modification of the method originally outlined by Kohler & Milstein (1975) using the protocol of Lerner (1981). Six-week old Balb/c mice (The Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally (ip) and subcutaneously with 20 μg of pure HeLa cell thymidylate synthase as an antigen in 50 μL of 0.2 M Tris-HCl buffer, pH 7.5, supplemented with 0.01 M 2-mercaptoethanol, mixed with an equal volume of complete Freund's adjuvant. The immunization procedure was repeated 3, 6, and 9 weeks after the first injection by using 25, 35, and 50 μg of pure enzyme, respectively. Four weeks after the last injection, the mice were boosted intravenously with 35 μg of thymidylate synthase in PBS. The mice were sacrificed 3 days later, their spleens were removed, and a single cell suspension was made and fused with the myeloma cell line P3-X63-Ag8 by using 50% poly(ethylene glycol), M_r 1500. Fused cells were selected in HAT (0.1 mM hypoxanthine, 0.8 μM aminopterin, and 16 μM thymidine) (Littlefield, 1964; Lerner, 1981) supplemented medium.

Selection of Monoclonal Antibodies. The clones secreting antibodies against thymidylate synthase were identified by screening the supernatant of centrifuged medium (600*g*, 10 min; 50 μL/well) by an enzyme-linked immunoassay (ELISA) using β-galactosidase conjugated with sheep $F(ab')_2$ against mouse IgG. Purified thymidylate synthase (15 ng in 30 μL of 0.2 mM Tris-HCl buffer, pH 7.5, supplemented with 0.01 M 2-mercaptoethanol) was adsorbed to the bottoms of wells of microtiter plates as the antigen of interest. Dihydrofolate reductase from various souces were used as controls. The clones with the highest positive reaction to thymidylate synthase and no reaction to the controls by ELISA were subcloned by limiting dilution, and other positive clones were frozen for future consideration.

Ascites Production. Balb/c mice, pretreated 7 days earlier with pristane (2,6,10,14-tetramethylpentadecane), were injected intraperitoneally with 5×10^6 hybridoma cells. The ascites fluid was harvested 3–5 times, 10–23 days after inoculation. It was clarified by centrifugation at 12500*g* for 15 min and stored at −70 °C.

Purification of Antibodies. The ascites fluid from mice and the supernatant of medium from growing hybridoma cells were used as sources of monoclonal antibodies. The antibodies were purified by two successive precipitations and 45% ammonium sulfate followed by chromatography on Sephadex G-200 and stored in 0.2 M sodium bicarbonate, pH 7.5.

Immunoglobulin Subclass Determination. Immunoglobulin subclass determination was done by Ouchterlony analysis (Ouchterlony, 1949) using subclass-specific rabbit anti-mouse immunoglobulin and about 20 times concentrated supernatants of medium.

Iodination of Antibodies. Ascites fluid containing monoclonal antibodies (1 mg of protein/mL) was added to a test tube with dried Iodo-Gen and radiolabeled according to a previously described method (Fraker & Speck, 1978).

Radioimmunoassay for Antibody Binding. The influences of substrates and effectors on thymidylate synthase were studied by using radioimmunoassay. Thymidylate synthase (0.4 μg/mL) alone or thymidylate synthase previously preincubated (1 h at 4 °C) with substrate (dUMP; 50 μM final concentration), coenzyme (5,10-CH_2-H_4PteGlu; 150 μM), of FdUMP (competitive inhibitor 0.5 μM) were used as antigens. Antigens (50 μL) were adsorbed to wells of poly(vinyl chloride) 96-well microtiter plates by overnight incubation at 4 °C. After the plates were washed with PBS and 3% BSA in Burridge buffer (5 mM Tris, 0.15 M NaCl, 8 mM sodium azide, pH 7.4), the ^{125}I antibody (5 μL of M-TS-4 or M-TS-9, diluted with 3% BSA in Burridge buffer to final volume of 100 μL/well) was added and incubated at room temperature for 4 h. The plates were washed with PBS and allowed to dry overnight and the wells counted in a γ counter.

Solid-Phase Cross-Linked Radioimmunoassay. This assay was used to indicate whether two monoclonal antibodies were competing for an antigenic site (Wright, 1983). One of the monoclonal antibodies (20 μg of protein/mL) in 0.1 M bicarbonate buffer, pH 9.6, was adsorbed to flat-bottomed wells (2 μg/well) of poly(vinyl chloride) microtiter plates overnight at 4 °C. The plates were rinsed with cold PBS, incubated for 1 h at 37 °C with 3% BSA in Burridge buffer, rinsed as above, and dried for 1 h at 37 °C. Antibody-coated wells were incubated for 2 h with thymidylate synthase from HeLa cells (25 ng/well) or with detergent-extracted HeLa cell lysate (5×10^6 cells) at room temperature and again washed with PBS. The second antibody (5 μg/well), which was previously radiolabeled with ^{125}I and diluted in 3% BSA in Burridge buffer to a final volume of 100 μL, was added and incubated 2 h at room temperature. The wells were then rinsed with PBS and dried overnight. The bottoms of the wells were cut, and the ^{125}I was counted in a γ counter.

ELISA Double Antibody Binding Assay. An ELISA double antibody binding assay in which saturating amounts of monoclonal antibodies were compared alone and in combination was used to test whether the monoclonal antibodies recognize different epitopes (Friguet et al., 1983). The antigen was first

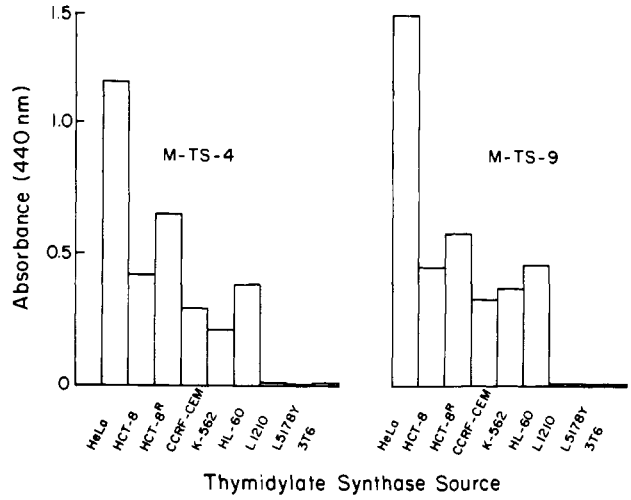

FIGURE 1: Binding of monoclonal antibodies (M-TS-4 and M-TS-9) to thymidylate synthase from different sources indicated by ELISA.

coated onto a microtiter plate. The monoclonal antibodies were then added either separately or simultaneously, and the amount of bound antibody was measured by use of immunoglobulin linked to β-galactosidase. There was additivity of the bound enzymatic activity when the monoclonal antibodies bound to distinct epitopes. An additivity index (AI) as defined by Friguet et al. (1983) was calculated from the absorbances obtained in the ELISA with two antibodies. This index allowed identification of antibodies binding to the same antigenic region (those with a low AI) and those binding to different regions (those with a high AI).

Results

Production of Monoclonal Antibodies against Human Thymidylate Synthase. Spleen cells from mice immunized with purified thymidylate synthase from human cervical carcinoma cells (HeLa) were fused with myeloma cells. The hybrid cells that exhibited significant growth in the HAT selective medium (85 wells) were screened for the production of thymidylate synthase antibodies by ELISA as described under Methods. Cells in 35 wells produced antibodies against thymidylate synthase from HeLa cells as indicated by an absorbancy of at least $2^1/_2$ times background; 10 of these were frozen. Cells from two wells with the highest antibody titer were subcloned by limiting dilution and rescreened by ELISA. One subclone from each group (M-TS-4 and M-TS-9) was selected for further study.

Characterization of Monoclonal Antibodies. (1) Immunoglobulin Subclass Determination. Medium containing M-TS-4 or M-TS-9 was examined by Ouchterlony immunodiffusion analysis to determine antibody subclass. M-TS-4 formed a precipitin band only when reacted against anti-mouse IgG1 and M-TS-9 only against anti-mouse IgM.

(2) Species Specificity of Monoclonal Antibody Binding to Thymidylate Synthase. The amount of monoclonal antibody binding to thymidylate synthase purified from different cell types was measured by ELISA (Figure 1). Monoclonal antibodies M-TS-4 and M-TS-9 were found to bind to thymidylate synthase purified from all human cell lines tested. The greatest binding was observed with the HeLa enzyme. There was less binding with thymidylate synthase from three human leukemic cell lines. Neither M-TS-4 nor M-TS-9 bound to thymidylate synthase purified from mouse cell lines.

(3) Epitope Specificity. Two types of assays (cross-linked immunoassay and ELISA addivity double antibody binding test) were done to determine the epitope specificity on the

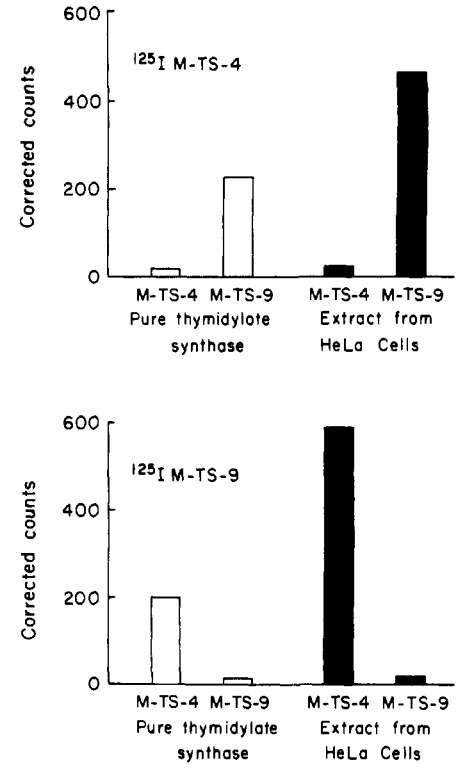

FIGURE 2: Cross-linked radioimmunoassay using purified HeLa thymidylate synthase and extract from HeLa cells. After M-TS-4 or M-TS-9 was absorbed to the plates, purified thymidylate synthase or HeLa cell lysate was incubated, and subsequent binding of ^{125}I-M-TS-4 (upper panel) or ^{125}I-M-TS-9 (lower panel) was measured as described under Experimental Procedures.

thymidylate synthase molecule. The monoclonal antibodies bound to different epitopes on the enzyme molecule as shown by a cross-linked immunoassay (Figure 2). When M-TS-4 was allowed by adhere to plates and then allowed to bind pure thymidylate synthase, subsequent bindng of ^{125}I-M-TS-4 was totally inhibited; however, there was substantial binding of ^{125}I-M-TS-9. When M-TS-9 was used as the primary antibody bound to enzyme, subsequent binding by ^{125}I-M-TS-9 was totally inhibited, while there was substantial binding of ^{125}I-M-TS-4. Similar results with both antibodies were observed when a crude extract from HeLa cells was used in place of purified thymidylate synthase. A relatively high value of the additivity index (AI = 55%) indicates that the antibodies can bind simultaneously to the antigen and therefore to distinct epitopes.

Effect of Monoclonal Antibody–Thymidylate Synthase Interactions on the Catalytic Activity of the Enzyme. The inhibition of HeLa thymidylate synthase activity by M-TS-4 or M-TS-9 was time dependent (Figure 3). No inhibition was observed without preincubation of the enzyme with the antibody. Increasing the preincubation temperature up to 18 °C did not significantly alter thymidylate synthase activity but did potentiate the inhibition effect of the antibodies on the enzyme activity (Table I). The pattern of dependency of thymidylate synthase inhibition on antibody concentration was of the "titration" type (Figure 4). However, even prolonged incubation of the enzyme with an excess of either antibody did not result in complete inhibition of enzyme activity. Maximum inhibitions by M-TS-4 and M-TS-9 were 54% and 70%, respectively.

Species Specificity of Thymidylate Synthase Inhibition. The effect of monoclonal antibodies on activity of thymidylate synthase from human and mouse cells are summarized in Table II. Enzyme activity from all human lines was partially

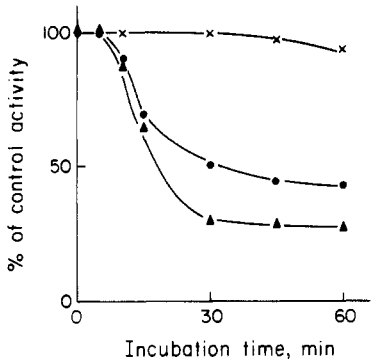

FIGURE 3: Time course of inhibition of thymidylate synthase by monoclonal antibodies. After preincubation of the enzyme (10^{-5} unit) with 3 μg/mL ascites fluid containing M-TS-4 (circles) or M-TS-9 (triangles) in a final volume of 50 μL for the time shown, thymidylate synthase activity was determined as described under Experimental Procedures. The reaction was started by adding 20 μL of preincubated enzyme–antibody mixture. (×) Control without antibodies.

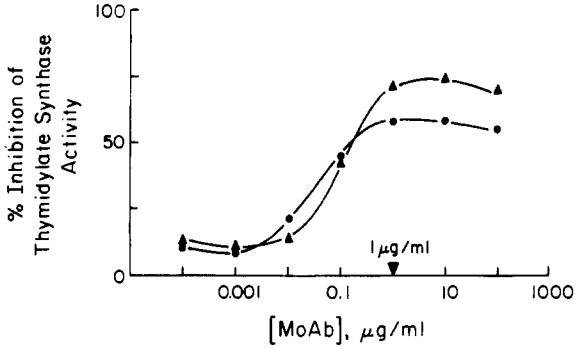

FIGURE 4: Inhibition of activity of HeLa thymidylate synthase by different concentrations of monoclonal antibodies. The enzyme (10^{-5} unit) was preincubated at room temperature with different concentrations of monoclonal antibodies, M-TS-4 (circles) or M-TS-9 (triangles), in a final volume of 50 μL. After 30 min thymidylate synthase activity was determined (in duplicate) as described under Experimental Procedures. The reaction was started by adding 20 μL of preincubated enzyme–antibody mixture.

Table I: Effect of Preincubation Temperature on the Inhibition of HeLa Cell Thymidylate Synthase Activity by Antibodies[a]

temp of preincubation (°C)	decrease of activity (%) of enzyme without antibodies	inhibition of activity[b] (%) of enzyme with antibodies	
		M-TS-4	M-TS-9
0	0	12	13
10	2	22	34
18	4	52	71
37	19	54	73
50	79	53	59

[a] Pure thymidylate synthase (10^{-5} unit) in 0.2 M Tris-HCl buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol, 0.1% Triton X-100, and 20% sucrose was preincubated 30 min with antibody containing ascites fluid (3 μg of protein/mL) in a final volume of 50 μL, at the temperature shown. After preincubation, enzyme activity was determined as described under Experimental Procedures. [b] Relative to activity of the enzyme preincubated alone at a given temperature.

inactivated whereas the enzyme from mouse cells was not affected. An equal amount of antibody was used in each experiment. Stronger inhibition was observed with M-TS-9 than with M-TS-4.

Influence of Substrates and Effectors on HeLa Thymidylate Synthase–Antibody Interaction. The effect of dUMP, 5,10-CH_2-H_4PteGlu, FdUMP or FdUMP, and coenzyme on the enzyme–antibody complex formation is summarized in Table III. The presence of the coenzyme did not result in changes

Table II: Human and Mouse Thymidylate Synthase Activity Inhibition by Monoclonal Antibodies against Thymidylate Synthase from HeLa Cells[a]

thymidylate synthase source	% inhibition of thymidylate synthase activity	
	M-TS-4	M-TS-9
HeLa	54	70
HCT-8	33	49
HCT-8^R	46	56
CCRF-CEM	40	44
K-562	34	42
HL-60	37	47
L1210	2	0
L5178Y	0	0
3T6	0	0

[a] Partially purified (after first affinity chromatography) thymidylate synthase (about 5×10^{-6} unit) from the respective cells was incubated at room temperature with antibody containing supernatant from medium (1 μg of protein/mL in a final volume of 50 μL) for 1 h, and the enzyme activity was subsequently measured and compared to the activity of thyidylate synthase without antibody (Experimental Procedures).

Table III: Effect of Substrate, Coenzyme, and/or Inhibitor on Antibody Binding to Thymidylate Synthase from HeLa Cells Indicated by Radioimmunoassay[a]

conditions	corrected counts (cpm) [mean ± SD (n = 4)]	
	^{125}I-M-TS-4	^{125}I-M-TS-9
thymidylate synthase alone	2430 ± 80	2680 ± 80
+coenzyme	2350 ± 100	2560 ± 60
+dUMP	2390 ± 50	1650 ± 110
+FdUMP	2320 ± 50	1500 ± 70
+FdUMP + coenzyme	2410 ± 90	990 ± 30

[a] Thymidylate synthase, with or without preincubation coenzyme and/or inhibitor, was allowed to adhere to microtiter plates, and ^{125}I-M-TS-4 or ^{125}I-M-TS-9 was added as described under Experimental Procedures.

Table IV: Protection of Thymidylate Synthase Activity from HeLa Cells by 5,10-CH_2-H_4Pte(Glu)$_n$ Cofactors[a]

monoclonal antibody	5,10-CH_2-H_4Pte(Glu)$_n$	% of control activity	
		2.5 μM cofactor	25 μM cofactor
M-TS-4	n = 1	45	46
	n = 2	47	46
	n = 3	53	67
	n = 5	63	82
M-TS-9	n = 1	34	33
	n = 2	44	48
	n = 3	46	66
	n = 5	65	85

[a] The enzyme (5×10^{-6} unit) was incubated for 1 h at room temperature with ascites fluid containing antibody (1 μg/mL) in the presence of the protecting agents in a final volume of 50 μL. Enzyme activity was determined as described under Experimental Procedures.

of the antibody–enzyme interaction. dUMP or FdUMP alone or FdUMP in the presence of the coenzyme did not inhibit M-TS-4 binding to the enzyme but strongly decreased (50% and 70%, respectively) M-TS-9 binding to the enzyme. Although 5,10-CH_2-H_4PteGlu did not protect HeLa enzyme from antibody inactivation, 5,10-CH_2-H_4Pte(Glu)$_n$ (n = 2, 3, 5) did protect this enzyme against antibody inactivation, with increased protection noted with increasing length of the polyglutamate chain (Table IV). Since the previous studies demonstrated that FdUMP with or without the 5,10-CH_2-H_4PteGlu could protect HeLa thymidylate synthase from M-TS-9 inactivation, the effects of these antibodies on the rate of [^{3}H]FdUMP binding to this enzyme were studied. Prein-

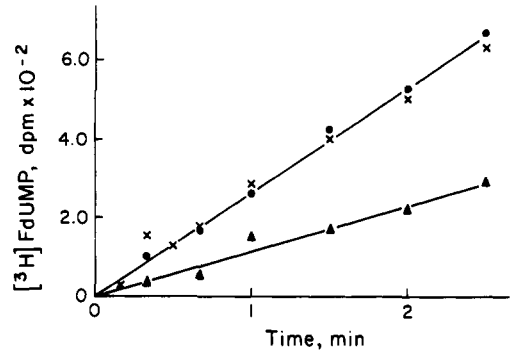

FIGURE 5: Rate of binding [^{3}H]FdUMP to HeLa thymidylate synthase in the presence of monoclonal antibodies. The enzyme (5×10^{-6} unit) preincubated 1 h at room temperature without (×) or with monoclonal antibodies (1 μg/mL), M-TS-4 (circles) or M-TS-9 (triangles), was added to the assay mixture as described under Experimental Procedures. The inactivation of the enzyme during preincubation was taken into account for calculating the ratio of [^{3}H]FdUMP binding.

cubation of the enzyme with M-TS-9 decreased the rate of [^{3}H]FdUMP binding by 50% in the presence of 5,10-CH_2-H_4PteGlu, whereas M-TS-4 had no effect upon binding of [^{3}H]FdUMP (Figure 5).

Discussion

Two murine hybridoma clones that secrete monoclonal antibodies (M-TS-4 and M-TS-9) against human thymidylate synthase obtained from HeLa cells are described in the present study. Both antibodies have similar profiles or specificity toward human thymidylate synthase purified from different cell lines, and neither antibody reacted with the enzyme from any of the tested murine tumor cell lines (Figure 1). Our results suggest that there are significant differences in antigenic structure of the thymidylate synthase molecule between the human and mouse enzyme.

Both the enzyme–antibody binding, measured by ELISA (Figure 1), and the antibody inhibition of enzyme activity (Table II) indicate that thymidylate synthase from HeLa cells is the most specific target. There is less antibody binding and less inhibition of enzyme activity with thymidylate synthase from HCT-8, HCT-8^R, CCRF-CEM, K-562, and HL-60 cell lines, respectively. One explanation for these findings is the presence of different forms of thymidylate synthase in various human tumor cell lines. A similar conclusion might also be inferred from the different properties of thymidylate synthase isolated from leukemia blast cells (Dolnick & Cheng, 1977), HeLa cells (Rode et al., 1980), CCRF-CEM, and FdUrd CCRF-CEM leukemia cells (Lockshin et al., 1979; Bapat et al., 1983).

Except for similarity of binding to thymidylate synthase, inhibition of enzyme activity, and protection of this inhibition by 5,10-CH_2-H_4Pte(Glu)$_n$ cofactors (Table IV), these two monoclonal antibodies are distinctly different. They belong to different subclasses as determined by Ouchterlony analysis (IgG1 and IgM, for M-TS-4 and M-TS-9, respectively). Data obtained from the cross-linked radioimmunoassay (Figure 2) indicate that M-TS-4 and M-TS-9 bind to different antigenic sites on the enzyme molecule.

M-TS-4 and M-TS-9 also differ significantly in their functional effect on the enzyme molecule. Initial binding of M-TS-9 to the enzyme decreased the rate of [^{3}H]FdUMP binding to the enzyme by about 2-fold, whereas M-TS-4 had no effect on the rate of [^{3}H]FdUMP binding (Figure 5). Decreased binding of M-TS-9 to the ternary complex of the enzyme with FdUMP and 5,10-CH_2-H_4PteGlu (Table III) indicates that the antibody may compete with the substrate analogue. However, we cannot exclude the possibility that, in the presence of FdUMP and coenzyme, the affinity of M-TS-9 for the ternary enzyme complex is markedly reduced, perhaps due to a conformational change. In contrast, preincubation of the enzyme with FdUMP and coenzyme did not affect the binding of M-TS-4 to thymidylate synthase. These results indicate that M-TS-9 binds to the enzyme on a different antigenic site than M-TS-4 and either binds to the active site and/or induces conformational changes on the enzyme molecule which affects its active site.

Evidence for binding of M-TS-9 to the active site comes from the data regarding the competition of M-TS-9 with FdUMP. This suggests that the antibody may bind directly at the FdUMP binding site (which is the active site on the thymidylate synthase molecule) and either blocks or modifies the function of that site. Lack of competition of M-TS-4 with FdUMP binding suggests that M-TS-4 may induce conformational changes of the enzyme affecting its active site(s) but did not bind directly to this site(s). A dissociation of active enzymes dimers into inactive monomers in the presence of antibody may also result in thymidylate synthase inhibition. This dissociation may be only partial, thus allowing some continued activity of the enzyme.

On the other hand, there is also evidence that both antibodies induce conformational changes on the active site without necessarily binding to that site. M-TS-4 and M-TS-9 only partially inhibit thymidylate synthase activity. The plateau of enzyme activity inhibition (about 50% of inhibition for M-TS-4 and 70% for M-TS-9) was reached with an antibody concentration of 1 μg/mL (Figure 4). A further 100-fold increase of the antibody concentration did not result in further inhibition of enzyme activity. Thus, both monoclonal antibodies may bind to thymidylate synthase at site(s) different from the active site, causing catalytically unfavorable conformational changes of the enzyme molecule, without completely blocking the activity of the site(s). FdUMP binding may also alter the enzyme conformation in a way that affects the M-TS-9 binding site, but not the M-TS-4 site.

HeLa thymidylate synthase had two FdUMP binding active sites, probably located on two subunits (Rode et al., 1980). Nonequivalence of these sites might explain some of our results. Danenberg et al. (1974) suggested that FdUMP in the presence of 5,10-CH_2-H_4PteGlu binds covalently with only one of the sites on *Lactobacillus casei* thymidylate synthase and forms a noncovalent complex with the other active site. Functional nonequivalence of active sites on Ehrlich ascites carcinoma thymidylate synthase molecule reflected by a biphasic Hill plot of the activity vs. dUMP concentration relationship has also been shown (Jastreboff et al., 1983b). Thus, M-TS-9 may bind to one of the subunits on the HeLa enzyme molecule blocking that active site, or alternatively, it may bind to a different site but alter the active site of one of the subunits by inducing a conformational change. This conformational change may result in one of the sites either changing its affinity for the inhibitor or becoming nonavailable for FdUMP binding. M-TS-4, which does not bind or affect the active site according to FdUMP binding studies, most likely inhibits enzyme activity by inducing a conformational change of the thymidylate synthase molecule.

Further studies utilizing these monoclonal antibodies are planned. The effect of M-TS-4 and M-TS-9 on thymidylate synthase isolated from other mammalian cell lines, the protection of inhibition by various polyglutamate coenzymes, and the intracellular localization of the thymidylate synthase–antibody complex are areas under active investigation. These

monoclonal antibodies may also be useful in characterizing the differences in thymidylate synthase from cells resistant to fluoropyrimidines vs. sensitive cells and may be useful as a probe for cloning the thymidylate synthase gene.

Registry No. Thymidylate synthase, 9031-61-2.

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