Multifactorial Resistance to 5,10-Dideazatetrahydrofolic Acid in Cell Lines Derived from Human Lymphoblastic Leukemia CCRF-CEM

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ABSTRACT

5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) is a potent antiproliferative agent in cell culture systems and in vivo in a number of murine and human xenograft tumors. In contrast to classical antifolates, which are dihydrofolate reductase inhibitors, DDATHF primarily inhibits GAR transformylase, the first folate-dependent enzyme along the pathway of de novo purine biosynthesis. The (6R) diastereomer of DDATHF (Lometrexol), currently undergoing clinical investigation, was used to develop CCRF-CEM human leukemia sublines resistant to increasing concentrations of the drug. Three cell lines were selected for ability to grow in medium containing 0.1 µM, 1.0 µM, and 10 µM of (6R)DDATHF, respectively. Impaired polyglutamylation was identified as a common mechanism of resistance in all three cell lines. A progressive decrease in the level of polyglutamylation was associated with diminished folylpolyglutamate synthetase activity and paralleled increasing levels of resistance to the drug. However, the expression of folylpolyglutamate synthetase RNA was not altered in the resistant cell lines compared to the parent cells. The most resistant cell subline also displayed an increased activity of y-glutamyl hydrolase.

INTRODUCTION

DDATHF is the first representative of a new class of antifolates, the dezatetrahydrofols, which have targets other than dihydrofolic acid reductase (1). DDATHF is a potent antiproliferative agent both in vitro on tumor cell lines or in murine models in vivo (2, 3). Deka-tetrahydrofols block de novo purine synthesis by inhibiting GAR and possibly AICAR transformylases (2, 4). This effect is translated into a rapid drop of intracellular ATP and GTP pools, but can be reversibly by the addition of a preformed purine such as hypoxanthine (5). DDATHF shares several properties with natural folates and antifolate analogues such as transport through the reduced folate transport system (6), and metabolism to polyglutamate forms by the enzyme folylpolyglutamate synthetase (7). There are two diastereomeric forms of DDATHF, (6R) and (6S). The (6R) diastereomer (Lometrexol), which is the focus of the present study, is currently undergoing clinical trials.

In this report we describe the development of CCRF-CEM human lymphoblastic leukemia sublines resistant to increasing concentrations of (6R)DDATHF. We define the mechanisms of resistance and investigate possible cross-resistance with other antifolates and analogues of DDATHF.

MATERIALS AND METHODS

(6R)DDATHF (Lometrexol), (6S)DDATHF, and 5-DATHF were kindly provided by Dr. Chuan Shih, Lilly Research Laboratories, Indianapolis, IN. DDATHF (glu) (n = 3, 5, and 7 indicating the total number of glutamate residues) were prepared by chemical synthesis in our laboratory as described previously. Methotrexate and trimetrexate were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD.

Horse serum, medium, and antibiotics were purchased from Gibco (Grand Island, NY). All other chemicals were from Sigma (St. Louis, MO).

Cell Lines. The CCRF-CEM human lymphoblastic leukemia parent cell line (8) and the resistant sublines, R17, R16, and R15, obtained after continuous exposure to increasing concentrations of (6R)DDATHF, were routinely cultured in RPMI 1640 supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a 5% CO2 atmosphere. All cell lines were tested for Mycoplasma using the Gen-Probe (San Diego, CA) method and were found consistently free.

Drug Treatments. Exponentially growing cells were treated with the indicated compounds and counted 72 h later using a Model B Coulter counter (Coulter Electronics, Hialeah, FL). For shorter exposures, cells were incubated for 4 or 24 h in the presence of the drug, washed, and then resuspended in drug-free medium and counted after a total of 72 h.

ED50 values were determined by plotting cell growth versus inhibitor concentration and interpolating to 50% inhibition.

Extract Preparation for GAR and AICAR Transformylases. Cells at a density of 3 × 10⁸/ml were harvested by centrifugation at 1000 x g for 10 min, washed twice with HBSS, and resuspended in 50 mM sodium phosphate (pH 6.8) containing 25% glycerol with 1 mM . Cells were disrupted by sonication (three times for 10 s; power level 3 at 4°C; Branson), and the extract centrifuged at 37,000 × g for 1 h at 4°C.

GAR Transformylase Assay and Purification. The cell extract was applied to a 10-formyl-5,8-dideazafolate-Sepharose column (9, 10). The column was washed initially with 50 mM sodium phosphate with 25% glycerol (pH 6.8) in the presence of 1 mM phenylmethylsulfonyl fluoride and then with 2 mM NaCl, 50 mM sodium phosphate (pH 7.4), and 25% glycerol. GAR-Tase was eluted with 2 mM urea, 50 mM sodium phosphate (pH 7.4), and 25% glycerol, dialyzed against 25 mM sodium phosphate (pH 7.4) and 25% glycerol, and then concentrated using Sephadex G200. The purification achieved was about 250-fold with a 30–35% yield.

Enzyme activity was determined by a spectrophotometric assay with 10-formyl-5,8-dideazafolate as a formyl donor. A solution of 0.25 mM GAR, 0.01 mM 10-formyl-5,8-dideazafolate in 50 mM potassium phosphate with 25% glycerol (pH 7.5) was incubated for 10 min at 37°C. After enzyme addition the assay was monitored by following the absorbance at 295 nm. The activity of GAR transformylase dramatically decreased with increased cell density, reaching a minimal value at a cell density above 5 × 10⁵ cells/ml.
AICAR Transformylase Assay. The enzyme from both sensitive and resistant cells was assayed using the substrate 10-formyltetrahydrofolate at a concentration of 0.1 mM. Assays were carried out in the presence of 32.5 mM Tris buffer (pH 7.4), 25 mM KCl, 5 mM 2-mercaptoethanol at 25°C under nitrogen. AICAR (0.05 mM) was added, and the formation of tetrahydrofolate at 298 nm was monitored.

Crude Enzyme Preparation for Other Assays. Cells were harvested at midlogarithmic growth and suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl, and 2 mM DTT. Crude extract was obtained by freeze-thawing the cells three times, followed by centrifugation at 16,000 × g for 30 min.

Dihydrofolate Reductase. Dihydrofolate reductase activity was determined spectrophotometrically, using a Gilford Model 2400 spectrophotometer, monitoring the decrease in absorbance at 340 nm that occurs when NADPH and dihydrofolate are converted to NADP and tetrahydrofolate, respectively (12).

Thymidylate Synthase. Thymidylate synthase was determined using the procedure described by Roberts (13). The reaction mixture, in a final volume of 50 μl, contained 1.1 μM [5-3H]UMP, 1 mM tetrahydrofolinic acid, 0.9 mM formaldehyde, 60 mM 2-mercaptoethanol, 25 mM NaF and a range of dilution of the crude extract. The mixture was incubated for 30 min at 37°C, and the reaction was terminated by the addition of 200 μl of a charcoal (Norit) suspension in 5% trichloroacetic acid. The suspension was centrifuged at 10,000 × g for 5 min, and 100 μl of supernatant was counted for radioactivity.

10-Methyltetrahydrofolate Reductase. 10-Methyltetrahydrofolate reductase was assayed as described by Kutzbach et al. (14). The reaction mixture contained 100 μl of crude extract, 2 μM flavin adenine dinucleotide, 160 mM NADPH, 20 mM 2-mercaptoethanol, 1 mM formaldehyde and 1 mM tetrahydrofolinic acid, in potassium phosphate (pH 7.2). The mixture was preincubated for 5 min, and the reaction was initiated by the addition of NADPH and incubated at 37°C.

Serine Hydroxymethyltransferase. The enzymatic activity was assayed by Taylor and Weissbach (15). The reaction system contained 0.25 mM [3-14C]-L-serine, 0.25 mM pyridoxal phosphate, and a range of dilution of the crude extract in potassium phosphate (pH 7.4). The reaction mixture was incubated for 15 min at 37°C and terminated by adding 1 mM sodium acetate (pH 4.5), 100 mM formaldehyde, and 400 mM 1,1-dimethyl-4-chloro-3,5-cyclohexanediene. The samples were boiled for 5 min and then cooled in an ice bath before the 1,1-dimethyl-4-chloro-3,5-cyclohexanediene derivative was extracted with 5 ml of toluene at room temperature.

Formyl-tetrahydrofolate Synthetase. The enzyme was assayed according to Bertino et al. (16). The assay mixture contained 10 mM 2-mercaptoethanol, 20 mM MgCl2, 1 mM tetrahydrofolinic acid, 10 mM formaldehyde, and enzyme in Tris-HCl buffer (pH 7.5). The mixture was incubated for 15 min at 37°C, and the reaction was terminated by the addition of 10% trichloroacetic acid. The 5,10-methylenetetrahydrofolate formed after acidification was determined spectrophotometrically at 355 nm.

5,10-Methylene-tetrahydrofolate Dehydrogenase. The enzyme activity was determined by incubating 1 mM tetrahydrofolinic acid, 5 mM formaldehyde, and 10 mM 2-mercaptoethanol in phosphate buffer (pH 7.5). The reaction was initiated by adding 0.6 mM NADP and crude extract, and the mixture was incubated for 15 min at 37°C. Trichloroacetic acid was added to precipitate the proteins and the 5,10-methylenetetrahydrofolate formed was determined spectrophotometrically at 355 nm (17).

Folylpolyglutamate Synthetase. FPGS was assayed using the method of McGuire et al. (18) with minor changes. 1-[3,4,6]-Hglutamic acid was used instead of the 1-[2,3]-Hglutamic acid. Potassium glutamate (50 mM), in addition to the 25 mM 2-mercaptoethanol, was used to stop the reaction at the end of the incubation period. These changes minimized background counts to 75 cpm/ml of column eluant. Activity was verified to be linear with respect to both time and enzyme concentration.

γ-Glutamyl Hydrodrolase. γ-Glutamyl hydrodrolase activity was determined as described by Rhee (19). Cells extract was prepared in 0.1 M Tris- HCl, (pH 7.0) after sonication and centrifugation at 15,000 × g for 20 min. The reaction was conducted at 37°C in the presence of DDAH FP glu, at a final concentration of 100 μM for 1 h. The reaction was stopped by boiling the samples for 3 min, and the resulting metabolites were identified by HPLC as described below.

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Northern Blot Analysis. An Escherichia coli plasmid containing a 2.2-kilobase human FPGS cDNA insert was generously provided by Dr. Barry Shane, University of California at Berkeley. The plasmid was purified using a Qiagen plasmid Purification kit (Qiagen, Inc., Chatsworth, CA). The 2.2-kilobase insert was obtained from the plasmid by digestion with EcoRI and purified with a Geneclean II kit (Bio 101, La Jolla, CA).

CCRF-CEM-sensitive and CCRF-CEM-resistant cells were washed with PBS; the RNA was then extracted with guanidinium thiocyanate followed by centrifugation in cesium chloride solution and phenol-chloroform extraction (20).

RNA (20 or 40 μg) was separated on a 1% agarose-formaldehyde gel and transferred to a Gene Screen nylon membrane. The Northern hybridization was carried out in the presence of heparin and dextran salt for 18 h at 65°C (21). The blots were extensively washed with 3 × SSC containing 0.1% SDS at 42°C. Autoradiograms were made on Kodak XAR-2 film with an intensifying screen at —70°C and quantitated by densitometry.

Analysis of (6R)DDATHF Polyglutamates. CCRF-CEM cells at a density of 1-2 × 10^5 cells/ml were incubated with 10 μM (6R)DDATHF in the presence of 2 μCi/ml of (3H)FDUMP. After 24 h, the cells were washed twice with ice-cold 0.9% saline and counted by centrifugation at 900 × g for 10 min. The cell pellet was suspended in 1 ml of 50 mM sodium phosphate (pH 6.0), containing 200 mM 2-mercaptoethanol, and boiled for 5 min. The cellular debris was removed by centrifugation at 4000 × g for 10 min. DDAH FP glu polyglutamate analysis utilized HPLC reverse-phase ion-pair chromatography. The 5-μm C18 Ultrasphere column (4.6 mm × 25 cm; Rainin, Woburn, MA) was eluted with a linear gradient from 20% methanol:acetone (2:1) to 40% methanol:acetone (2:1) in 100 mM ammonium phosphate and 1.5 mM tetrabutylammonium bromide (pH 6.5) at 1 ml/min. The column effluent was collected in 1 ml fractions. Radioactivity was determined by scintillation counting after the addition of scintillation cocktail (Optifluor; Packard Instrument Co., Downers Grove, IL). The various DDAH FP glu polyglutamates were identified by comparison of their retention volumes with those unlabeled and chemically synthesized, as described previously (7).

Measurement of Intracellular Follates and Glutamate Chain Length. Reduced folates were measured by the ternary complex method, which is based on entrapment of methylenetetrahydrofolate by thymidylate synthase and [(3H)FDUMP (22). For quantitative estimation of individual reduced folates, cells were washed twice with cold PBS and suspended in an extraction buffer that contained 50 mM Tris- HCl, 50 mM sodium ascorbate, and 1 mM EDTA (pH 7.4) giving a final concentration of 2 × 10^7 cells/ml. Cell suspensions were placed in a boiling water bath for 3 min to achieve lysis and centrifuged at 15,000 × g for 10 min at 4°C. The resultant supernatants (25–90 μl) were used to estimate the following reduced folates as previously described: CH2FH4 (23); FH4 (24); FH2 (25); 5-CH2FH4 (26); 10-CHOFH4 (27); and 5-CHOFH4 (28). Polyglutamate chain length distribution of intracellular CH2FH4 and FH4 were measured by electrophoresis of the undenatured ternary complexes and autoradiography (23). Cells (1 × 10^7) were suspended in the above extraction buffer to give an intracellular CH2FH4 and FH4 concentration of 35 nm. Cells were lysed by freezing and thawing 3 times in dry ice/acetone in the presence of 125 nm [(3H)FDUMP. 6.5 mM formaldehyde, and 20 μM thymidylate synthase, to minimize interference caused by hydrolysis of polyglutamates during sample preparation.

Intracellular Folyopolyglutamates by HPLC. Cells were cultured in folic acid-free medium for 48 h and then incubated for 24 h in complete RPMI 1640 in the presence of [(3H)Folic acid at 37°C. The cells were harvested by centrifugation (1000 × g for 5 min), and the cell pellet was washed twice with ice-cold 0.9% saline solution. The folate derivatives were extracted by the addition of boiling 25 mM sodium phosphate (pH 6.0), containing 200 mM 2-mercaptoethanol, and boiled for 2 min. Cellular debris was removed by centrifugation at 10,000 × g for 15 min at 4°C. The polyglutamates were separated using a reversed phase-ion-pair chromatography system, consisting in an Ultrasphere C18 column (25 cm × 4.6 mm) eluted with a gradient of methanol:acetone (2:1, v/v), from 10 to 25%, in 100 mM ammonium acetate with 1.5 mM terti butyl acetate, pH 6.5 (29).

Chemically synthesized fo2polyglutamates [p'teqlu, ...] were added to the radiolabeled samples to provide an internal control. Effluent was collected in 1 ml fractions, and radioactivity was counted as above.

Intracellular Folate Pools by HPLC. An aliquot of the supernatant obtained for the determination of intracellular polyglutamates was treated
with partially purified hog kidney polyglutamate hydrolase. The hydrolysis was performed in the presence of 0.1 M sodium acetate (pH 4.5)—0.1 M glutamic acid for 30 min at 37°C (30). Following the enzymatic treatment the samples were again boiled for 2 min and centrifuged.

The samples were analyzed using a Spherisorb ODS column (25 cm x 4.6 mm) eluted with a gradient of acetonitrile in 0.1 M sodium acetate (pH 5.5) with a flow rate of 2 ml/min (31). Fractions were collected at 30-s intervals, and radioactivity was determined as described above.

**Transport Experiments.** Transport determinations were carried out using a cell suspension (1-3 x 10^9 cells/ml) previously washed with buffer containing 135 mM NaCl, 16 mM NaHCO_3, 4.4 mM KCl, 1.1 mM NaH_2PO_4, 19 mM CaCl_2, and 1 mM MgCl_2 at 0°C and resuspended into the same buffer at 37°C (32). After incubation for the indicated period of time, the samples were processed as described previously (6).

**De Novo Purine Metabolism.** The synthetic activity related to de novo purine biosynthesis was assessed by measurement of the incorporation of [2-14C]glycine into cellular purines. Cells were cultured in complete RPMI 1640 supplemented with 10% dialyzed horse serum and 3 μM thymidine. The incorporation of [2-14C]glycine into ATP and GTP pools was determined after a 2-h exposure to the drug. No substantial morphological or size changes were observed in these mutants by phase contrast light microscopy. Exposure to the drug. No substantial morphological or size changes were observed in these mutants by phase contrast light microscopy.

**RESULTS**

**Development of Resistance to (6R)-DDATHF.** CCRF-CEM-resistant cells were sequentially selected on progressive exposure to increasing concentrations of (6R)-DDATHF over a 1-year period. Three major sublines were derived: R17, R16, and R15, able to proliferate in 0.1 μM, 1.0 μM, and 10 μM (6R)-DDATHF, respectively. In Table 1 the ED_50 values are reported for the different cell lines after a 72-h exposure to the drug. No substantial morphological or size changes were observed in these mutants by phase contrast light microscopy. Similarly, no karyotypic changes were seen on standard metaphase spreads. In drug-free medium, the resistance was stable for more than 1 year. The doubling time was not substantially different from the parent cell line, which was about 20 h.

**Cross-Resistance.** The (6R)-DDATHF-resistant cell lines were tested for cross-resistance with other deazatetrahydrofolates as shown in Table 1. The two analogues, (6S)-DDATHF and 5-DATHF, showed less efficacy on the resistant mutants than on the sensitive cell line. The degree of resistance was similar to (6R)-DDATHF for both the (6S) isomer and 5-DATHF.

The effect of MTX and trimetrexate, antifolates whose inhibitory target is DHFR, was compared in the (6R)-DDATHF-sensitive and (6R)-DDATHF-resistant lines for various drug exposure times (Table 2). No cross-resistance was seen using a 72-h MTX exposure, and a very modest cross-resistance was seen using a 24-h exposure. However, for a 4-h exposure, the 3 cell lines were highly resistant to MTX cytotoxicity. No cross-resistance was found in the case of the lipophilic DHFR inhibitor, trimetrexate.

**DDATHF Polyglutamylmaion.** Both diastereomers of DDATHF have been shown to be excellent substrates for FPGS (36). Previous studies have indicated that the conversion of DDATHF to higher polyglutamates is an essential step in the development of deazafolate antiproliferative activity (7).

CCRF-CEM parent, R17, R16, and R15 cell lines were incubated for 24 h in the presence of 10 μM [3H](6R)-DDATHF, and the cell extract was analyzed for the presence of (6R)-DDATHF metabolites by HPLC, using a reverse-phase ion-pair chromatography. A dramatic progressive decrease in the capacity to accumulate (6R)-DDATHF polyglutamate forms is evident in all the resistant cell lines, and the extent of this effect increases with the degree of resistance (Fig. 1). The R17 and R16 cell lines were also incubated with 0.1 μM and 1 μM DDATHF, respectively. The effect on DDATHF accumulation was even more evident compared to the sensitive cells (Fig. 2). The progressive impairment in polyglutamate accumulation directly correlated with decreased FPGS activity in cell extracts whether (6R)-D- DATHF, aminopterin, or tetrahydrofolate were used as a substrate (Table 3). We also examined the kinetic characteristic of FPGS partially purified from the CCRF-CEM R17 cell line, comparing it to FPGS from the parent line. As reported in Table 4, we observed modestly decreased V_max values for all the substrate used for the resistant enzyme but no significant alteration in the K_m values.

FPGS gene expression was examined by Northern blot analysis in sensitive and resistant CCRF-CEM cell lines. No significant difference in expression was detected between sensitive and resistant cells (Fig. 3).

Since multiple mechanisms of resistance to deazafolates are clearly possible, we investigated additional biochemical parameters that could be indicative of alternate modes of resistance.

**γ-Glutamyl Hydrolase Activity.** The activity of γ-glutamyl hydrolase was investigated in the parent and resistant cell lines using DDATHF(7), with some glu5, as the substrate for the enzyme, after preparation of the cell extracts. The R15 cell line showed a 2–3-fold elevated capacity to cleave the DDATHF polyglutamate substrate compared to the sensitive and the other resistant cell lines (Table 5). We also examined the kinetic characteristic of FPGS partially purified from the CCRF-CEM R17 cell line, comparing it to FPGS from the parent line. As reported in Table 4, we observed modestly decreased V_max values for all the substrate used for the resistant enzyme but no significant alteration in the K_m values.

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**γ-Glutamyl Hydrolase Activity.** The activity of γ-glutamyl hydrolase was investigated in the parent and resistant cell lines using DDATHF(β-glut), with some glu5, as the substrate for the enzyme, after preparation of the cell extracts. The R15 cell line showed a 2–3-fold elevated capacity to cleave the DDATHF polyglutamate substrate compared to the sensitive and the other resistant cell lines (Table 5). We also examined the kinetic characteristic of FPGS partially purified from the CCRF-CEM R17 cell line, comparing it to FPGS from the parent line. As reported in Table 4, we observed modestly decreased V_max values for all the substrate used for the resistant enzyme but no significant alteration in the K_m values.

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Fig. 1. (6R)DDATHF polyglutamates accumulation in sensitive and resistant CCRF-CEM cell lines. Cells were incubated with 10 μM [1H-6R]DDATHF, and after 24 h cells were harvested and washed twice in ice-cold PBS. The HPLC analysis was performed as described in "Materials and Methods." Data represent the average of two determinations of extracts from a constant number of cells.

Enzyme Activities. The CCRF-CEM R16 cell line, representing an intermediate degree of resistance, was studied in regard to levels of folate-requiring enzymes. Compared to the parent cell line, two of the enzymes showed modest alteration in their activity (Table 7). 5,10-Methylenetetrahydrofolate dehydrogenase activity was 3.5-fold higher than in the parent cell line. In the resistant cell line the activity of the primary enzyme target for DDATHF, GAR transformylase, was elevated 2-fold over the parent cell line, at a cell density of 2.5 × 10^5/ml. Kinetic analysis showed only minor alterations in the characteristic of the enzyme purified from the resistant cells, K_m values of 5.6 ± 0.9 μM for GAR and 2.2 ± 0.3 μM for 10-formyl-5,8-dideazatetrahydrofolate for the parent enzyme and 4.9 ± 1.1 μM and

Table 3 Activity of FPGS from parental and resistant cell lines for different folate and antifolate substrates

FPGS activity was assayed using the method of McGuire et al. as described in "Materials and Methods." The values represent the average with SD of at least three experiments conducted in duplicate.

Table 4 Kinetic constants for FPGS from parental and R17 resistant cells using different folate substrates

The assays were performed on partially purified FPGS according to the method of McGuire et al. as described in "Materials and Methods." The values represent the average of three to five experiments.

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5.6 ± 0.8 μM for the CCRF-CEM R16 enzyme. The $K_v$ value for inhibition of GARTase by (6'R)DDATHF was 0.3 μM for the enzyme from the sensitive cells and 0.9 μM for the enzyme from the R16-resistant cells. The activity of GAR transformylase in the CCRF-CEM R17- and CCRF-CEM R15-resistant cell lines was found to be 2.6 and 6.3 nmol/min/mg of protein, respectively.

**Alteration in Purine Anabolism.** The antiproliferative effects of deazatetrahydrofolates result from inhibition of the de novo purine biosynthesis pathway and can be completely ablated by supplying enough exogenous purines to maintain intracellular pools via the salvage pathway. Cells of various types are known to differ in their relative dependence on de novo versus salvage purine synthesis. Mutations are thus conceivable which would shift overall cellular purine economy away from reliance on de novo purine synthesis, decreasing their sensitivity to deazatetrahydrofolates. To investigate this possibility, the extent of [14C]glycine incorporation into intracellular purine nucleotides was compared in the sensitive and R16 resistant line, as shown in Fig. 5. These results showed no significant differences in the base-line degree of [14C]glycine incorporation between the sensitive and resistant lines. Since the sizes of the total intracellular adenine and guanine (nucleotide) pools were essentially unchanged, this indicated similar contribution to intracellular purine pools via de novo pathway. Importantly, however, [14C]glycine incorporation is markedly suppressed by (6'R)DDATHF in the sensitive line but remains unaffected in the R16-resistant cells.

**Transport.** Transport studies with [3H](6'R)DDATHF at 1.0 μM concentration, the approximate transport $K_m$ value in CCRF-CEM cells for this deazatetrahydrofolate (6), in sensitive and resistant cells showed no significant differences and indicated that this parameter was not involved in the resistance to DDATHF (data not shown). The level of folate-binding protein, as indicated by the binding of [3H]folic acid at 25 nM concentration, was also not affected in the resistant cell lines (data not shown).

**DISCUSSION**

In this paper we report the development and characterization of CCRF-CEM sublines with increasing degrees of resistance to (6'R)DDATHF. These cell lines were developed upon prolonged exposures to increasing concentrations of drug. Stable resistant cell lines were developed with resistance to (6'R)DDATHF more than 10,000-fold compared to the parental CCRF-CEM cells.

The three resistant sublines we investigated showed cross-resistance with other deazatetrahydrofolates, such as the diastereomer, (6S)DDATHF and 5-DATHF. Furthermore, we observed cross-resistance to the classical antifolate methotrexate for the shorter exposures to the drug (4 and 24 h). When the resistant sublines were incubated with methotrexate for 72 h or exposed to the lipophilic antifolate trimetrexate, we observed complete sensitivity to these DHFR inhibitors.

This pattern of sensitivity and resistance to classical and nonclassical antifolates confirms several observations that we and others have reported previously (29, 37-38). Polyglutamylation is essential for the intracellular activation of (6'R)DDATHF. Decreased formation of polyglutamate metabolites makes this drug less effective since the polyglutamate forms are more potent inhibitors of the enzyme targets GARTase and AICARTase than the monoglutamate form. This is in contrast to the case of methotrexate, which otherwise has characteristics very similar to (6'R)DDATHF in terms of transport and metabolism by FPGS. Polyglutamylation of methotrexate is more important for intracellular drug retention rather than is increasing its affinity...
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Fig. 4. Glutamate chain length distribution of \( \text{CH}_2\text{FH}_4 \), and \( \text{FH}_4 \) from extracts of CCRF-CEM-sensitive and CCRF-CEM-resistant cells. Lanes 1 and 4: glu2, glu4, and glu6 standards; Lanes 2 and 7, glu3, glu5, and glu7 standards; Lane 3, CCRF-CEM-sensitive cells; Lanes 4, 5, and 6, CCRF-CEM R17, R16, and R15 cells, respectively.

Table 7 Activities of various folate-dependent enzymes in parental and CCRF-CEM R16 cell lines

<table>
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<th>Enzyme</th>
<th>CCRF-CEM</th>
<th>CCRF-CEM R16</th>
<th>R16: parental ratio</th>
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<td>Serine hydroxymethyltransferase</td>
<td>1.65</td>
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The procedure for each enzyme assay is reported in “Materials and Methods.” The values indicate the average of two to six experiments.

toward its primary target DHFR. Thus, cross-resistance to methotrexate is seen for shorter drug exposures where accumulation of polyglutamate forms becomes critical for subsequent intracellular drug retention. At longer drug exposures, intracellular drug levels can be maintained through continuous influx, and the polyglutamylation defect in the resistant cells has much less influence (39).

The main mechanism of resistance common to the three different cell sublines studied appear to be a progressive impairment in the ability to accumulate polyglutamate metabolites of the drug. This phenomenon directly correlated with diminished FPGS activity in vitro. The decreased activity of FPGS was apparent not only when (6R)DDATHF was used as a substrate but also when aminopterin or tetrahydrofolate were utilized, indicating a generalized decrease in activity rather than an altered affinity specifically for (6R)DDATHF.

The analysis of \( \gamma \)-glutamyl hydrolase activity revealed an increased catabolic activity only in the R15 subline. This observation correlates with the almost total absence of DDATHF polyglutamates accumulated and with the substantial presence of a reduced folate monogluta- mate form. These findings represent another example of this mechanism of resistance recently described in a H35 hepatoma cell line resistant to DDATHF (19).

Impaired polyglutamylation as a mechanism of resistance to methotrexate has been described previously in CCRF-CEM cells (29). It might be argued that this cell line is particularly susceptible to development of this mechanism of resistance. However, numerous and varied attempts to develop other cell lines resistant to (6R)D- DATHF resulted only in sublines with diminished capacity to accumulate polyglutamates. In addition to CCRF-CEM cells, experiments with HL-60 and K562 cells (data not shown), found defective polyglutamylation as the only major mechanism of resistance among the several determinants we investigated. Subsequently, in an attempt to obtain more highly resistant cells from the CCRF-CEM R15 described here, a cell subline with both an impaired polyglutamylation and an impaired transport system for reduced folates was selected (40).

It was possible to isolate and kinetically characterize FPGS from the R17 cell line. No major differences in the kinetic parameters were found. This of itself would suggest decreased expression of a normal FPGS protein. However, measurement of FPGS mRNA levels showed no significant differences in FPGS message expression between sensitive and resistant cells, suggesting a functionally impaired FPGS. Until it becomes possible to measure the amount of the FPGS protein independent of activity, such as by immunoreactivity, these possibil-

Fig. 5. Incorporation of \([2-^14\text{C}]\)glycine in CCRF-CEM and CCRF-CEM R16 cell lines. Cells treated with 10 \( \mu \text{M} \) (6R)DDATHF and untreated were incubated with \([2-^14\text{C}]\)glycine for 2 h. The incorporation of \([2-^14\text{C}]\)glycine in ATP and GTP pools was determined after HPLC separation as described in “Materials and Methods.” The values represent the average of two separated experiments.

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ities cannot be readily distinguished. It must be born in mind that all the cell lines, parental and resistant, are tetraploid, thus the cells possess a minimum of four FPGS genes. Mutations resulting in dysfunctional proteins could occur in one or more of these, leaving a diminished amount of functionally normal FPGS with apparently normal levels of FPGS mRNA. This possibility is being further investigated.

A number of other possible mechanisms of resistance were investigated because multimodal resistance is a likely possibility. Among these, alteration in drug influx via the reduced folate transport system was ruled out in the studies described here. We have, however, described transport defects as the basis for cross-resistance to deazatetrahydrofolates in cells selected for resistance to methotrexate (7). Moreover, transport-based resistance did ultimately develop as a superimposed alteration in our polyglutamylation deficient lines upon further prolonged exposure to (6R)DdATF. The results of these limited cell culture experiments should not, however, be interpreted as an indication of how frequently transport versus polyglutamylation defects might occur as modes of clinical resistance to deazatetrahydrofolates. We have also determined previously that deazatetrahydrofolates may gain entry to the cells via the folate-binding protein receptor system. We did not detect differences in the levels of this receptor between the sensitive and resistant cell lines.

Major alterations in overall dependence on the de novo rather than the salvage pathway for the maintenance of intracellular purine nucleotide pools seem unlikely in view of the lack of changes in operation of the de novo pathway as measured by $[14C]$glycine uptake.

Our inability to generate resistant mutants which overproduce GARTase, the primary target of deazatetrahydrofolates, is somewhat surprising in view of the frequency with which this mode of resistance develops to DHFR inhibitors (41–45). At most only 2-fold increases in levels of GARTase were detected. Whether this finding is fortuitous or represents an inherent lack of susceptibility toward amplification for the GARTase gene is unknown. It may be relevant, however, that the GARTase gene is likely to be considerably larger than that for DHFR as the former also encodes two other activities of the de novo purine pathway, GAR synthetase and AIR synthetase. In passing, it was noted that the levels of GARTase varied with the cell culture density, being lowest during the stationary phase. This suggests some degree of cell cycle dependence for the expression of GARTase activity.

Our finding that the elevation of reduced folate pools in R17 and R16 lines but not in the more highly resistant R15 subline is perplexing and unexplained at this time. The distribution of polyglutamate chain lengths appears to be shifted toward shorter chain polyglutamates in the resistant line which is consistent with the decreased FPGS activity in these cells. Further, the increase in 10-formyl-tetrahydrofolate, the natural substrate for both GARTase and AICAR-Tase, that occurs in conjunction with the increase in total folates in R17 and R16 cells could contribute to deazatetrahydrofolate resistance. The observation of increased 10-formyl-tetrahydrofolate is consistent with the finding of a 3.5-fold increase in 5,10-methylenetetrahydrofolate dehydrogenase activity, as this enzyme represents one of two major routes to 10-formyl-tetrahydrofolate. Of note is the fact that we did not observe a concomitant increase in 10-formyl-tetrahydrofolate synthetase activity, although both activities are present in mammalian cells along with cyclohydrolase on a single trifunctional protein.

In summary, the primary mechanism of deazatetrahydrofolate resistance detected in these studies appears to be the significantly diminished accumulation of polyglutamate forms of the drug. The basis for this decreased accumulation is mainly a decrease in FPGS activity, although additional effects from altered reduced folate pools and elevated γ-glutamyl hydrolase may also contribute. These results further underscore the importance of deazatetrahydrofolate polyglutamates as active metabolites and indicate that their presence is essential for the action of these drugs.

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Multifactorial Resistance to 5,10-Dideazatetrahydrofolic Acid in Cell Lines Derived from Human Lymphoblastic Leukemia CCRF-CEM

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