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Mutations Leading to Antifolate Resistance in Chinese Hamster Ovary Cells after Exposure to the Alkylating Agent Ethylmethanesulfonate

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SUMMARY

Chinese hamster ovary cells with a single allele for dihydrofolate reductase were used as a model system to study the effect of exposure to an alkylating agent, ethylmethanesulfonate, on rates and types of mutations at the dihydrofolate reductase locus leading to antifolate resistance. After overnight exposure to 400 μg/ml ethylmethanesulfonate, cells were allowed to recover for 3 days, and resistant colonies were selected in 8×10^{-8} M trimetrexate. Trimetrexate, rather than methotrexate, was used as the selecting agent to increase the probability of obtaining mutations in dihydrofolate reductase, rather than in the reduced folate transport carrier protein. Seven of several hundred surviving colonies were selected at random, and cell lines were established. Cell lines 1–3 were maintained in culture in the presence of 8×10^{-8} M trimetrexate and were 66–170-fold resistant to the drug. Cell lines 4–7 were initially expanded in 8×10^{-8} M trimetrexate but were then maintained in the absence of the drug. These cell lines were 4.4–26-fold resistant to the drug, compared with the parental cell line. Cell line 1 was found to have an increase in dihydrofolate reductase activity, a corresponding increase in mRNA for dihydrofolate reductase, and amplification of this gene. Cell lines 2 and 6 had a mutated dihydrofolate reductase with altered trimetrexate- and methotrexate-binding properties. Cell line 3 had a 3-fold increase in dihydrofolate reductase activity. In cell lines 4, 5, and 7 the mechanisms of resistance to trimetrexate remain unknown.

MTX, in combination with other chemotherapeutic agents, has been successfully used in the treatment of malignancies that include acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteogenic sarcoma, choriocarcinoma, breast cancer, and head and neck cancer (1). Although response to treatment may occur in a high percentage of patients, many patients still experience relapse due to development of resistance (2).

In the chemotherapy of some of the aforementioned malignancies, MTX is usually administered in combination chemotherapy, either at the same time as or soon after administration of an alkylating agent, e.g., cyclophosphamide (1). To test the hypothesis that pretreatment with alkylating agents might result in an increase of resistance to subsequently used antimetabolites and to study the effect of pretreatment with alkylating agents on the mutations leading to antifolate resistance, we developed a model system in tissue culture, using the DHFR locus in CHO cells as a target. Antifolate-resistant mutants can be readily selected from a hemizygous cell line (CHO UA2) that carries a single allele for the DHFR gene (3). CHO UA2 cells were exposed to EMS and then selected with TMTX, a nonclassical antifolate. EMS, a monofunctional alkylating agent, has been found to be mutagenic in a wide variety of genetic test systems (reviewed in Ref. 4). TMTX, currently undergoing phase II clinical trials, enters the cell by a route distinct from the classical reduced folate transport system and also cannot be polyglutamylated (5). This compound rather than MTX was used as the selecting agent to decrease the incidence of transport-related mutations or decreased polyglutamylation mutants (2). Thus, an increase in DHFR activity or an alteration of the target enzyme (DHFR) would be expected to be the major causes of resistance in this study.

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ABBREVIATIONS: MTX, methotrexate; CHO, Chinese hamster ovary; EMS, ethylmethanesulfonate; TMTX, trimetrexate; DHFR, dihydrofolate reductase; H_2folate, dihydrofolate; PCR, polymerase chain reaction; MDR, multidrug resistance; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; kb, kilobase(s); APRT, adenosine phosphoribosyl transferase; MATS, {2-(*N*-morpholino)ethanesulfonic acid}-acetic acid-Tris.

Materials and Methods

Cell lines and culture conditions. The parental cell line used in this study, CHO UA2 (a hemizygous mutant for the DHFR gene), was kindly provided by L. Chasin of Columbia University (3). Stock cultures of all cell lines were maintained under identical conditions in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin, in a humidified atmosphere of 5% CO_2 at 37°. Cells in exponential growth phase were used in these experiments.

Drugs and chemicals. TMTX (glucuronate salt) and [^{14}C]TMTX (specific activity, 101 mCi/mmol) were kindly provided by Warner Lambert Parke-Davis (Ann Arbor, MI). A stock solution (10^{-2} M) was prepared by dissolving the drug in sterile water and was stored at 4°. Subsequent dilutions were made in RPMI medium plus 10% dialyzed fetal bovine serum. MTX was purchased from Lederle Labs (Pearl River, NY). Doxorubicin was purchased from Adria Labs (Columbus, OH) and vinblastine sulfate from Eli Lilly & Co. (Indianapolis, IN). Folic acid, NADPH (enzymatically reduced, type III), and bovine serum albumin (crystallized and lyophilized) were obtained from Sigma Chemical Co. (St. Louis, MO). H_2folate was prepared from folic acid as described by Blakley (6) and was stored at −80° in 1 mM HCl containing 50 mM β-mercaptoethanol (J. T. Baker Chemicals, Phillipsburg, NJ). All other reagents were high purity reagents from commercial sources.

Sensitivity to EMS. The sensitivity of the cell line to EMS (Sigma) was determined in the following manner. CHO UA2 cells (3×10^6/150-mm dish or 10^3/six-well plate) were incubated for 24 hr and then exposed overnight to a range of EMS concentrations. After exposure to EMS the cells were washed three times with medium and incubated for 14 days. Colonies were fixed in 3.7% formaldehyde and stained with crystal violet, and those consisting of >50 cells were counted. Inhibition of colony formation in treated dishes was expressed as a percentage of colony formation in untreated controls.

Sensitivity to TMTX. To determine the optimum concentrations of TMTX for selection of resistant mutants, the cell line CHO UA2 was exposed to increasing concentrations of the drug. Cells (6×10^6) not treated with EMS were plated and incubated in TMTX for 14 days. Surviving colonies with >50 cells were counted. The minimal concentration of TMTX that resulted in no colony formation in cells not exposed to EMS was 8×10^{-8} M.

Mutagenesis and selection of resistant clones. Cells (6×10^6) growing in logarithmic phase were exposed overnight (16 hr) to 400 μg of EMS/ml of culture medium, a concentration that resulted in 60% inhibition of colony formation. The cells were washed free of EMS and allowed to grow for 3 days, to allow phenotypic expression. Cells were then grown with continuous exposure to 8×10^{-8} M TMTX. After 14 days approximately 1000 colonies (>50 cells/colony) were obtained. Eighteen individual colonies were isolated with a ring cylinder and grown to larger numbers in the presence of TMTX. Seven of these colonies were randomly selected, expanded to cell lines, and maintained in tissue culture both in the presence and in the absence of TMTX.

Cytotoxicity assay. The degree of resistance of the parent line and the resistant sublines to TMTX and two other drugs (vinblastine and doxorubicin) was determined by a growth inhibition assay. The degree of resistance to vinblastine and doxorubicin was examined in these cell lines to determine whether resistance to TMTX was due to expression of the MDR phenotype. Aliquots of cells in suspension, at a final concentration of $3–4 \times 10^4$ cells/ml, were added to 25-cm^2 tissue culture flasks (final volume, 8 ml), to which 0.1 ml of graded concentrations of drug in medium was added. For controls, medium without drug was used. Cells were incubated for 3 days, after which the number of viable cells was determined. Drug concentration versus cell growth inhibition (dose-response) curves were obtained by calculating the percentage of viable cells in drug-treated flasks, compared with that in control flasks without drug. The ED_{50} was determined from the dose-response curve. The relative resistance for each drug was expressed as the ratio of the ED_{50} of the resistant cells to the ED_{50} of the sensitive parental CHO UA2 cells.

TMTX uptake and efflux studies. TMTX uptake was studied in cells 24 hr after plating of 5×10^5 cells/35-mm dish (Costar). The medium was decanted and replaced with prewarmed complete tissue culture medium containing [^{14}C]TMTX (1 μM, 25 cpm/pmol). The plates were then incubated at 37°. To determine uptake, triplicate plates were quickly cooled on ice at each time point and washed twice with ice-cold phosphate-buffered saline. Cells were detached by incubation with 1 N NaOH (1 ml) at 37°. After 45 min, 1 ml of 1 N HCl was added to each plate, 1-ml aliquots were added to 15 ml of scintillation fluid, and radioactivity was determined in a Beckman liquid scintillation counter. To determine drug efflux, cells were incubated with drug for 60 min, cooled on ice, washed three times with ice-cold medium, and incubated at 37° with prewarmed drug-free medium. At appropriate time points triplicate dishes were processed as described for uptake.

2-[5′-^{3}H]Deoxyuridine tritium release assay for determination of thymidylate synthesis in intact cells. Antifolate resistance was tested by using an assay for the *in situ* determination of thymidylate synthesis activity, as described previously (7). Cells were suspended to a density of $1–2 \times 10^6$ cells/ml in complete medium and were divided into 400-μl aliquots. Aliquots were incubated for 3 hr in the presence of no drug, 1 μM MTX, or 1 μM TMTX. 2-[5′-^{3}H]Deoxyuridine was added to a final concentration of 1 μCi/ml, and 100-μl samples were obtained at 0, 15, 30, and 45 min, added to 200 μl of a 15% activated charcoal suspension in 4% trichloroacetic acid, mixed, and centrifuged for 5 min at 16,000 × *g*. One hundred microliters of the supernatant were added to 5 ml of scintillation fluid and quantitated using a liquid scintillation counter (Beckman LS5). The scintillation counts were analyzed by calculating the slopes of the linear tritium release curves, using linear regression. Inhibition is expressed as the percentage of the slope of the curve for treated versus untreated cells.

Northern blot analysis of DHFR mRNA. Total cellular RNA was prepared by guanidinium isothiocyanate extraction (8). RNA samples were electrophoresed in 1.5% denaturing formaldehyde-agarose gels. The gels were washed for 15 min in 10× SSC (1.5 mM NaCl, 0.15 M sodium citrate) and blotted to nylon filters (Nytran, Schleicher and Schuell, Keene, NH) for 24 hr, with 10× SSC as blotting solution. After blotting, RNA was immobilized to the filter by UV cross-linking. Prehybridization solution contained 5× SSC, 50% (v/v) formamide (deionized), 5× Denhardt's solution, 0.1% SDS, water, and 50 μg/ml denatured salmon sperm DNA. Filters were prehybridized for 2–4 hr at 42°. For hybridization the final concentration of Denhardt's solution was 1×. Hybridization was done at the same temperature for 12–16 hr. The unbound probe was removed by washing the filters for 30 min in 1× SSC/0.1% SDS at room temperature and for 10 min in 0.1× SSC/0.1% SDS at 55°. Radioactive DNA probes were prepared by random priming using gel-purified full length cDNA generated by PCR amplification. Two different probes were used. CHO DHFR cDNA (specific activity, 1.7×10^9 cpm/μg) was used for hybridizations at a concentration of 5×10^6 cpm/ml. P36 phosphoprotein cDNA was used for hybridizations as a control single-copy gene (9). Filters were exposed at −70° to X-ray films with an intensifying screen. Scanning densitometric analysis was performed to quantitate the signal intensity.

Slot blot analysis of RNA. RNA was blotted on nitrocellulose filters using a Bio-Rad slot blot apparatus. Increasing amounts of RNA (5, 10, 20, and 40 μg) were diluted in the same final volume (100 μl) with diethylpyrocarbonate-treated water, 37% formaldehyde, 20× SSC, and were added to the slots. Conditions for prehybridization, hybridization, and washing were identical to those used in the Northern analysis.

Southern blot analysis of DNA. Genomic DNA was isolated from the parental line and the seven TMTX-resistant CHO cell lines. DNA was restricted with the following enzymes: *Eco*RI, *Pst*I, *Bam*HI, and *Hind*III. Fifty micrograms of the overnight digest were electrophoresed in a 1.2% agarose gel. This was transferred to a nylon filter (Nytran; S&S) with 10× SSC as the blotting solution (10). Prehybridization, hybridization, and washing were done as described for the Northern

blot analysis. CHO DHFR cDNA was used for hybridizations at a concentration of 15×10^6 cpm/ml. *Eco*RI- and *Pst*I-restricted DNA were prehybridized with a probe for APRT as a control (11).

Slot blot analysis of DNA. DNA (10 μg for each resistant cell line and 5–35 μg for the parental cell line) diluted in 100 μl was treated with an equal volume of 0.3 M NaOH and then with an equal volume of 2 M ammonium acetate. The total 400-μl solution was applied to a Bio-Rad slot blot apparatus and transferred to a nitrocellulose filter. Prehybridization, hybridization, and washing were done as described above. The filter was hybridized sequentially with human DHFR cDNA, CHO DHFR cDNA, and CHO APRT cDNA. The filter was exposed at −70° to X-ray film with an intensifying screen. The radioactive bands on the filter were also quantitated directly with a Betascope (Betagen), and the copy number of the DHFR gene was determined by linear regression analysis (using Enzfitter software).

DNA sequence analysis. Total cellular RNA was extracted and first-strand cDNA was synthesized by reverse transcriptase (12). Briefly, 100 ng of total RNA were reverse transcribed at 42° for 1 hr, using the cDNA kit from Invitrogen (San Diego, CA). DHFR cDNA from each cell line was amplified by PCR as described (12, 13). Single-stranded DNA was generated via asymmetric PCR, and the product was used for direct sequence analysis by chain termination reaction, using a modified T7 DNA polymerase (Sequenase, version 2.0; United States Biochemicals, OH) (12).

DHFR characterization. For crude extract preparation, approximately $3–15 \times 10^8$ cells were washed three times in cold saline and resuspended in 50 mM sodium phosphate buffer, pH 7.0, containing 10% glycerol, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 500 Kallikrein inhibitory units/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. Cells were then lysed by six 20-sec sonification bursts with a Vibracell sonicator (Sonics and Materials, Inc., Danbury, CT). Suspensions were centrifuged at $100,000 \times g$ for 30 min at 4°, and the resulting supernatant was recovered for immediate analysis.

Enzyme activity. DHFR activity was measured by a standard spectrophotometric assay. NADPH and 20–50 μl of cell extract were added to 0.8 ml of MATS assay buffer (14) that had been prewarmed to 25°, in a 1-ml quartz cuvette. The decrease in absorbance at 340 nm was monitored for several minutes using a Shimadzu UV-2101 PC spectrophotometer. This rate serves as a background rate and presumably incorporates any endogenous NADPH-oxidizing activity of the crude cell extract. The DHFR reaction was then started by addition of H_2folate. The total assay volume was 0.9 ml, and the final concentrations of NADPH and H_2folate were 50 and 20 μM, respectively. Protein was determined by the method of Bradford (15), using the Bio-Rad protein assay dye reagent.

Cell extracts were also analyzed for the presence of a putative DHFR-activating factor, as follows. Aliquots of sample extract (containing 1 mg of total cellular protein) were boiled for 7 min, chilled, and centrifuged in an Eppendorf centrifuge to sediment aggregated protein. The resulting supernatants were then placed into cuvettes containing MATS assay buffer and 50 milliunits of highly purified recombinant mouse (wild-type) DHFR. After 15 min at 23°, the remaining assay components were added to the cuvettes and the activity was monitored. This entire procedure was performed in duplicate for each cell line extract examined.

Enzyme characterization. The K_m values for H_2folate were determined by measuring DHFR initial reaction velocities at variable concentrations of H_2folate. The NADPH concentration in assays was at a saturating level of 50 μM. Whereas assay conditions were identical to those described above, 10-cm-pathlength cuvette cells (total assay volume, 23 ml) were used to more accurately monitor reaction rates at low (0.2–10 μM) H_2folate concentrations. Bovine serum albumin was added to the assay mixture to minimize absorptive losses of enzyme to cuvette surfaces. Kinetic parameters were ultimately determined by fitting the data to the Michaelis-Menten equation using an iterative, nonlinear, least squares, regression program (16). A simple qualitative test to assess the binding strength of MTX and TMTX was performed by monitoring the rate of enzyme reactivation after formation of enzyme-NADPH-inhibitor complexes (ternary complexes), with reactivation for the different cell lines being compared with that obtained for the parental cell line. The methodology used was similar to that described by Jackson *et al.* (17). Briefly, a volume of crude cell extract containing 5.5 milliunits of DHFR activity was incubated with MATS assay buffer containing 20 μg/ml bovine serum albumin, 1 mM β-mercaptoethanol, 1 mM dithiothreitol, 20 μM NADPH, and either 120 pmol of MTX, TMTX, or no inhibitor. After a 10-min incubation at 23°, duplicate portions of each mixture were added to cuvettes containing spectrophotometric assay components as described above but with elevated concentrations of NADPH and H_2folate (200 μM and 150 μM, respectively). After quick manual mixing, the reaction time course was followed for at least 90 min. Incubations conducted in the absence of inhibitor compounds produced linear reaction progress curves.

Results

EMS sensitivity and frequency of TMTX-resistant clones. After several preliminary experiments using different amounts of cells, a concentration of 8×10^{-8} M TMTX (continuous exposure) was chosen for selection of resistant clones, because at this or higher concentrations no colony formation was noted in cells exposed to TMTX in the absence of EMS. Exposure to 400 μg/ml EMS followed by TMTX selection resulted in approximately 1000 TMTX-resistant colonies (i.e., 1.66 colonies/10^6 cells). Thus, EMS increased the frequency of mutations leading to MTX resistance by approximately 1000-fold.

Cytotoxicity studies. The effect of TMTX on the growth rates of the parental line and the seven selected resistant cell lines is shown in Fig. 1 and Table 1. Clones 1 and 2 had high levels of resistance to TMTX (170- and 125-fold); clones 3–7 had lower levels of resistance (4.4–66-fold). The TMTX-resistant cell lines showed no cross-resistance to vinblastine and doxorubicin, indicating that resistance was not due to the MDR phenotype (data not shown). Moreover, the TMTX-resistant cell lines were also cross-resistant to MTX (data not shown).

Tritium release assay. The effect of MTX and TMTX on *in situ* thymidylate synthesis was measured in the parental line and five of the resistant sublines. The parental cell line was sensitive to both antifolates after a 3-hr incubation in the presence of drug (Table 2). In contrast, thymidylate synthesis activity in cell lines 1–3 was not inhibited by the same concentrations of these antifolates, and cell lines 5 and 7 were also less sensitive than the parental cell line; however, there was some inhibition of thymidylate synthesis at these concentrations. These results suggested that an increased or altered DHFR level in these cell lines was the probable cause of resistance to TMTX.

Uptake and efflux of TMTX. When cells were incubated with radiolabeled TMTX, the intracellular TMTX concentration increased linearly for at least 10 min before plateauing in all the cell lines (Fig. 2). A higher concentration of TMTX was achieved at 60 min in cell lines 1, 2, and 3 (20-, 19-, and 12-fold higher, compared with the parental line), proportionally to the increase in DHFR activity. TMTX efflux was rapid in both sensitive and resistant CHO cells. The intracellular drug concentration remaining 1 hr after efflux was also higher in the three cell lines with increased DHFR activity, compared with the parental cell line and cell lines 4–7, with normal levels of DHFR.

Genomic organization of DHFR genes in CHO UA2

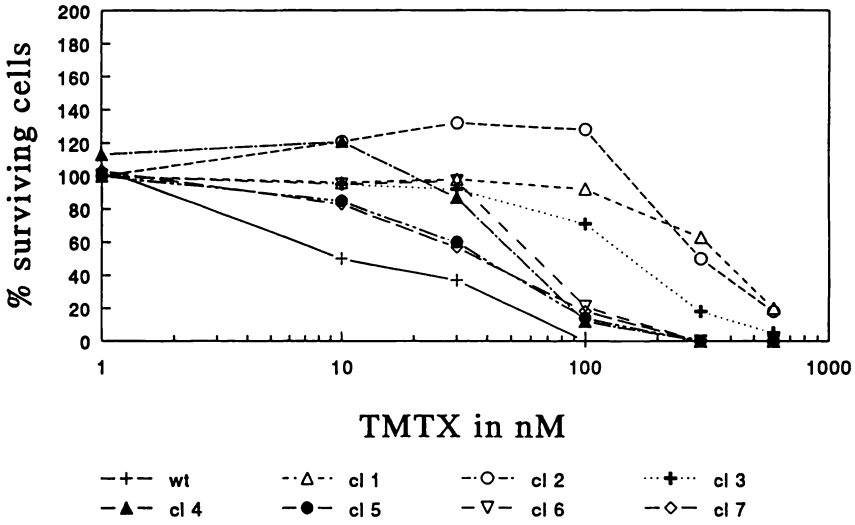

Fig. 1. Cytotoxicity of TMTX in parental CHO UA2 cells and the seven TMTX-resistant cell lines. Cells were exposed to the indicated doses of TMTX for 3 days, after which the number of viable cells was determined. *wt*, wild-type; *cl*, cell line.

TABLE 1

Specific activities and DHFR kinetic parameters of CHO cell extracts and degree of resistance of sensitive and antifolate-resistant cells

Errors indicated are 1 SE.

Cell line	DHFR specific activity[a]	Dihydrofolate K_m	TMTX K_i	ID_{50}[b]	Relative resistance to TMTX[c]
	milliunits/mg of protein	*μM*	*pM*	*M*	
CHO UA2 (parental)	3.8 ± 1.4 (2)	0.11	20.1 ± 6.8	2.4×10^{-9}	1
CHO UA2/TMTX^{R1}	44.8 ± 15.3 (2)	0.17	22.7 ± 5.8	4.1×10^{-7}	170
CHO UA2/TMTX^{R2}	19.8 ± 3.7 (2)	0.21	76.4 ± 15.4	3.0×10^{-7}	125
CHO UA2/TMTX^{R3}	9.3 ± 1.2 (1)	ND[d]	ND	1.6×10^{-7}	66
CHO UA2/TMTX^{R4}	3.7 ± 0.6 (1)	ND	ND	4.8×10^{-8}	20
CHO UA2/TMTX^{R5}	4.5 ± 0.7 (1)	ND	ND	1.8×10^{-8}	4.4
CHO UA2/TMTX^{R6}	3.8 ± 1.8 (2)	0.28	84.9 ± 19.0	6.3×10^{-8}	26
CHO UA2/TMTX^{R7}	3.7 ± 0.6 (1)	ND	ND	2.2×10^{-8}	9.2

[a] At least triplicate measurements of activity. Numbers in parentheses, number of different extract preparations used in determining specific activity.
[b] ID_{50} equals the concentration of drug necessary to inhibit cell growth by 50%.
[c] Mean of three determinations.
[d] ND, not determined.

TABLE 2

Inhibition of whole-cell thymidylate synthesis by antifolates in CHO UA2 parental and resistant cell lines

Cells were incubated for 3 hr in the absence or presence of 1 μM MTX or 1 μM TMTX. At the end of this time thymidylate synthesis was measured as described in Materials and Methods.

Cell line	Tritium release	
	MTX	TMTX
	% of control	
CHO UA2 parental	10.7	12.9
CHO UA2/TMTX^{R1}	91.2	93.8
CHO UA2/TMTX^{R2}	93.2	89.7
CHO UA2/TMTX^{R3}	97.1	103.3
CHO UA2/TMTX^{R5}	39.0	79.5
CHO UA2/TMTX^{R7}	40.9	74.8

cells and the resistant sublines. The Southern blot patterns of restricted DNAs from the parental line and seven resistant cell lines hybridized with ^{32}P-labeled CHO DHFR cDNA are shown in Fig. 3. The relative intensities of fragments hybridizing with the probe revealed an approximately 6-fold amplification of the DHFR gene in cell line 1, compared with the parental line. Slot blot hybridization confirmed amplification of the DHFR gene in cell line 1 (Table 3).

The Southern blot patterns of *Hin*dIII-, *Bam*HI-, and *Pst*I-digested DNAs from parental and resistant cell lines were identical (Fig. 3, A, B, and D, respectively). However, the DHFR gene in two TMTX-resistant cell lines exhibited an *Eco*RI restriction fragment length polymorphism (Fig. 3C). In the parental cell line *Eco*RI digestion resulted in bands of 4.6, 3.2, 2.6, 1.5, and 1.3 kb in size (in accordance with data from Ref. 3, except for the absence of a 0.8-kb band representing a noncoding region of exon 6, which is not expected to hybridize with a full length cDNA, and a 0.3-kb band that is undetectable on a 1.2% agarose gel). In cell lines 2 and 6 Southern blot hybridization after *Eco*RI digestion displayed a new band of 1.6 kb, accompanied by disappearance of the 1.3-kb fragment. The organization of the DHFR gene in the CHO cell line shows that a 1.3-kb fragment and an approximately 0.3-kb fragment are joined at an *Eco*RI site located in the coding region (second exon) of the gene. Thus, the 1.6-kb fragment in cell lines 2 and 6 arose through the loss of this site.

Sequence analysis of DHFR cDNA. Analysis of cDNA derived from cell lines 1, 3, 4, 5, and 7 showed the same sequence as the parental cell line. In cell lines 2 and 6 a single base change (thymidine to cytidine on the coding strand; adenosine to guanosine on the noncoding strand) from the parental coding sequence at nucleotide position +92 was detected. This thymidine to cytidine transition at position +92 results in the sub-

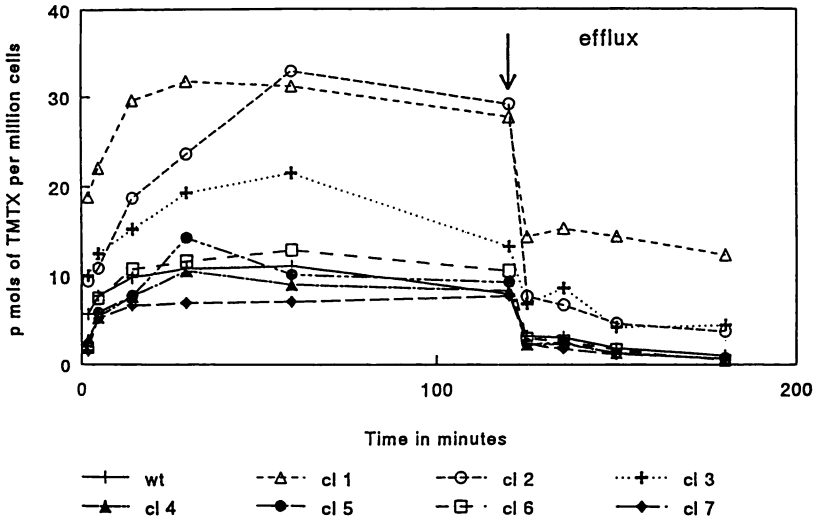

Fig. 2. Uptake and efflux of TMTX in sensitive and resistant CHO UA2 cells. Cells (5×10^5/well in 24-well plates) were incubated in the presence of 1 μM TMTX (25 pmol). Intracellular drug accumulation at the time points indicated was analyzed as described in Materials and Methods. After a 1-hr incubation, cells were washed and resuspended in fresh medium. Efflux was determined by harvesting and processing cells at the times indicated, as described for uptake analysis. Each *point* represents the average of three samples in a single representative experiment.

stitution of serine for phenylalanine at residue 31 of DHFR in these two cell lines (Fig. 4). This change also results in the loss of the *Eco*RI recognition sequence. No other base changes were detected in the coding region of the DHFR cDNA for any of the cell lines.

Northern blot analysis. Hybridization of the major 2.35-kb DHFR transcript (18) from the parental line (Fig. 5, *lane 2*) and four resistant cell lines is shown in Fig. 5. An increase in DHFR-specific transcript, proportional to the increase in DHFR gene copies, for cell line 1 (Fig. 5, *lane 3*) was seen. Densitometric analysis of the DHFR transcript of the parental line and cell lines 1 and 2 revealed an 8-fold increase in the steady state levels of DHFR mRNA in cell line 1 and a 6-fold increase in cell line 2. The level of DHFR transcript in cell line 6 (Fig. 5, *lane 1*) was found to be 3-fold greater than that in the parental cell line. RNA from the CHO Pro MTX R^{III} cell line (Fig. 5, *lane 5*) was used as a positive control for increases in DHFR message, because this line is known to have approximately 15 copies of the DHFR gene.

DHFR characterization. DHFR specific activities and kinetic parameters for the seven cell lines are presented in Table 1. Cell lines 4, 5, 6, and 7 all had enzyme activities similar to that of the parental cell line, whereas cell lines 1, 2, and 3 had increased DHFR activities. Cell line 1, which had the highest activity (11-fold greater than the parental cell line), was also the most antifolate-resistant subline. Because cell lines 2 and 3 had increased DHFR activity (2–3-fold) without evidence of gene amplification, a mixing experiment was performed to eliminate the possibility that a soluble activating component was present, giving rise to increased DHFR activities. Boiled extracts of parental cells and cell lines 1, 2, and 6 were added to a uniform amount of purified wild-type mouse DHFR, but in no instance was activation (or inhibition) of the added enzyme observed (data not shown).

DHFR from lines 2 and 6 (identified by sequence analysis as carrying the same point mutation in DHFR), the parental cell line, and cell line 1 was further investigated in terms of binding affinity for substrate and antifolates. As presented in Table 1, the Michaelis constant for H_2folate was slightly elevated for cell lines 2 and 6, in comparison with the values obtained for parental cells and cell line 1. The values for the latter cell lines are comparable to values quoted in the literature for vertebrate DHFRs (19). Inhibitor studies with enzyme from these four cell lines yielded more interesting data (Fig. 6). In all instances where inhibitor (MTX or TMTX) was present in preincubation mixtures, the monitored progress curves featured an initial slow enzymatic reaction rate that increased with time. Such a feature is consistent with reactivation of enzyme as a consequence of enzyme-inhibitor complex dissociation (18). Although the early portion of reactivation could not be accurately defined and, in some instances, substrate exhaustion was apparent before reactions reached an apparent steady state condition, two definite observations were made. 1) For all extracts examined, the rate of reactivation and the final achieved catalytic rate were quicker for TMTX-preincubated mixtures than for the MTX-treated ones. This is undoubtedly due to a quicker off-rate and associated lower affinity of DHFR for TMTX, in comparison with MTX, inhibition complexes. A weaker binding affinity of TMTX for DHFR has been a general observation made for several species of eukaryotic DHFR (19). 2) For both inhibitor compounds tested, final rates of catalysis for DHFR from cell lines 2 and 6 were greater, in comparison with enzyme from parental cells and cell line 1. This observation supports the contention that cell lines 2 and 6 harbor an altered DHFR with reduced antifolate-binding ability.

Discussion

The purpose of this study was to determine whether pretreatment with a known mutagen increased the frequency of resistance to TMTX and to determine whether point mutations leading to an altered DHFR with decreased binding of this antifolate were produced. Except for the altered DHFR described by Flintoff *et al.* (20), using this approach with MTX selection, there have been no studies reported using mutagens and antifolate selection that have resulted in altered DHFRs with decreased affinity for antifolates in mammalian cells. A few MTX-resistant cell lines have been described that were selected after exposure to multiple stepwise increases in MTX and that have altered DHFR enzymes with decreased affinity for MTX (21–26). However, the major mechanisms of acquired resistance to MTX found either with or without exposure to mutagenic agents have been gene amplification leading to an increase in this enzyme activity or decreased uptake of the drug (25, 26). In contrast, mutations leading to a decreased affinity of DHFR for the antifolate pyrimethamine, used in the treat-

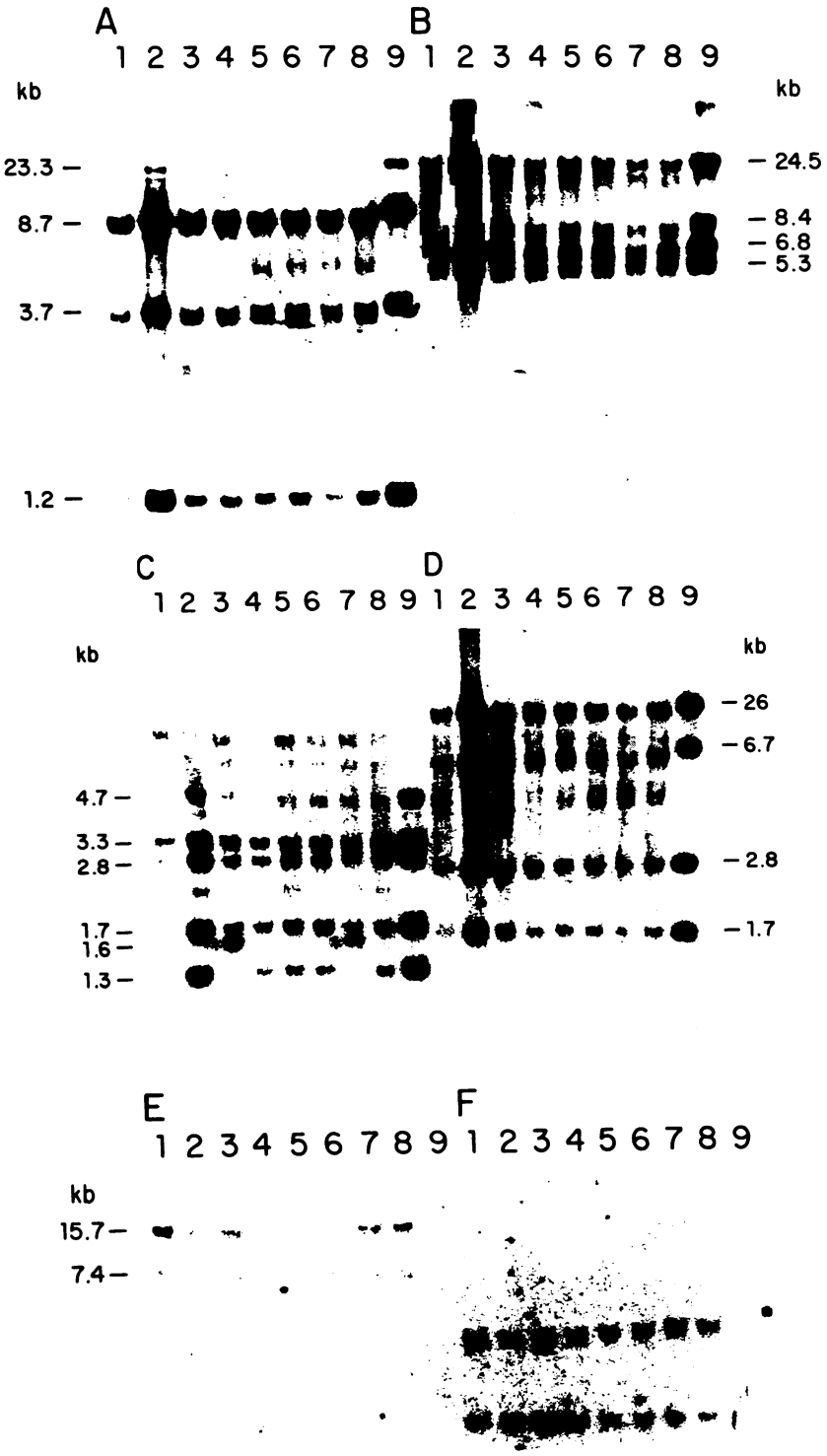

Fig. 3. Southern blot analysis of DNA from drug-resistant and -sensitive cell lines restricted with *Hind*III (A), *Bam*HI (B), *Eco*RI (C), and *Pst*I (D). *Lane 1*, 50 μg of DNA from the parental line; *lanes 2-8*, 50 μg of DNA from lines 1–7; *lane 9*, 10 μg of DNA from the CHO Pro MTX R^{III} cell line, which has approximately 15 DHFR gene copies and was used as a positive control. CHO cDNA was used as the probe. E and F, Same blot as in C and D but probed with the hamster APRT gene. Hamster APRT gene is a single-copy gene and serves as a control for loading and transfer of DNA. All lane assignments are identical. *, Bands of interest.

ment of malaria, is the major cause of resistance to this drug (27). We reasoned that if we selected resistant clones with TMTX after treatment with EMS we would see an increased frequency of point mutations leading to altered DHFRs, because this drug, like pyrimethamine, enters the cell by passive diffusion and does not require polyglutamylation for retention, thus eliminating these possible resistance mechanisms (28). Sharma *et al.* (29) examined the effects of X-rays on the frequency and mechanisms of resistance to TMTX in cells; however, they did not examine the resistant cell lines obtained for evidence of altered DHFR enzymes with impaired TMTX binding.

EMS treatment followed by TMTX selection of the CHO cell line, used in the current study, resulted in the generation of a larger number of resistant clones (approximately 1000-fold greater) than did selection with TMTX alone. Both gene amplification (cell line 1) and a point mutation in DHFR (cell lines 2 and 6) were identified as mechanisms of resistance in the seven clonal expanded cell lines examined. Cell lines 2 and 3 also had a 2–3-fold increase in DHFR activity, and low level

TABLE 3

Quantitation of DHFR gene copy number in parental CHO UA2 cells and seven TMTX-resistant cell lines

Cell line	Relative value		
	Gene copy number[a]	mRNA[b]	Enzyme activity[c]
CHO UA2	1	1	1
TMTX^{R}1	8	8	12
TMTX^{R}2	2	6	3
TMTX^{R}3	1.2	1	3
TMTX^{R}4	2.8	1	1
TMTX^{R}5	2.8	1	1.2
TMTX^{R}6	1.2	3	1
TMTX^{R}7	1	1	1

[a] From Southern analysis (Fig. 3) and slot blots.
[b] From Northern analysis (Fig. 5) and slot blots.
[c] From Table 1.

gene amplification may have been missed because of the sensitivity limits of the slot blot and Southern assays. It is of interest that the uptake studies confirmed these data, in that higher levels of TMTX were retained in these cells after efflux, presumably due to the increase in DHFR levels. Cell line 1 had an 11-fold increase in DHFR activity and an 8-fold increase in gene copy number and DHFR mRNA. There was no evidence for cytoplasmic activators in this or other cell lines with increased DHFR activities. Both cell lines 2 and 6 had the same point mutation (thymidine to cytidine) at nucleotide 92, leading to the phenylalanine to serine substitution. An identical point mutation at nucleotide 92 was also found in a colon cancer HCT-8 subline resistant to MTX (HCT-8/R), selected with stepwise increases of MTX alone (21). Another cell line with a mutation at this codon (phenylalanine to tryptophan) has also been recently reported (30).

The finding of the same point mutation in two different resistant clones, as well as in another MTX-resistant cell line (20), suggests that certain areas of the DHFR gene are "hot spots" for mutations. Modeling studies using the crystal structure coordinates for human DHFR (which are nearly identical for all vertebrate species DHFRs) have revealed Phe-31 to be an active site residue participating in hydrophobic interactions with substrate and inhibitors (22). Evidence that this amino acid change could affect ligand binding was afforded by equilibrium dialysis and enzyme kinetic experiments that revealed that the dissociation constant for MTX binding was 100-fold greater for the altered enzyme, compared with wild-type DHFR (21, 22). Kinetic characterization of the altered CHO DHFR from cell lines 2 and 6 in the current study has likewise indicated a reduced affinity for substrate (Table 1) and antifolates (Fig. 6), in comparison with enzyme from line 1 and the parental cell line. Furthermore, it appears likely that the combination of both a higher level of DHFR and a Phe-31 to Ser-31 mutation in DHFR explains the greater degree of resistance noted for cell line 2, compared with cell line 6. (A 3-fold increase in DHFR message was seen in cell line 6, compared with an 8-fold increase in DHFR message in cell line 2.) The selection of TMTX-resistant clones was carried out as a single-step selection in a modest concentration of TMTX (8×10^{-8} M TMTX). It is conceivable that any mutation in the DHFR gene would not result from the TMTX treatment but would actually be selected by the TMTX selection step. The prior exposure of these cells to a known mutagen, EMS, helped to increase the frequency of mutational events, and the TMTX selection step selected the antifolate-resistant clones from the milieu. Previous studies on TMTX resistance in MOLT-3 acute lymphoblastic leukemia lines reported development of the MDR phenotype along with antifolate resistance (31). However, Miyachi *et al.* (32) in a preliminary report recently showed that the TMTX-resistant MOLT-3/TMQ200 line had a mutation in the DHFR gene at codon 31, in which the parental phenylalanine (TTC) was changed to serine (TCC). This finding further strengthens the argument that codon 31 in the DHFR gene represents a hot spot, as does codon 22, where other mutations have also been reported.

The mechanism of resistance to TMTX for clones 4, 5, and 7 remains undefined. Impaired uptake of TMTX due to the MDR phenotype (32, 33) and other membrane defects have been described in TMTX-resistant cell lines. However, sublines 4, 5, and 7 were still sensitive to the MDR drugs doxorubicin and vinblastine and were cross-resistant to MTX, a compound that utilizes a different uptake mechanism; therefore, impaired uptake as the mechanism of resistance to this drug in these sublines is unlikely. It is of interest that Sharma *et al.* (29), in their study of the effect of X-rays on TMTX resistance, also found that the TMTX-resistant clones resulting after X-ray treatment did not contain amplified DHFR genes, nor were they impaired in their uptake of the drug. However, those authors did not examine the TMTX-resistant lines for evidence of impaired TMTX binding. We are continuing to examine

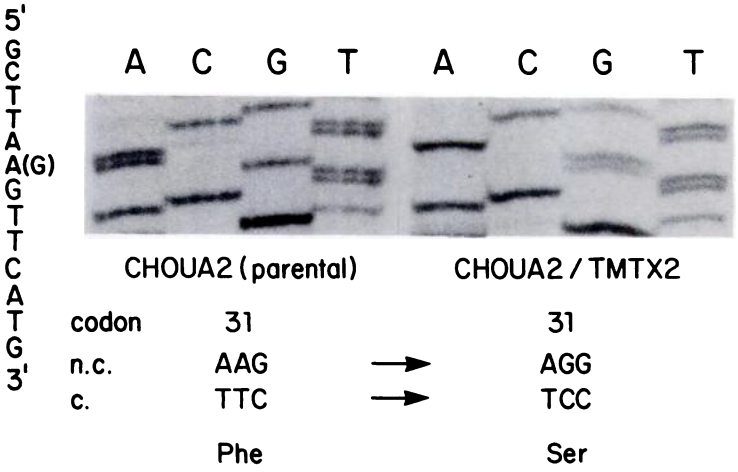

Fig. 4. Nucleotide sequence of parental line and resistant line DHFR cDNA. Sequence analysis of the region between bases 85 and 97 is shown for the parental line and cell line 2. A point mutation is seen at nucleotide 92 (adenosine to guanosine), resulting in the substitution of phenylalanine for serine (*arrow*). This base transition results in the loss of a *Eco*RI site in CHO UA2/TMTX^{R}2 and six cell lines, which was confirmed by Southern analysis; n.c., noncoding; c, coding.

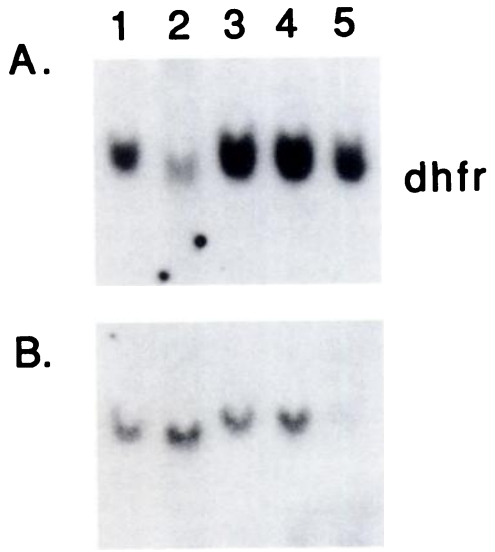

Fig. 5. Northern blot analysis of DHFR-specific transcript in drug-sensitive and -resistant cells. A, *Lanes 1-4*, 25 μg of total cellular RNA from cell line 6, parental UA2 line, cell line 1, and cell line 2, respectively; *lane 5*, 5 μg of total cellular RNA from the CHO Pro MTX R^{III} cell line, which has approximately 15 copies of the DHFR gene. B, Same blot as in A but rehybridized with 36B4 ribosomal control probe (9) to adjust for loading and transfer of RNA.

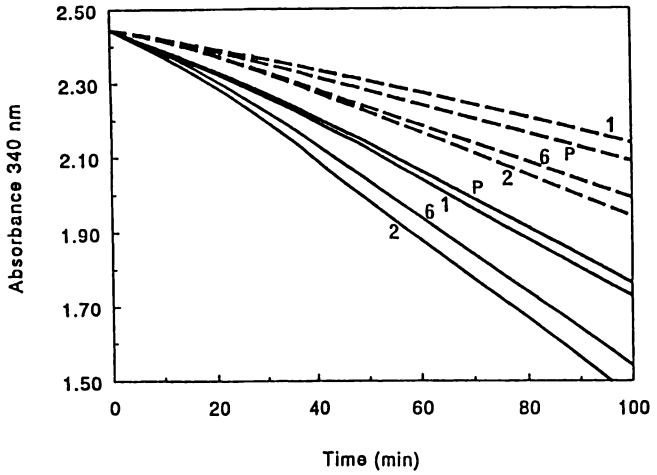

Fig. 6. Representative progress curves obtained for reactivation of DHFR in crude CHO cell extracts after preincubation with NADPH and either MTX or TMTX. Preincubation and assays were conducted as described in Materials and Methods. The *curves* represent regain of activity on dilution of ternary enzyme-cofactor-inhibition complexes (uniform amounts of enzyme for all cell lines, based on original activity) into a reaction assay mixture containing 200 μM NADPH and 150 μM H_2folate. Represented are the "average" of duplicate progress curves obtained for each experimental condition; *P*, parental cell line; *1*, cell line 1; *2*, cell line 2; *6*, cell line 6. – – –, MTX inhibition complexes; ——, TMTX inhibition complexes. Progress curves obtained from dilution of enzyme preincubated in the absence of inhibitors were superimposable for each cell line (not depicted).

sublines 4, 5, and 7 for possible new mechanisms of resistance to TMTX.

The current study provides support for the possibility that an altered DHFR may account for the antifolate-resistant phenotype in tumors that may result after alkylating agent and antifolate administration in patients. The fact that the same mutation (Phe-31 to Ser-31) has now been identified in two different antifolate-resistant cell lines supports the general hypothesis that hot spots for mutations may exist in the DHFR gene and that, under appropriate conditions, they may mutate more readily to impart a resistant phenotype. Mutations in the codon for Leu-22 (leucine to phenylalanine and leucine to arginine) have also been described, indicating that this active site hydrophobic amino acid may also be a hot spot (23, 24). Additional studies using cell culture model systems and human malignancies should delineate the frequency and nature of these and other possible DHFR mutations.

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