Characterization of the Coexisting Multiple Mechanisms of Methotrexate Resistance in Mouse 3T6 R50 Fibroblasts*

(Received for publication, September 29, 1991)

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We have studied the discrepancy in the degree of methotrexate (MTX) resistance that exists between two clonal cell lines, mouse 3T6 R50 cells and Chinese hamster ovary B11 0.5 cells that overexpress comparable levels of dihydrofolate reductase, yet exhibit a 100-fold difference in MTX resistance while maintaining similar sensitivity to the lipophilic antifolates trimetrexate and piritrexim. These data suggested that R50 cells may possess additional mechanism(s) of antifolate resistance, such as MTX transport alteration. Flow cytometric analysis using fluorescein methotrexate revealed comparable levels of fluorescein MTX displacement with lipophilic antifolates in viable R50 and B11 0.5 cells, but marked insensitivity of R50 cells to MTX competition, thus suggesting a poor uptake of MTX into R50 cells. Analysis of the kinetic parameters of dihydrofolate reductase from R50 cells neither showed alterations in enzyme affinities for various antifolates nor in the Michaelis constant for folic acid and NADPH nor a change in the pH activity optimum. R50 cell-free extracts contained wild-type levels of folylpoly-γ-glutamyl synthetase activity. However, following metabolic labeling with [14C]MTX, no MTX polyglutamates could be detected in R50 cells. We conclude that the high level of MTX resistance in R50 cells is multifactorial, including overexpression of dihydrofolate reductase, reduced MTX transport, and possibly altered formation of MTX polyglutamates. The potential interactions between the different modalities of MTX resistance in R50 cells are being discussed.

Since the introduction of the 4-aminofolate aminopterin (APT) (Farber et al., 1948), its structural homolog methotrexate (MTX) has gained wide-spread clinical use as an important component of chemotherapeutic regimens employed in the treatment of various malignancies including lymphoma, Hodgkin's disease, choriocarcinoma, mammary and bladder carcinoma, head and neck cancer and osteogenic sarcoma (Jolivet et al., 1983). MTX is a potent inhibitor of dihydrofolate reductase (DHFR; EC 1.5.1.3) and thus exerts its cytotoxic effect via interference with the biosynthesis of tetrahydrofolic acid, a key cofactor in a variety of metabolic pathways involving one-carbon transfer. The successful cytotoxic action of hydrophilic antifolates such as MTX depends on the intact function of at least three processes, including 1) intracellular drug accumulation against a concentration gradient, via an energy-dependent carrier-mediated system (Goldman, 1969); 2) the presence of an intact high binding affinity intracellular target enzyme, DHFR; 3) prolonged intracellular retention due to the action of a specific enzyme, folylpoly-γ-glutamate synthetase (FPGS), that concatenates γ-glutamyl residues to MTX (McGuire and Bertino, 1981). However, two major impediments toward successful chemotherapy are inherent and acquired drug resistance. Quantitative or qualitative alterations in each of the above cellular processes have been widely recognized as important determinants of MTX resistance. These alterations include 1) increased activity of the target enzyme DHFR (Alt et al., 1978) as a result of DHFR gene amplification (Schimke, 1988); 2) diminished (Sirotnak et al., 1981) or abolished uptake of MTX (Assaraf and Schimke, 1987); 3) reduced affinity of DHFR for MTX (Haber et al., 1981; Srimatkandada et al., 1989); and 4) defective formation of MTX polyglutamates, resulting in a decreased cellular retention of this antifolate (Cowan and Jolivet, 1984; Frei et al., 1984; Wright et al., 1987).

The existence of each of the various modes of MTX resistance was extensively described in rodent and human tumor model cell lines established in the laboratory using different drug selection protocols. The simultaneous existence of two or more modes of MTX resistance has also been described, albeit to a lesser extent. For example, Sirotnak et al. (1981) have described the presence of both DHFR enzyme overproduction and MTX transport alterations in clonal isolates of MTX-resistant mouse leukemia cells derived in vivo. Haber et al. (1981) have reported the coexistence of a profoundly structurally altered DHFR in MTX-resistant mouse 2T6 fibroblasts (R400) that highly overexpress this enzyme. Cowan and Jolivet (1984) have characterized a MTX-resistant breast carcinoma cell line bearing multiple defects including decreased MTX transport, diminished thymidine synthase activity, and markedly reduced MTX polyglutamation. Frei
et al. (1984) and Rosowsky et al. (1985) have described several MTX-resistant human squamous cell carcinoma sublines with either decreased MTX transport and diminished formation of MTX polyglutamates or increased DHFR activity and defective MTX transport. The present study was initiated by the observed discrepancy in the degree of MTX resistance that exists between murine 3T6 R50 fibroblasts and Chinese hamster ovary (CHO) B11 0.5 cells that overexpress comparable levels of DHFR, maintain similar levels of sensitivity to the lipophilic antifolates trimetrexate (TMTX) and pterixim (PTX), but yet surprisingly exhibit a 100-fold difference in MTX resistance. Thus, we find that in mouse 3T6 R50 clonal cells that exhibit over 13,000-fold MTX resistance, only a fraction could be attributed to the 145-fold DHFR overexpression; flow cytometric analysis using viable F-MTX labeling and its competition with antifolates revealed a significant decrease in MTX uptake into R50 cells. Furthermore, although wild-type activity levels of FPGS were present in R50 cell-free extracts, none of the glutamate conjugates of MTX could be detected in viable cells even when intracellular DHFR molecules were saturated with the nonpolyglutamylatable antifolate, TMTX. Parallel characterization of MTX-resistant CHO B11 0.5 cells that overproduce comparable levels of DHFR and display normal MTX accumulation provided a means of assessing the relative contribution of the multiple defects in R50 cells to the overall MTX-resistance phenotype. This study supports the conclusion that multiple modalities of MTX resistance can coexist in a given cell population following antifolate selective pressure. Thus, MTX-resistant phenotypes in mammalian cells are frequently complex and hence require detailed analyses to identify resistance mechanisms and determine their relative contribution to the overall resistance phenotype.

EXPERIMENTAL PROCEDURES

Drugs—MTX was obtained from the National Cancer Institute, TMTX (glucuronate salt) was kindly provided by Warner Lambert/Parke Davis, Ann Arbor, MI, and PTX (isethionate salt) was a gift from Burroughs Wellcome, Research Triangle Park. F-MTX was purchased from Molecular Probes, Inc., Eugene, OR. Folic acid and enzymatically reduced NADPH were obtained from Sigma. TMTX (gluconate salt) was kindly provided by Warner Lambert/Parkes Davis, Ann Arbor, MI, and PTX (isethionate salt) was a gift from Burroughs Wellcome, Research Triangle Park. F-MTX was purchased from Molecular Probes, Inc., Eugene, OR. Folic acid and enzymatically reduced NADPH were obtained from Sigma.

Cell Cultures—Clones of murine 3T6 cells (clone 5) and Chinese hamster ovary (CHO) K1 cells (clone 1) subline (Kellens et al., 1979) derived by multiple step selection with MTX to a 50 µM concentration were maintained in Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum (dFBS, from GIBCO), 2 mM glutamine, and 10 µg/ml gentamicin but lacking glycine, hypoxanthine, and thymidine. B11 0.5 growth medium also contained trexim (PTX), but yet surprisingly exhibit a 100-fold difference in MTX resistance. Following 8-10 h of F-MTX labeling (1985) have described several modes of MTX-Resistance in R50 Cells

Concomitant Assay for Antifolate Cytotoxicity—Parental and their MTX-resistant sublines were grown in antifolate-free medium for at least five doublings and then plated (104 mouse 3T6 cells and 105 hamster cells/60-mm dish) in medium containing different concentrations of antifolates. After 7-10 days of incubation, colonies were briefly washed with cold PBS, fixed with methanol, stained with crystal violet, and counted (colony ≥ 50 cells). The mean plating efficiencies (n = 6-10) of parental 3T6, R50, CHO K1, and B11 0.5 cells were 51, 41, 76, and 65%, respectively.

Determination of Gene Copy Number and mRNA Levels—Genomic DNA and cytoplasmic RNA were isolated as described (Maniatis et al., 1982). Mouse and hamster DHFR gene copy numbers were determined by Southern blot analysis of EcoRI-digested DNA using a "P-oligolabeled (Feinberg and Vogelstein, 1983) 1-kb EcoRI/HindIII fragment of a genomic plasmid pSP64-T10-containing mouse 5'-DHFR sequence and a 622-base pair BamHI/EcoRI fragment of the CHO genomic plasmid pBS13–7-containing 3'-DHFR sequence (Carothers et al., 1983), respectively. Steady-state levels of mouse and hamster DHFR mRNA were determined by Northern blot analysis using a 1.9-kb EcoRI/HindIII fragment of the mouse cDNA clone pD11 (Setzer et al., 1980) and a 0.93-kb PstI fragment of a hamster cDNA clone MQ19–97, respectively (Melera et al., 1984). Steady-state levels of mouse thymidylate synthase were detected by a 300-base pair Pstl-BamHI insert of pSP64-TS (Feder et al., 1990), which contains the 5'-most sequences of a mouse thymidylate synthase cDNA mPMTS-3 (Goeddel and Johnstone, 1984). cDNA fragments were gel-purified prior to oligolabeling. Gene copy numbers and mRNA levels were determined by scanning densitometry of linear exposures of hybridization signals normalized to the actual amounts of DNA and RNA loaded by scanning Polaroid negatives (Type 665, Sigma). The ethidium bromide staining of the gels.

Enzymatic Assay for DHFR Activity—Cells were grown in drug-free medium for at least five generations, detached by trypsinization, washed with excess cold PBS, counted, and suspended in ice-cold medium containing 0.1 mM potassium phosphate, pH 7.0, at a cell density of 106 cells/ml (R50 and B11 0.5 cells) or 105 cells/ml (parental 3T6 and CHO K1 cells). Crude cytosolic extracts were then prepared as previously described (Assaraf et al., 1989a), used immediately or aliquotted, frozen in liquid nitrogen, and stored at −85 °C until analysis. DHFR activity was determined by the [14C]folic acid reduction assay of Rothenberg (1986) with the modification of Nakamura and Littlefield (1972), in a standard reaction mixture (0.2 ml, as detailed elsewhere (Assaraf et al., 1989a), except that the pH of the reaction mixture for mouse DHFR activity assay was 4.3, whereas for hamster DHFR, the pH was 5.0.

Measurement of Km Values of DHFR for Folic Acid and NADPH—In order to determine Km values for folic acid (0.4 mM) and NADPH (0.03–0.5 mM) in parental 3T6 cells and MTX-resistant cells for folic acid, initial velocities of folate reduction were measured in reaction mixtures in which the concentrations of nonisotopic folate were varied from 25 to 350 µM for cytosolic extracts from parental 3T6 and R50 cells, and 25-400 µM for K1 and B11 0.5 extracts. To determine the Km values for NADPH, enzymatic reactions containing NADPH-supplemented folate free, folate containing the concentration of NADPH was varied from 25 to 500 in 3T6 and R50 cytosols and, for K1 and B11 0.5 cytosols, it was varied from 25 to 400 µM. The concentration of [14C]folate in both experiments was 17.5 nM. The Km values were derived from double reciprocal plots (Lineweaver and Burk, 1934).

pH Profile of Folate Reductase Activity—The pH dependence of folic acid reduction was measured using three different overlapping buffer systems at 0.1 M concentration. A sodium acetate buffer was used from pH 3.2 to 6.5, a sodium phosphate buffer was used from pH 6.5 to 8.1, and a Tris buffer from pH 8.1 to 9.0.

Determination of DHFR Affinity for Various Antifolates—To determine the affinity of DHFR from parental 3T6 and CHO K1 cells and their MTX-resistant sublines (R50 and B11 0.5) for the different antifolates, equal amounts of enzyme units were preincubated in the dark with various concentrations of MTX, TMTX, and PTX at 4 °C for 10 min, after which the remaining folate reductase activity was determined as previously described (Assaraf et al., 1989a). The 50% inhibitory concentrations (IC50) were graphically derived from each antifolate as previously described (Cheng and Prusoff, 1973).
values of MTX were then derived as described above. Then, based on the moles of MTX present in a given ICh on Avagadro's constant, and on the number of cells used in each assay, the number of active DHFR molecules/cell was calculated.

**Materials Labeling with L-[35S]Metionine and Preparation of Cell Extracts—For preparation of cell extracts, their MTX-resistant R50 subline, parental CHO K1 cells, and their MTX-resistant variant B11.0.5 were seeded in growth medium (containing 100 μM MTX for R50 and 0.5 μM MTX for B11.0.5 cells) at a density of 2-4 x 10^5 cells/10-cm dish. Two days later, 2 ml of medium containing 100 μCi/ml of L-[35S]Metionine (5000 Ci/mmol, Du Pont-New England Nuclear) and 5% DBS, but lacking nonisotopic methionine, were added to each plate. After pulse-labeling for 30 min at 37°C, cells were rinsed twice with ice-cold PBS containing 1 mM nonradioactive methionine and 5% DBS. Cells were then lysed with buffer (0.5 ml/plated cell) containing 100 μM Tris.Cl at pH 7.2, 150 mM NaCl, and 0.5% Nonidet P-40 (Sigma), and centrifuged at 15,000 x g for 5 min at 4°C. The incorporation of radiolabeled methionine into solubilizable cellular proteins (in the supernatant) was determined by trichloroacetic acid precipitation of triplicate aliquots (2 μl) of cell lysates. The supernatants of the cell lysates were frozen in liquid nitrogen and stored at -85°C until analysis.

**Nonequilibrium pH Gradient Electrophoresis—Radiolabeled proteins from total cellular extracts were analyzed by two-dimensional gel electrophoresis according to the method of O'Farrel et al. (1977) as modified by Jones (1980). Aliquots of cell lysates containing 10^5 acid-stable counts/min were electrophoresed in the first dimension (nonequilibrium pH gradient electrophoresis) buffer containing 9.5 M urea, 2% Nonidet P-40, 1.6% amphicline, pH 5-7, 0.4% ampholine, pH 3.5-10 (Pharmacia LKB Biotechnology Inc.) and 5% 2-mercaptoethanol, followed by electrophoresis at 500 V for 6 h at room temperature. The gels were equilibrated in sodium dodecyl sulfate sample buffer for 2 h, followed by electrophoresis on a 12% polyacrylamide gel containing sodium dodecyl sulfate (Laemmli, 1970). Gels were then fixed with 50% trichloroacetic acid, washed with excess distilled water, fluorographed by impregnation in 1 M sodium salicylate for 30 min, dried, and exposed to x-ray film.

**Polyglutamylation Determinations—Polyglutamylation determinations were made by the method of Bradford (1976) with Bio-Rad reagents and by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

**Assay for Polyglutamylation of MTX in Viable Cells—In order to assess the capability of parental 3T6 cells and their R50 subline to polyglutamylate MTX, cells were incubated for 24 h in growth medium (supplemented with 10% DBS) containing 2 μM of HPLC purified [3,5,5',7'-3H]MTX (0.85 Ci/mmol, Amersham Corp.), 2 mM glutamine, and 30 μM each of glycine, hypoxanthine, and thymidine. Cells were then washed twice with warm PBS, lysed by sonication for 10 seconds in 0.5 M ammonium acetate buffer containing 0.2 μmol of nonisotopic peak markers, namely MTX and each of its polyglutamates (G1-G6). The mixture was then analyzed by HPLC on a Nova-Pak C18 column eluting at a linear gradient of 0-5% acetonitrile in 1 M ammonium acetate buffer at pH 6.0 over 70 min at a rate of 1.0 ml/min. Fractions of 0.5 ml were collected directly in minivials for scintillation counting. Data from the liquid scintillation counting were plotted versus time in order to determine the correspondence of the radioactive peak with those obtained by ultraviolet detection of nonradioactive standards.

**Cell-free Assay for Polyglutamate Synthetase—To determine the levels of PFGs, exponentially growing cells were washed (20 ml/100-mm dish) three times with prewarmed PBS, lysed on the plates with buffer (1 ml/dish) containing 20 mM potassium phosphate at pH 7.0 and 50 mM Tris.Cl, 100 mM β-mercaptoethanol, scraped off the plates with a rubber cell harvester, and frozen in liquid nitrogen. Cells were then sonicated for 10 s and centrifuged, and FPGs activity in the supernatant was measured by the incorporation of radiolabeled glutamine into folate and MTX products, as described by Cichowicz and Shanes (1987). Assay mixtures consisted of 100 mM Tris, 50 mM glycine buffer at pH 9.5 (22°C), 40 μM 6-amido-tetrahydrofolate or 200 μM MTX, 0.5 mM L-[3H]glutamate (2.5 μCi; specific radioactivity, 10 mCi/mmol, Amerham), 5 mM ATP, 10 mM MgCl2, 20 mM KCl, 100 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, and 100 μl enzyme extract, in a total volume of 0.5 ml. The reaction tubes were capped and incubated at 37°C for 2 h. The reactions were then terminated by the addition of 1.5 ml of ice-cold solution of 30 mM β-mercaptoethanol and 10 mM nonisotopic glutamate, after which the reaction mixtures were applied onto a 2 x 0.7 cm DEAE cellulose (Whatman DE52) column, equilibrated with 10 mM Tris buffer, pH 7.5, containing 80 mM NaCl and 30 mM β-mercaptoethanol. The column was then washed three times with 5 ml of equilibration buffer to remove labeled glutamate. Radiolabeled folate or MTX polyglutamate products were eluted with 5 ml of 0.1 M HCl, and the radioactivity was determined by liquid scintillation counting.

**RESULTS

**DHFR Gene Copy Number, Steady-state mRNA, and Enzyme Levels—To determine DHFR gene copy numbers, various amounts of EcoRI-digested genomic DNA purified from parental 3T6 and CHO K1 cells and their MTX-resistant R50 and B11.0.5 sublines were subjected to Southern blot analysis using 32P-oligolabeled probes. Mouse DHFR gene was detected with two probes: a genomic probe that recognizes a mouse 5'-DHFR sequence and a mouse DHFR cDNA probe. Hamster DHFR gene was detected by a genomic probe directed to a hamster 3' -DHFR sequence. Scanning densitometry of the resulting mouse genomic 3.4-kb fragment (Fig. 1A), as well as of the 14.8-kb fragment that hybridized to the cDNA probe (Fig. 1B) in several linear exposures of the autoradiograms, revealed approximately 180 copies of the DHFR gene in R50 cells, as compared with the single DHFR gene present in parental 3T6 cells (Fig. 1, A and B). Scanning densitometric analysis of the 4.1-kb fragment that hybridized to the hamster 3'-DHFR genomic probe showed that B11.0.5 cells contained approximately 73 copies of the DHFR gene, as compared with the single DHFR gene present in parental CHO K1 cells (Fig. 1C). In order to measure the steady-state levels of DHFR mRNA, various amounts of cytoplasmic RNA isolated from parental cells and their MTX-resistant sublines were subjected to Northern blot analyses on formaldehyde-agarose gels and probed with mouse and hamster cDNA sequences. Scanning densitometry of the major 1.6-kb mouse DHFR transcript showed that R50 cells contained an average increase of 194-fold in the steady-state levels of the DHFR mRNA relative to their MTX-sensitive parental 3T6 cells (Fig. 2A). Scanning densitometry of the major 2.4-kb hamster DHFR transcript revealed a 100-fold elevation in B11.0.5 cells, as compared with the level of DHFR mRNA present in wild-type CHO K1 cells (Fig. 2B). Thus, the marked DHFR gene amplification in R50 and B11.0.5 cells was accompanied by a consistent elevation in the steady-state levels of DHFR mRNA.

Since previous reports have identified that an inverse relationship exists in some cell lines between the activity of thymidylate synthase and the relative sensitivity to MTX (Morgan et al., 1979; Cowan and Jolivet, 1984), we have determined the steady-state mRNA levels of thymidylate synthase from 3T6 and R50 cells. Northern blot analysis demonstrates that the 1.3-kb mRNA of thymidylate synthase (Geyer and Johnson, 1984) is equally expressed in 3T6 and R50 cells (Fig. 2C).

To estimate the expression of DHFR enzyme in parental cells and their MTX-resistant sublines, we have chosen to titrate the actual number of catalytically active DHFR molecules/cell. Crude cytosolic extracts were prepared from a known number of cells, and DHFR activity was stoichiometri-
Modes of MTX-Resistance in R50 Cells

Fig. 1. Autoradiogram of Southern blots containing DNA from wild-type cells and their MTX-resistant sublines probed with DHFR sequences. Genomic DNA extracted from nuclei obtained from wild-type 3T6 and its MTX-resistant R50 subline with a 9'-oligolabeled mouse DHFR genomic clone pSP64RT10.0.5 cells (C) was digested with EcoRI, fractionated on agarose gels, and transferred to a Zetabind filter membrane. Blots were hybridized with a 32P-oligolabeled mouse DHFR genomic clone pSP64RT10 (EcoRI-HindIII fragment, panel A), a mouse DHFR cDNA clone pD11 (EcoRI-HindIII insert, panel B), and a genomic clone of hamster DHFR, pB13-7 (BamHI-EcoRI fragment, panel C). Following hybridization for 2 days at 42°C, blots were washed under high stringency conditions. The ethidium bromide staining of the gel-fractionated DNA digests is shown to indicate the different amounts of DNA hybridizing fragments. Genomic DNA (pg) 10^0 1.0 2.5 0.5 0.25 0.1 10

Fig. 2. Autoradiograms of Northern blots containing RNA from wild-type and MTX-resistant cells probed with dihydrofolate reductase and thymidylate synthase cDNA sequences. Cytoplasmic RNA from mouse 3T6 and R50 cells (A and C) from CHO K1 and B11 0.5 cells (B) was fractionated on agarose-formaldehyde gels, transferred to a Zetabind membrane, and hybridized to a [32P]dATP-labeled EcoRI-HindIII fragment of a mouse cDNA clone pD11 (panel A), a PstI fragment of a hamster DHFR cDNA clone MQ19-97 (panel B), and a PstI-BamHI fragment of a mouse thymidylate synthase cDNA clone pSP64-TS (panel C). The various amounts of RNA loaded onto the gels are demonstrated by the ethidium bromide staining. Arrows indicate size in kb of the hybridizing fragments. Parental CHO K1 and H11

ricularly inhibited by the high affinity folate antagonists, MTX, and by its structural homolog, APT. Superimposable dose-dependent inhibition curves of folate reductase activity derived from 10^7 parental CHO K1 cells were obtained with MTX and APT and similarly with 3 x 10^9 B11 0.5 cells at higher concentrations of MTX and APT (Fig. 3). DHFR activity from 10^7 parental 3T6 cells and 5 x 10^6 R50 cells was also inhibited with MTX in a dose-response manner, revealing a typical sigmoid curve that shifted to a higher range of antifolate concentration in extracts from R50 cells (Fig. 3). Thus, based on the number of cells used in each enzymatic assay, the antifolate concentration producing 50% inhibition of initial enzyme activity (IC50), the volume of each enzymatic assay, as well as Avogadro’s constant, the number of active (e.g., capable of binding MTX) DHFR molecules/cell were calculated for each cell line. Parental CHO K1 and 3T6 cells were found to contain an average of 1.3 x 10^8 and 1.6 x 10^9 enzyme molecules/cell, respectively, whereas their MTX-resistant sublines B11 0.5 and R50 cells contained approximately 1.4 x 10^7 and 2.3 x 10^7 DHFR molecules/cell, respectively (Table I). Thus, R50 cells expressed 145-fold more DHFR enzyme molecules than parental 3T6 cells in agreement with the prominent DHFR gene amplification and elevation of the mRNA levels (Table I). B11 0.5 cells had an approximately 104-fold increase in the number of DHFR molecules/cell, as compared with parental CHO K1 cells, in concordance with their DHFR gene copy number and steady-state mRNA levels. These results indicate that R50 and B11 0.5 cells overexpressed comparable amounts of catalytically active DHFR enzyme molecules.

Cytotoxicity of Antifolates to Parental and Drug-resistant Cells—Since R50 and B11 0.5 cells contained a marked elevation in DHFR activity, their sensitivity and that of their parental cells to various antifolates was assessed in a clonogenic assay. When the clonal surviving fraction of R50 and 3T6 cells was determined for various concentrations of the lipid-soluble antifolates TMTX and PTX, it became evident that approximately 2 orders of magnitude of antifolate resistance was obtained with R50 cells (Fig. 4), thus being in agreement with the degree of DHFR enzyme overexpression (Table I). However, the concentration of the hydrophilic antifolate, MTX, required to reduce R50 cell survival by 90% was over 4 orders of magnitude higher than that needed to obtain the same effect with parental 3T6 cells (Fig. 4). Table II depicts the 50% antifolate lethal doses (LD50) and the resulting levels of resistance obtained with R50 and B11 0.5 cells, as compared with their parental 3T6 and CHO K1 cells. Consistent with the 104- and 145-fold enzyme overexpression in R50 and B11 0.5 cells, respectively (Table I), was their 82-96-fold resistance to TMTX and PTX, as compared with parental cells (Table II). In contrast, R50 cells were surpris-
ingly 13,250-fold resistant to MTX, relative to parental cells. Thus, only a small fraction of this MTX resistance in R50 cells could be attributed to DHFR enzyme overexpression, while leaving a large extent of MTX resistance unexplained.

Displacement of Cellular F-MTX Labeling by Antifolates as a Measure for Intracellular Accumulation of MTX—We have previously reported (Assaraf and Schinike, 1987) the use of cellular F-MTX labeling and its specific displacement by competition with hydrophilic and lipophilic antifolates in the identification of mammalian cells bearing transport alterations that impair or completely abolish the intracellular accumulation of MTX. F-MTX enters mammalian cells by passive transport and/or by facilitated diffusion (Assaraf et al., 1988b), thus being distinct from MTX uptake, whose carrier-mediated active transport is subject to various alterations (Sirotznak et al., 1981). Additionally, the efficiency of the displacement of cellular F-MTX labeling by antifolates directly depends on the intracellular accumulation and, thus, concentration of the competing antifolate (Assaraf et al., 1989c). Inasmuch as transport measurements (initial velocities in particular) are difficult to perform in monolayer cells (especially those containing high levels of DHFR) and the F-MTX assay is rapid, facile, and reproducible (Assaraf et al., 1989a, 1989b, 1989c), this F-MTX assay was used here to test whether the uptake of MTX in R50 cells was impaired.

Following saturation of intracellular DHFR with F-MTX in parental 3T6 and R50 cells, F-MTX labeling was subjected to competition with MTX, a hydrophobic folate antagonist that enters mammalian cells by an energy-dependent carrier-mediated process (for a review, see Sirotznak et al., 1979), as well as with TMTX and PTX, lipophilic antifolates that appear to enter mammalian cells via diffusion (Duch et al., 1982; Kamen et al., 1984; Fry and Besserer, 1988). Similar dose-response curves for F-MTX displacement by MTX, TMTX, and PTX were obtained with 3T6 and CHO K1 cells (data not shown). In contrast, displacement of F-MTX in R50 cells started to occur only at a concentration of 10 μM MTX, whereas B11 0.5 cells lost all their fluorescence at this concentration; complete loss of fluorescence in R50 cells required 5 mM MTX (data not shown). The ratio of the 50% displacement concentration (DC_{50}) obtained with hydrophilic (e.g. MTX) and lipophilic antifolates (e.g. TMTX and PTX) is indicative of the degree of the MTX transport impairment. Mean DC_{50} values of F-MTX were derived from several independent experiments performed with various antifolates. The mean DC_{50} values of TMTX and PTX were identical in parental 3T6 and CHO K1 cells (Table I). Similarly, the DC_{50} values of these lipophilic antifolates TMTX and PTX were comparable for the DHFR-overexpressing cell lines R50 and B11 0.5 (Table III). Thus, the R50/3T6 and B11 0.5/CHO K1 ratios of the DC_{50} values for TMTX and PTX were comparable and generally consistent with the extent of DHFR overproduction (Table I). Similarly, the DC_{50} values of MTX in parental 3T6 and CHO K1 cells were comparable (Table III). Consistent with the displacement data obtained with the lipophilic antifolates and in good agreement with the 104-fold overproduction of DHFR enzyme in B11 0.5 cells (Table I) was the 106-fold increase in the DC_{50} of these cells as compared with parental CHO K1 cells (Table III). Thus, MTX accumulation in B11 0.5 cells appeared to be intact. In contrast, R50 cells that overexpressed 145-fold DHFR enzyme surprisingly required over 11,000-fold higher MTX concentration than did the parental 3T6 cells to achieve the same DC_{50}. Furthermore, although R50 and B11 0.5 cells contained comparable amounts of DHFR enzyme molecules/cell (Table I), R50 cells required an over 290-fold higher MTX concentration than B11 0.5 cells to achieve the same DC_{50} (Table III). These results agree well with the high level of resistance to MTX (but not to TMTX and PTX) in R50 cells (Table II) and provide evidence that R50 cells possess a transport defect that significantly impairs intracellular accumulation of MTX.

Kinetic Properties of DHFR from Parental and MTX-resistant Cells—R50 cells are the precursor cells of the R400 cell line that was found to highly overproduce a structurally altered DHFR enzyme (Haber et al., 1981) with a 270-fold reduction in the binding affinity for MTX, a basic shift in its pH activity profile. Thus, to address the question of whether R50 cells contained a structurally altered DHFR that could similarly contribute to the high level of MTX resistance, total

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**Table I**

| DHFR | 3T6 | R50 | R50/3T6 | CHO K1 | B11 0.5 | B11 0.5/K1 |
|------|-----|-----|---------|--------|---------|-----------|
| Gene copy number | 1   | 180 | 280     | 1      | 73      | 73        |
| mRNA levels | 1   | 194 | 194     | 3      | 100     | 100       |
| Molecules/cell | 1.6 × 10^4 | 2.32 × 10^7 | 145 | 1.3 × 10^6 | 1.35 × 10^7 | 104       |
Modes of MTX-Resistance in R50 Cells

**TABLE II**

| Antifolate | 3T6 | R50 | Resistance | CHO K1 | B11 0.5 | Resistance |
|------------|-----|-----|------------|--------|---------|------------|
| Methotrexate | 200 | 265,000 | 13,250 | 18 | 2,500 | 139 |
| Trimetrexate | 5.5 | 460 | 84 | 17 | 1,000 | 96 |
| Piritrexim | 40 | 3,000 | 90 | 43 | 3,500 | 82 |

*Numbers represent the antifolate concentration (nM) that produces 50% cell killing (LD_{50}).

**TABLE III**

| Antifolate | 3T6 | R50 | Resistance | CHO K1 | B11 0.5 | Resistance |
|------------|-----|-----|------------|--------|---------|------------|
| Methotrexate | 24 | 265,000 | 11,042 | 17 | 1,800 | 106 |
| Trimetrexate | 0.5 | 62 | 124 | 0.5 | 34 | 68 |
| Piritrexim | 1.4 | 54 | 36 | 1.5 | 60 | 40 |

*Antifolate concentration (nM) that produces 50% displacement (DC_{50}) of cellular F-MTX labeling.

Clonogenic assays were performed as detailed under "Experimental Procedures." Cell survival frequency was corrected for the individual plating efficiency of each cell line obtained in drug-free medium. Curves shown are representative of three independent experiments.

![Graphs showing clonal survival of sensitive and MTX-resistant cells in the presence of various antifolates.](image)

**Fig. 4. Clonal survival of sensitive and MTX-resistant cells in the presence of various antifolates.**

Clonogenic assays were performed as detailed under "Experimental Procedures." Cell survival frequency was corrected for the individual plating efficiency of each cell line obtained in drug-free medium. Curves shown are representative of three independent experiments.

Cell extracts were prepared from [35S]methionine-labeled parental 3T6 and MTX-resistant R50 cells, after which the soluble supernatants were subjected to two-dimensional gel electrophoresis. This was performed using nonequilibrium pH gradient gel electrophoresis in the first dimension, as this method is better suited for the resolution of basic proteins (such as DHFR), even though it does not yield an exact isoelectric point. Fig. 5 shows the fluorograms of the protein migration patterns obtained with cell lysates from 3T6 and R50 cells; comparison of the fluorograms from the overproducing R50 cells reveals the position of DHFR as a major protein spot in R50 cells as compared with the low levels present in parental 3T6 cells (Fig. 5; arrows). Both DHFR enzymes from 3T6 and R50 cells appear to have the same molecular weight and migrate to the same basic point relative...
The cells were then lysed in buffer containing 10 mM Tris, pH 7.2, 0.15 NaCl, 0.5% Nonidet P-40 and sedimented at 15,000 x g for 5 min at 4°C. Samples of 10% acid-insoluble counts were electrophoresed on the gels. The first dimension was a nonequilibrium pH gradient, and the second was a sodium dodecyl sulfate-containing polyacrylamide gel. Metabolically labeled, both the DHFR spots in close-up sections of the fluorograms after long exposure. This became more evident when identical DHFR-containing close-up sections of longer exposures of the fluorograms were compared; the same molecular weight and the same basic point of migration were obtained for neighboring spots. The long arrows indicate the position of DHFR spots, whereas the short arrows in the small panels on the right indicate the position of the DHFR spots in close-up sections of the fluorograms after long exposure.

![Fig. 5. Fluorograms of two-dimensional gel electrophoresis of total soluble proteins. Parental 3T6 cells and their MTX-resistant R50 cells were metabolically labeled for 30 min with $[^{35}S]$ methionine (100 $\mu$Ci/ml) in L-methionine-free medium. Radiolabeled cells were then lysed in buffer containing 10 mM Tris, pH 7.2, 0.15 NaCl, 0.5% Nonidet P-40 and sedimentsed at 15,000 x g for 5 min at 4°C. Samples of 10% acid-insoluble counts were electrophoresed on the gels. The first dimension was a nonequilibrium pH gradient, and the second was a sodium dodecyl sulfate-containing polyacrylamide gel. Metabolically labeled, both the DHFR spots in close-up sections of the fluorograms after long exposure. This became more evident when identical DHFR-containing close-up sections of longer exposures of the fluorograms were compared; the same molecular weight and the same basic point of migration were obtained for neighboring spots. The long arrows indicate the position of DHFR spots, whereas the short arrows in the small panels on the right indicate the position of the DHFR spots in close-up sections of the fluorograms after long exposure.]

**Table IV**

| Kinetic properties of DHFR enzyme from wild-type and antifolate resistant cells |
|---|
| **K_m (µM)** | **pH** | **Affinity for Antifolates (IC_{50})** |
| **Folate** | **NADPH** | **MTX** | **APT** | **TMTX** | **PTX** |
| 3T6 | 60 | 350 | 4.3 | 13 | 9 | 15 | 35 |
| R50 | 70 | 330 | 4.3 | 15 | 8 | 16 | 38 |
| CHO K1 | 55 | 150 | 5.0 | 11 | 10 | 14 | 34 |
| B11 0.5 | 70 | 200 | 5.0 | 14 | 10 | 13 | 35 |

The apparent Michaelis constants for folate and NADPH ranged between 55-70 µM. The apparent Michaelis constants for DHFR from 3T6 and R50 cells and at pH 5.0 for DHFR from CHO K1 and B11 0.5 cells. Thus, K_m values for folate and NADPH obtained with DHFR from R50 and B11 0.5 cells were very similar; both enzymes retained wild-type affinity for various antifolates, indicating that MTX resistance in R50 and B11 0.5 cells could not be explained on the basis of reduced enzyme affinity for MTX.

**Polyglutamylation of MTX in Viable Cells**—As decreased polyglutamylation of MTX has been documented as one of the mechanisms that may contribute to reduced intracellular retention of MTX and therefore increased MTX resistance (Cowan and Jolivet, 1984; Pizzorno et al., 1988), the ability of viable parental 3T6 and R50 cells to convert [3H]MTX to its various polyglutamate forms (G_1-G_9) was examined. Following 24 h of incubation in medium containing 2 µM purified [3H]MTX and 30 µM glycine, hypoxanthine, and thymidine (to protect cells from the cytotoxic effect of MTX), cell lysates were prepared, and radiolabeled MTX and its polyglutamate conjugates were fractionated by HPLC and identified using nonlabeled markers. Fig. 6 shows a representative pattern of [3H]MTX polyglutamates obtained with parental 3T6 cells. MTX (2 µM) was efficiently taken up by wild-type 3T6 cells and converted to its various polyglutamate derivatives. The di-, tri-, and tetraglutamate forms of MTX were the predominant population of MTX conjugates, whereas the mono- and pentaglutamates of MTX were less represented. In two independent experiments with parental 3T6 cells, the di-, tri-, and tetraglutamate forms of MTX reproducibly constituted 94% of the polyglutamate forms (Fig. 6). The triglutamate was predominant and comprised of 50% of the total. Thus, in accord with previous results in human cells (Wright et al., 1987), parental 3T6 cells displayed a typical pattern of MTX polyglutamylation. However, in two independent experiments, the amount of [3H]MTX associated with R50 cells was strikingly low, and none of the MTX polyglutamates could be detected (Fig. 6). It was likely that any MTX that penetrates into R50 cells would be sequestered by high affinity binding to the overexpressed DHFR, leaving no available substrate for FPGS. In one experiment aimed to overcome this potential obstacle, we first preincubated R50 cells in medium containing 50 µM TMTX (~100 times the LD_{50}) for 3 h in order to saturate intracellular DHFR. TMTX was chosen as the DHFR-saturating antifolate in R50 cells for several considerations. 1) TMTX and MTX possess very similar binding affinity for DHFR (Table IV; Assaraf et al., 1989a). 2) TMTX is a lipophilic antifolate that enters cells by diffusion and thus will accumulate in R50 cells, despite their MTX transport impairment. 3) TMTX lacks a glutamate side chain present in MTX and thus cannot undergo polyglutamylation. This implies that the use of excess TMTX should not interfere with polyglutamylation of MTX. Following 3-h preincubation with TMTX, R50 cells were incubated for 24 h in medium containing 5 µM [3H]MTX (4-fold higher concentration than producing cells; the IC_{50} values were in the high affinity range (9-16 nM) for MTX, APT, and TMTX, whereas PTX exhibited a slightly weaker inhibition (34-38 nM), in good agreement with a previous report (Assaraf et al., 1989a). Using double reciprocal plots, the K_m values for folate and NADPH were determined. The apparent Michaelis constants of DHFR from wild-type 3T6, CHO K1 cells, and from the MTX-resistant R50 and B11 0.5 sublines for folate were similar and ranged between 55-70 µM. The apparent Michaelis constants of DHFR from 3T6 and R50 cells for NADPH were 330-350 µM, and those of CHO K1 and B11 0.5 cells were 150-200 µM. In addition, a superimposable sharp acidic peak of folate reductase activity at pH 4.3 was obtained from DHFR from 3T6 and R50 cells and at pH 5.0 for DHFR from CHO K1 and B11 0.5 cells. Thus, K_m values for folate and NADPH obtained with DHFR from R50 and B11 0.5 cells were very similar; both enzymes retained wild-type affinity for various antifolates, indicating that MTX resistance in R50 and B11 0.5 cells could not be explained on the basis of reduced enzyme affinity for MTX.

In order to rule out the possibility that R50 cells contained a structurally altered DHFR that could not be identified by the above analyses (e.g. arising from a neutral amino acid substitution) but would decrease affinity for MTX, the affinities for folic acid as a substrate for the cofactor NADPH, as well as for various antifolates, were determined, using crude cytosolic preparations of DHFR from parental and MTX-resistant cells. Table IV summarizes the kinetic properties of DHFR from parental 3T6 and CHO K1 cells and their resistant R50 and B11 0.5 sublines. It is evident that the ranges of IC_{50} values for the hydrophilic antifolates MTX and APT and those for the lipophilic antifolates TMTX and PTX are very similar for wild-type enzyme and for DHFR from the over-
with 3T6 cells) and 25 μM TMTX (to maintain intracellular DHFR molecules continuously occupied by TMTX). Despite the high concentration of radiolaabeled MTX used and the presence of excess TMTX, the amount of radioactive MTX associated with R50 cells was persistently low, and no MTX polyglutamates could be detected. The poor accumulation of MTX in R50 cells was consistent with the insensitivity of F-MTX labeling to MTX competition observed in these cells.

Several lines of evidence suggest that DHFR from R50 cells maintained wild-type physical and catalytic properties. 1) DHFR from R50 cells appeared to retain the molecular weight and pl of parental 3T6 enzyme. 2) DHFR from R50 and parental 3T6 cells exhibited the typical sharp pH optimum for reductase activity at 4.3. 3) DHFR from R50 cells exhibited wild-type affinity for several hydrophilic and lipophilic antibiotics. Consistently, R50 cells efficiently bound F-MTX. 4) The enzyme from R50 cells had a similar Michaelis constant for reductase activity at 4.3. 5) Two-dimensional gel electrophoresis of metabolically labeled DHFR molecules from R50 and B11 0.5 cells (relative to their parental counterparts) were comparable (145- and 104-fold, respectively). 4) Two-dimensional gel electrophoresis of metabolically labeled cellular proteins revealed a predominant and consistent overproduction of DHFR in R50 and B11 0.5 cells, as compared with parental cells. 5) The -fold increase of the mean saturation levels of F-MTX labeling in R50 and B11 0.5 cells raised the possibility that additional mechanisms of MTX resistance may exist in R50 cells. The various analyses comparing R50 and B11 0.5 and their wild-type cells suggest that the resistance to MTX in R50 cells is multifactorial, including overproduction of DHFR, reduced transport of MTX, and possible alteration in MTX polyglutamylation. Overproduction of comparable DHFR levels in R50 and B11 0.5 cells is supported by the following findings. 1) R50 and B11 0.5 cells contained a significant increase in DHFR gene copy number as compared with their parental cells. 2) Steady-state levels of DHFR mRNA were consistently elevated in R50 and B11 0.5 cells. 3) The increased numbers of catalytically active DHFR molecules in R50 and B11 0.5 cells (relative to their parental cells) were comparable (145- and 104-fold, respectively). 4) Two-dimensional gel electrophoresis of metabolically labeled cellular proteins revealed a predominant and consistent overproduction of DHFR in R50 and B11 0.5 cells, as compared with parental cells. 5) The -fold increase of the mean saturation levels of F-MTX labeling in R50 and B11 0.5 cells raised the possibility that additional mechanisms of MTX resistance may exist in R50 cells. The various analyses comparing R50 and B11 0.5 and their wild-type cells suggest that the resistance to MTX in R50 cells is multifactorial, including overproduction of DHFR, reduced transport of MTX, and possible alteration in MTX polyglutamylation. Overproduction of comparable DHFR levels in R50 and B11 0.5 cells is supported by the following findings. 1) R50 and B11 0.5 cells contained a significant increase in DHFR gene copy number as compared with their parental cells. 2) Steady-state levels of DHFR mRNA were consistently elevated in R50 and B11 0.5 cells. 3) The increased numbers of catalytically active DHFR molecules in R50 and B11 0.5 cells (relative to their parental cells) were comparable (145- and 104-fold, respectively). 4) Two-dimensional gel electrophoresis of metabolically labeled cellular proteins revealed a predominant and consistent overproduction of DHFR in R50 and B11 0.5 cells, as compared with parental cells. 5) The -fold increase of the mean saturation levels of F-MTX labeling in R50 and B11 0.5 cells raised the possibility that additional mechanisms of MTX resistance may exist in R50 cells. The various analyses comparing R50 and B11 0.5 and their wild-type cells suggest that the resistance to MTX in R50 cells is multifactorial, including overproduction of DHFR, reduced transport of MTX, and possible alteration in MTX polyglutamylation.

**DISCUSSION**

The intracellular DHFR content in 3T6 R50 cells that were made resistant by progressively increasing MTX concentrations in multiple steps up to 50 μM was found to account for 1.3% of total soluble protein in exponentially growing R50 cells (Kellem et al., 1979). This high degree of target enzyme overexpression would seem to provide the basis for the antifolate resistance of this cell line. However, the initial observation that 3T6 R50 cells and another MTX-resistant rodent cell line, CHO B11 0.5, stained similarly with F-MTX (see below), and yet surprisingly, that R50 cells could grow in medium containing 100-fold more MTX than did B11 0.5 cells raised the feasibility that additional mechanisms of MTX resistance may exist in R50 cells. The various analyses comparing R50 and B11 0.5 and their wild-type cells suggest that the resistance to MTX in R50 cells is multifactorial, including overproduction of DHFR, reduced transport of MTX, and possible alteration in MTX polyglutamylation. Overproduction of comparable DHFR levels in R50 and B11 0.5 cells is supported by the following findings. 1) R50 and B11 0.5 cells contained a significant increase in DHFR gene copy number as compared with their parental cells. 2) Steady-state levels of DHFR mRNA were consistently elevated in R50 and B11 0.5 cells. 3) The increased numbers of catalytically active DHFR molecules in R50 and B11 0.5 cells (relative to their parental cells) were comparable (145- and 104-fold, respectively). 4) Two-dimensional gel electrophoresis of metabolically labeled cellular proteins revealed a predominant and consistent overproduction of DHFR in R50 and B11 0.5 cells, as compared with parental cells. 5) The -fold increase of the mean saturation levels of F-MTX labeling in R50 and B11 0.5 cells raised the possibility that additional mechanisms of MTX resistance may exist in R50 cells. The various analyses comparing R50 and B11 0.5 and their wild-type cells suggest that the resistance to MTX in R50 cells is multifactorial, including overproduction of DHFR, reduced transport of MTX, and possible alteration in MTX polyglutamylation.
with the abundance of DHFR activity may shift the equilibrium of folate and dihydrofolate reduction toward the formation of increased levels of tetrahydrofolate. Consequently, FPGS may be presented with excess tetrahydrofolate concentrations, thus efficiently competing on polyglutamation of any MTX available for FPGS. 3) Another possible explanation for the lack of detectable MTX polyglutamates in R50 cells may be the potential presence of elevated levels of a hydrolytic activity that could cleave any MTX polyglutamates formed. The overproduction of such an enzymatic activity could be the result of a co-amplification process of a gene located near the DHFR gene that has undergone a prominent gene amplification process in R50 cells.

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