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A Structurally Altered Human Reduced Folate Carrier with Increased Folic Acid Transport Mediates a Novel Mechanism of Antifolate Resistance*

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CEM/MTX is a subline of human CCRF-CEM leukemia cells which displays >200-fold resistance to methotrexate (MTX) due to defective transport via the reduced folate carrier (RFC). CEM/MTX-low folate (LF) cells, derived by a gradual deprivation of folic acid from 2.3 μM to 2 nM (LF) in the cell culture medium of CEM/MTX cells, resulted in a >20-fold overexpression of a structurally altered RFC featuring; 1) a wild type K_m value for MTX transport but a 31-fold and 9-fold lower K_m values for folic acid and leucovorin, respectively, relative to wild type RFC; 2) a 10-fold RFC1 gene amplification along with a >20-fold increased expression of the main 3.1-kilobase RFC1 mRNA; 3) a marked stimulation of MTX transport by anions (*i.e.* chloride); and 4) a G → A mutation at nucleotide 227 of the RFC cDNA in both CEM/MTX-LF and CEM/MTX, resulting in a lysine for glutamate substitution at amino acid residue 45 predicted to reside within the first transmembrane domain of the human RFC. Upon transfer of CEM/MTX-LF cells to folate-replete medium (2.3 μM folic acid), the more efficient folic acid uptake in CEM/MTX-LF cells resulted in a 7- and 24-fold elevated total folate pool compared with CEM and CEM/MTX cells, respectively (500 *versus* 69 and 21 pmol/mg of protein, respectively). This markedly elevated intracellular folate pool conferred a novel mechanism of resistance to polyglutamatable (*e.g.* ZD1694, DDATHF, and AG2034) and lipophilic antifolates (*e.g.* trimetrexate and pyrimethamine) by abolishing their polyglutamylation and circumventing target enzyme inhibition.

The reduced folate carrier (RFC)[1] has long been recognized for its essential role in mediating the cellular uptake of reduced folate cofactors required for several biosynthetic processes (1–4). Furthermore, RFC-mediated transport is also known to be essential for the cytotoxic activity of folate-based chemotherapeutic drugs such as methotrexate (MTX) (5–7). One of the characteristic transport kinetic properties of the RFC is its poor affinity for folic acid (K_m 200–400 μM) as compared with reduced folate cofactors and MTX (K_m 1–10 μM) (3, 4, 8–10). Human RFC is a transmembrane glycoprotein that undergoes a prominent glycosylation resulting in an apparent molecular mass of 80–120 kDa (11–13).

Insight into the molecular aspects of RFC was obtained from recent studies reporting the isolation of rodent and human cDNA clones encoding for a protein, RFC1, that is involved in the uptake of folates and antifolates (13, 14–17). Correlates between RFC1 and RFC function have been implicated from observations that transfection with RFC1 cDNA could restore MTX uptake and drug sensitivity in RFC transport-defective murine, hamster, and human cells (13, 14–18). Moreover, RFC1 gene amplification and consequent mRNA overexpression has been demonstrated in human CEM-7A leukemia and K562.4CF cells, which overexpressed RFC protein following gradual adaptation to low extracellular concentrations of leucovorin (11, 13, 19–21).

Defective transport via the RFC as a result of qualitative and/or quantitative defects has been recognized as a common and frequent mechanism of antifolate resistance (4, 6, 7, 22–24). The biochemical and molecular basis of RFC transport defects is starting to be recently unraveled (11, 12, 16, 24–28) and includes structural alterations of the RFC protein (11, 13), additional membrane protein changes along with a translocation defect of the RFC (29, 30). In the rodent RFC gene a number of different mutations have been reported which resulted in single amino acid substitutions in either the first (26, 67), fourth (27), or the loop between the 7th and 8th predicted transmembrane domains of the RFC protein (28). These mutations resulted in a marked defect in MTX transport (26, 27, 67). Interestingly, however, a serine to asparagine substitution at amino acid 46 (26) and a glutamate to lysine substitution at amino acid 45 of the mouse RFC gene (67) selectively enhanced transport of natural folates (leucovorin, folic acid) over folate

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[1] The abbreviations used are: RFC, reduced folate carrier; DHFR, dihydrofolate reductase; DHF, dihydrofolate; CH_2THF, 5,10-methylenetetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; MTX, methotrexate; EDX, edatrexate; TMQ, trimetrexate; PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-(hemiphtaloyl-L-ornithine); GW1843, (S)-2(5-(((1, 2-dihydro-3-methyl-1-oxobenzo(*f*)quinazolin-9-yl)-methyl)-amino) 1-oxo-2-isoindolinyl)-glutaric acid; ZD9331, (2*S*)-2-{*O*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino] benzamido}-4-(tetrazol-5-yl) butyric acid; DDATHF, 5,10-dideza-5,6,7,8,-tetrahydrofolic acid; AG2034, 4-[2-(2-amino-4-oxo-4,6,7,8-tetrahydro-3*H*-pyrimido[5,4-6]thiazin-6-yl)-(*S*)-ethyl-2,5-thienoylamino-L-glutamic acid; HBSS, Hepes-buffered saline solution; FdUMP, 5-fluoro-2′-deoxyruidine 5′-monophosphate; PCR, polymerase chain reaction; THF, tetrahydrofolate; LF, low folate; FCS, fetal calf serum; HPLC, high performance liquid chromatography.

analogues such as MTX. In contrast and thus far in the human RFC gene, only N-terminal premature translation termination mutations, which disrupt RFC protein expression, were reported (25).

This study is a first report on the identification of a mutation in the human RFC gene that results in marked changes in the kinetic properties of RFC-mediated transport of (anti)folates. Upon growth in low folate (LF) medium, CEM/MTX cells expressed high levels of a structurally altered RFC that is functionally characterized by a 9- and 31-fold increased affinity (decreased K_m) for transport of reduced folate cofactors and folic acid, respectively. In folate-replete medium, CEM/MTX-LF cells can accumulate intracellular folates to such a high level that it confers resistance to lipophilic antifolate inhibitors of dihydrofolate reductase and to some polyglutamatable antifolates due to an abolishment of polyglutamylation.

EXPERIMENTAL PROCEDURES

Chemicals

RPMI 1640 medium, with or without folic acid, and (dialyzed) fetal calf serum were obtained from Life Technologies, Inc. (6*S*)-5-Formyltetrahydrofolate and (6*S*)-5-methyltetrahydrofolate were a generous gift from Eprova AG, Schaffhausen, Switzerland. Folic acid was purchased from Sigma. MTX-diglutamate (MTX-Glu_2) was obtained from Schircks Chem. Co., Jona, Switzerland. The unlabeled antifolate drugs used were gifts from the following sources: MTX (Pharmachemie, Haarlem, The Netherlands); edatrexate (31), Ciba Geigy (Basel, Switzerland); trimetrexate (32), Parke-Davis; PT523 (33), Dr. W. T. McCulloch (Sparta Pharmaceuticals, Research Triangle Park, NC); GW1843U89 (34), Dr. R. Ferone (Glaxo Welcome); ZD1694 (35) and ZD9331 (36), Dr. A. Jackman (Institute of Cancer Research, Sutton, UK); DDATHF (37), (Lilly); AG2034 (38), Dr. R. C. Jackson (Agouron Pharmaceuticals, San Diego, CA); and pyrimethamine (39), Sigma.

The radiochemicals [3′,5,′7′-^{3}H]MTX (23.4 Ci/mmol), [3′,5′,7,9-^{3}H]-(6**S**)leucovorin (50 Ci/mmol), [3′,5′,7,9-^{3}H]folic acid (25.5 Ci/mmol), [^{3}H]FdUMP (20 Ci/mmol), and [5-^{3}H]-2′-deoxycytidine (28.5 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA. [3′5′,7,9-^{3}H]edatrexate (15 Ci/mmol) was prepared as a special customer synthesis (40, 41). All labeled antifolates were purified prior to use by thin layer chromatography as described previously (42). [2,3-^{3}H]glutamic acid (22 Ci/mmol) (0.01 N HCl formulation) was obtained from NEN Life Science Products, The Netherlands.

The monoclonal antibody MOv18, directed against an epitope of membrane folate receptors, was a obtained from Dr. S. O. Warnaar, Centocor, Inc. Leiden, The Netherlands. All other chemicals were of the highest purity available.

Cell Lines

Human CCRF-CEM leukemia cells and a variant CEM-7A subline, exhibiting 95-fold increased V_{max} for MTX influx along with a 30-fold overexpression of RFC protein, were cultured as described previously (12, 20). CEM/MTX cells were originally isolated by Rosowsky *et al.* (43) by stepwise selection for MTX resistance and displayed a 200-fold resistance to MTX due to defective transport via the RFC (5, 22). CEM/MTX cells are maintained in folate-replete RPMI 1640 medium, supplemented with 10% FCS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin, in the presence of 1 μM MTX.

Selection of CEM/MTX-LF Cells

CEM/MTX cells were transferred to folate-free medium supplemented with 10% dialyzed FCS in which the folic acid concentration was gradually deprived from 2.3 μM to 2 nM over a 5-month period. In addition, 50 nM MTX (5-fold the IC_{50} of wild type CCRF-CEM cells) were also present during the folate deprivation of CEM/MTX cells to prevent the emergence of possible revertants of wild type CCRF-CEM cells. The CEM/MTX cells selected to grow at 2 nM folic acid were cloned and will be further designated CEM/MTX-LF (for "low folate").

Growth Inhibition Experiments

One ml of CCRF-CEM, CEM/MTX, and CEM/MTX-LF cells were plated at an initial density of 1.25×10^5/ml in the individual wells of a 24-well tissue culture plate containing a 2-log range of antifolate drug concentrations. Following 72 h of continuous drug exposure, viability cell counts were determined with a hemocytometer and trypan blue exclusion. IC_{50} values are defined as drug concentrations at which cell growth is inhibited by 50% compared with untreated controls (5).

Enzyme Assays

All enzyme assays were carried out on cells collected in their exponential growth phase. Cells were washed three times with a HEPES-buffered saline solution (HBSS) containing 107 mM NaCl, 20 mM HEPES, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 7.0 mM D-glucose, adjusted to pH 7.4 with NaOH. The final cell pellets were either used immediately or stored at −80 °C.

Folylpolyglutamate synthetase activity was determined according to a procedure as described previously (44). Two hundred micrograms of protein extract were assayed per experiment using 250 μM MTX as a substrate for the folylpolyglutamate synthetase reaction.

Folylpolyglutamate hydrolase activity was measured essentially as described by O'Connor *et al.* (45). Twenty five micrograms of cellular protein extract and 100 μM MTX-Glu_2 were used as substrate for the folylpolyglutamate hydrolase reaction assay. The assay pH was 6.9. The reaction products were quantitated by HPLC (8, 41).

Thymidylate synthase catalytic activity and the number of FdUMP binding sites was determined as described by Van der Wilt *et al.* (46). Dihydrofolate reductase (DHFR) levels were quantitated in a [^{3}H]MTX binding assay as described by Schuetz *et al.* (29).

Transport Studies

(Anti)folate transport studies were carried out with (variant) CEM cells in the mid-log phase of growth. CCRF-CEM, CEM/MTX, and CEM/MTX-LF cells were washed and resuspended in HBSS transport buffer (20) to a final density of 1.5×10^7 cells/ml; for CEM-7A cells a density of 3×10^6 cells/ml was used. Incubation times varied from 1.5 min (for CEM-7A cells) to 3 min (for other cells) at extracellular concentrations of radiolabeled folate (analogues) ranging from 0.25 to 250 μM. Transport of radiolabeled folate (analogues) was stopped by the addition of 10 ml of ice-cold transport buffer, after which the cell suspension was centrifuged ($500 \times g$, 5 min, 4 °C) and the cell pellet was washed once more with 10 ml of ice-cold transport buffer. The final cell pellet was suspended in water and processed for radioactivity counting.

Affinity Labeling

Quantitation of folate transporter level, using 100 nM of an *N*-hydroxysuccinimide ester of [^{3}H]MTX or [^{3}H]folic acid, followed by SDS-polyacrylamide gel electrophoresis analysis of labeled protein, was carried out as described previously (20, 42).

Membrane Folate Receptor Expression Analysis by MOv18 Flow Cytometric Analysis

Flow cytometric analysis for the cellular expression of membrane folate receptors by a monoclonal antibody directed to membrane folate receptors (MOv18) was carried out as described previously (47, 48).

Analysis of MTX/EDX Polyglutamate Formation

CCRF-CEM cells and CEM/MTX-LF cells, maintained either in folate-deplete (2 nM folic acid) and folate-replete (2.3 μM folic acid) medium, were incubated in 25-cm^2 tissue culture flasks (10 ml of cells, density 1×10^6/ml) in the presence of 1 μM [^{3}H]MTX or 0.5 μM [^{3}H]EDX (specific activity, 1,000 dpm/pmol). After 24 h of incubation, cells were washed and analyzed for polyglutamate forms of [^{3}H]MTX and [^{3}H]EDX by HPLC as described previously (8, 40, 41).

Determination of RFC1 Gene Copy Number and mRNA Levels

High molecular weight genomic DNA was extracted from CCRF-CEM, CEM-7A, CEM/MTX, and CEM/MTX-LF cells maintained in folate-deplete and folated-replete media. DNA was digested with *Eco*RI, *Hin*dIII, *Pst*I, and *Bam*HI, fractionated by electrophoresis on 0.8% agarose gels, transferred to a Zetaprobe (Bio-Rad) nylon membrane, and UV cross-linked. RFC1 gene copy number was determined by Southern blot analysis using a ^{32}P-labeled (49) human RFC1 cDNA probe isolated from a cDNA library from CEM-7A cells. Poly(A)$^+$ RNA was size-fractionated on 1.5% agarose/formaldehyde gels, blotted onto a Gene Screen$^+$ (NEN Life Science Products) nylon membrane and UV cross-linked. Human RFC1, β-actin (50), and MDR1 cDNAs (51) were labeled by random hexamer priming (49). Southern and Northern blot hybridizations and post-hybridization washes were carried out under high stringency conditions as described previously (23, 52). Southern

and Northern blots were quantitated with a BAS 1000 Bio Imaging Analyzer (Fujix).

Genomic and cDNA Screening of Mutations and Sequencing

In order to identify the putative alteration(s) present in the RFC1 gene in CEM/MTX and CEM/MTX-LF cells we have undertaken several experimental approaches.

Construction of a cDNA Library from CEM/MTX Cells and Screening of RFC cDNA Clones—A cDNA library was constructed from CEM/MTX poly(A)$^+$ mRNA (2 μg) as described previously (52) and screened with a ^{32}P-oligo-labeled human RFC cDNA probe. Several clones were isolated of which a full-length cDNA clone was fully sequenced thus revealing only a single G to A substitution at nucleotide position 227 (based on the nucleotide numbering of Prasad *et al.* (15), GenBank™ accession no. UI15939).

Reverse Transcriptase-Polymerase Chain Reaction (PCR) of the Entire RFC Coding Region—1-μg aliquots of total RNA isolated from parental CEM, CEM/MTX, and CEM/MTX-LF cells were reverse transcribed using Super-Script II reverse transcriptase (Life Technologies, Inc.). Subsequently a PCR-based amplification of total cDNA was performed using the high fidelity *Taq* polymerase according to the manufacturer's protocol (Boehringer Mannheim); this was performed using primers previously described by Gong *et al.* (25). The reverse transcriptase-PCR cDNA products were cloned into the pGEM-T Easy vector system (Promega), and several clones from each cell line were fully sequenced (see Table III under "Results").

Genomic PCR-Single Strand Conformational Polymorphism and Isolation of Multiple Clones—High molecular weight genomic DNA from parental CEM, CEM/MTX and CEM/MTX-LF cells was amplified with *Taq* polymerase in 1xPCR buffer supplied by the manufacturer (Promega) in the presence of [α-^{32}P]dATP. PCR was performed using the conditions described by Gong *et al.* (25); the downstream primers for exon 2 and exon 3 were those previously described (25), whereas the upstream primers were various 5′-intronic-based oligonucleotides. Aliquots of the PCR products were resolved on nondenaturing polyacrylamide gels using the single strand conformational polymorphism analysis as described previously (53, 54). Following thymidylate synthase screening for changes in single strand DNA fragment mobility, the PCR products were cloned into the pGEM-T Easy vector system (Promega) after which a large number of wild type and altered clones were sequenced (see Table III under "Results").

Genomic PCR and Direct Sequencing—We have also undertaken a direct sequencing approach of PCR-amplified genomic DNAs from CEM, CEM/MTX, and CEM/MTX-LF cells. This PCR was performed with nonradioactive deoxynucleotides triphosphate. DNA sequence analysis of multiple genomic PCR products, genomic PCR clones and cDNA clones was performed by the fluorescent dideoxy chain termination method using ABI 373A and 377 sequencers (DNA Sequencing Unit, The Weizmann Institute, Rehovot, Israel).

Determination of Intracellular Folate Pools

Measurement of reduced folates was made by a series of assays that are based on enzymatic cycling of reduced folates to N^5,N^{10}-methylenetetrahydrofolate (CH_2THF) followed by entrapment into a stable ternary complex with excess thymidylate synthase and [^{3}H]FdUMP (20 Ci/mmol) (55, 56). Frozen cell pellets were thawed (25×10^6 cells/ml in an extraction buffer containing 50 mM Tris-HCl, 50 mM sodium ascorbate, and 1 mM EDTA, pH 7.4. Cells were lysed by boiling for 3 min and centrifuged at $15{,}000 \times g$ for 10 min at 4 °C. Aliquots (20–60 μl) of the supernatant were used to determine CH_2THF, THF, DHF, leucovorin, and 5-CH_3THF. The 10-CHO-THF pool was assayed in the presence of 0.4 milliunit of 10-CHO-THF dehydrogenase and 10 μM NADP instead of the previously reported 10-CHO-THF deacyclase system. Protein concentration was determined by the method of Bradford (57).

RESULTS

Folate Dependence of CEM/MTX Cells—Defective transport via the RFC has been associated with >200-fold resistance to MTX in human CEM/MTX leukemia cells (5, 22, 43). However, previous studies from our laboratory (58) and others (59–61) have shown that CEM/MTX cells surprisingly exhibited only a minor (3–10-fold) cross-resistance to 5,10-dideazatetrahydrofolate (DDATHF), a folate-based inhibitor of glycinamide ribonucleotide formyltransferase. Moreover, since the presumed defective RFC did not compromise growth of CEM/MTX cells in folate-replete medium (containing 2.3 μM folic acid), we investigated whether alternative routes for folic acid and DDATHF may be present in CEM/MTX cells. In order to obtain further insight into the mechanistic basis of folate-dependent growth of RFC-defective CEM/MTX cells, we rationalized that a gradual deprivation of folic acid could impose a selective pressure to increase the expression of the folate transporter that is responsible for this phenotype.

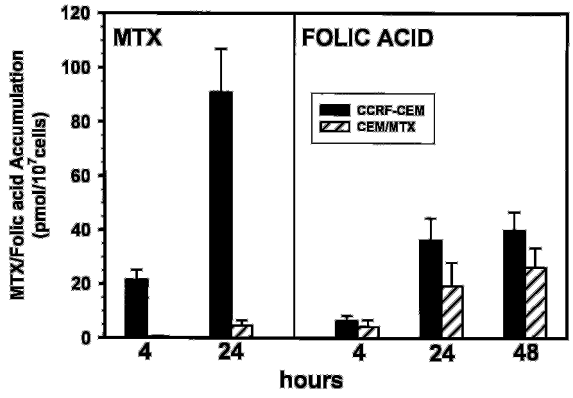

FIG. 1. **Accumulation of [^{3}H]MTX and [^{3}H]folic acid by CCRF-CEM and CEM/MTX cells.** CCRF-CEM and CEM/MTX cells were seeded (15 ml) in 80-cm^2 tissue culture flasks at a density of 7.5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FCS and 1 μM [^{3}H]MTX (specific activity, 1,500 dpm/pmol) (*left panel*) or 2 μM [^{3}H]folic acid (specific activity, 1,500 dpm/pmol) (*right panel*). After 4, 24, and 48 h of incubation in 5% CO_2 atmosphere, CCRF-CEM (*black bars*) and CEM/MTX (*hatched bars*) cells were harvested by centrifugation, washed twice with HBSS buffer, pH 7.4, and analyzed for radioactivity. Incubations with [^{3}H]MTX were not extended beyond 24 h because of its cytotoxic effect to CCRF-CEM cells. Results are presented as the mean ± S.D. of three to four experiments.

We noted that folic acid rather than purines/pyrimidines in undialyzed fetal calf serum was essential for cell growth of CEM/MTX cells (58). This was illustrated by the fact that growth of CEM/MTX cells was unaffected in medium containing 2.3 μM folic acid, 10% dialyzed FCS, whereas cessation of cell growth was observed when CEM/MTX cells were transferred to medium containing 2 nM folic acid and 10% undialyzed FCS (data not shown). Another line of evidence for folic acid transport capacity in CEM/MTX cells was obtained from the marked protection by folic acid against growth inhibition by DDATHF; 11- and 330-fold protection in medium supplemented with 20 and 200 μM folic acid, respectively (data not shown). Finally, Fig. 1 shows that while the accumulation of [^{3}H]MTX in CEM/MTX cells was 40-fold (after 4 h) and 20-fold (after 24 h) lower than in CCRF-CEM cells, the accumulation of [^{3}H]folic acid in CEM/MTX cells over a 4–48-h time period was preserved and was comparable to that in wild type CCRF-CEM cells.

[^{3}H]Folic acid accumulation in CEM/MTX-LF cells, the subline of CEM/MTX that was adapted to grow at 2 nM folic acid (see "Experimental Procedures") is shown in Fig. 2. Accumulation of [^{3}H]folic acid in CEM/MTX-LF cells followed a pattern that was time- and concentration-dependent. After 48 h of incubation at extracellular concentrations of 2 nM and 2 μM folic acid, CEM/MTX-LF cells accumulated 2.4 and 284 pmol/10^7 cells, respectively. This latter value is 10-fold higher than the accumulation of [^{3}H]folic acid in CEM/MTX cells under the same conditions (Fig. 1).

Affinity Labeling of Folate Transporter—In order to assess whether the increased accumulation of [^{3}H]folic acid in CEM/MTX-LF cells is associated with an increased expression of a specific folate transport protein we determined the amount of transport protein by affinity labeling with an *N*-hydroxysuccinimide ester of [^{3}H]MTX and [^{3}H]folic acid. Specific incorporation of [^{3}H]MTX was below the limit of detection in CEM/MTX

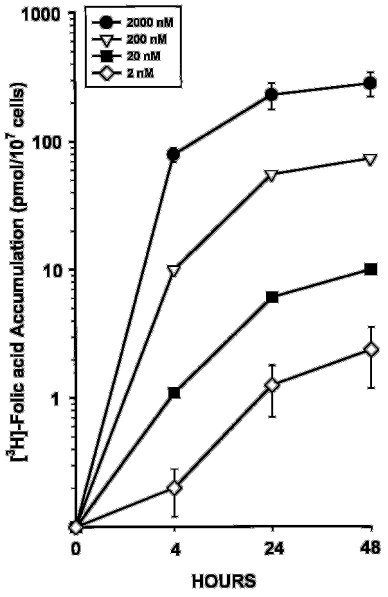

FIG. 2. **Accumulation of [^{3}H]folic acid by CEM/MTX-LF cells.** Cells were distributed (15 ml) into 80-cm^2 tissue culture flasks at a density of 7.5×10^5 cells/ml in folate-free RPMI 1640 medium supplemented with 10% dialyzed FCS and [^{3}H]folic acid (specific activity, 1,500 dpm/pmol) at extracellular concentrations of 2 nM, 20 nM, 200 nM, and 2 μM. After 4, 24, and 48 h of incubation in a 5% CO_2 atmosphere, cells were harvested by centrifugation, washed twice with HBSS buffer, pH 7.4, and analyzed for radioactivity. Results are presented as the mean ± S.D. of two to three experiments.

cells but for CEM/MTX-LF cells it was 17-fold higher than in wild type CEM cells (Table I). Affinity labeling of RFC in CEM-7A cells (which display a 30-fold overexpression of RFC protein over wild type CEM cells) (20) was only 2-fold higher than for CEM/MTX-LF cells. The increased expression of the folate transporter was stably retained when CEM/MTX-LF cells were grown for a period of 1 month in folate-replete medium (2.3 μM folic acid). Although wild type RFC has a 2 orders of magnitude lower affinity for folic acid than for MTX (2, 4, 10), we used *N*-hydroxysuccinimide-[^{3}H]folic acid as a labeling reagent for CEM/MTX-LF cells. As expected, specific affinity labeling with this reagent was below the limit of detection for CEM cells and CEM/MTX cells, whereas CEM/MTX-LF cells surprisingly contained 2.1 pmol of [^{3}H]folic acid/mg of protein, a value which is even higher (1.5-fold) than that observed for the highly RFC overexpressing CEM-7A cells.

SDS-polyacrylamide gel electrophoresis analysis of *N*-hydroxysuccinimide-[^{3}H]MTX labeled CEM-7A and CEM/MTX-LF cells, as well as *N*-hydroxysuccinimide-[^{3}H]folic acid-labeled CEM/MTX-LF cells showed a broadly migrating band which peaked at a molecular mass of 75–80 kDa for both CEM-7A cells and CEM/MTX-LF cells (results not shown). CEM/MTX-LF cells did not express membrane folate receptors (8, 62, 63) as judged from the lack of a high affinity binding of [^{3}H]folic acid and the absence of immunoreactivity with the membrane folate receptor-specific monoclonal antibody MOv18 (47, 48, 63). Flow cytometric analysis with MOv18 was found negative for CEM/MTX-LF cells as compared with folate receptor overexpressing nasopharyngeal KB-3-1 cells (results not shown). Furthermore, no evidence was found for the presence of a low pH folate transport route (64, 65) as pH 7.6 was optimal for [^{3}H]folic acid and [^{3}H]MTX transport in CEM/MTX-LF cells.

RFC1 Gene Amplification and mRNA Overexpression—The expression of the RFC1 gene was analyzed in CEM/MTX-LF cells and compared with CCRF-CEM, CEM/MTX, and CEM-7A cells. Northern blot analysis (Fig. 3) with poly(A)$^+$ RNA from

TABLE I
Affinity labeling of folate transporters in CCRF-CEM cells and its various sublines

Intact cells (2.5×10^7/ml for CCRF-CEM and CEM/MTX, 1.0×10^7/ml for CEM/MTX-LF and CEM-7A cells) were affinity labeled with a concentration of 100 nM NHS-[^{3}H]MTX or 100 nM NHS-[^{3}H]folic acid.

Cell line	Affinity label	
	NHS [^{3}H]MTX	NHS-[^{3}H]folic acid
CCRF-CEM	0.23 ± 0.11[a]	<0.02
CEM/MTX	<0.02	<0.02
CEM/MTX-LF	4.0 ± 1.3	2.1 ± 0.4
CEM-7A	8.1 ± 1.0	1.4

[a] Labeling of transport protein is expressed as picomoles of [^{3}H]MTX/[^{3}H]folic acid incorporated/mg of protein. Affinity labeling in the presence of 1 mM unlabeled MTX or folic acid served as a control for labeling specificity. Results are expressed as the mean ± S.D. of two to four separate experiments.

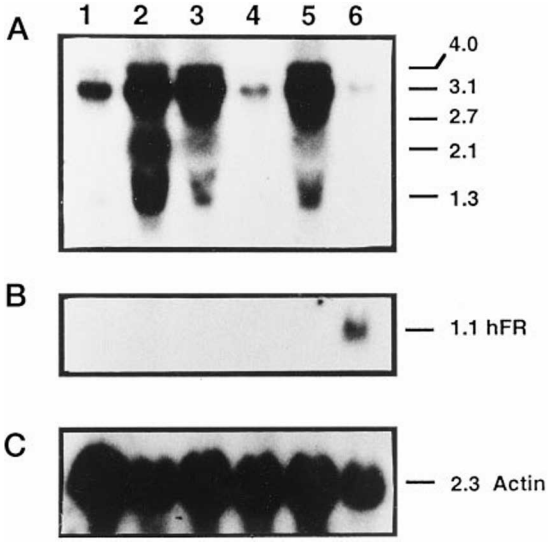

FIG. 3. **Autoradiogram of a Northern blot performed with RNA isolated from CCRF-CEM cells and its various sublines.** Samples (20 μg/lane) of total RNA obtained from parental CCRF-CEM cells (*lane 1*), CEM-7A cells (*lane 2*), CEM/MTX-LF cells (maintained at 2 nM folic acid) (*lane 3*), CEM/MTX cells (*lane 4*), CEM/MTX-LF cells grown for 1 month in folate-replete medium (containing 2.3 μM folic acid) (*lane 5*), and human nasopharyngeal KB-3-1 carcinoma cells (*lane 6*) were fractionated on a 1.5% agarose/formaldehyde gel, transferred to a Zetabind nylon membrane (Bio-Rad), and hybridized with a ^{32}P-oligo-labeled RFC1 cDNA clone, hRFC25 (S. Drori, G. Shemer, G. Jansen, and Y. G. Assaraf, manuscript in preparation) (*panel A*). Following stripping, the blot was hybridized with a human folate receptor (*hFR*) cDNA sequence (*panel B*). To normalize for the actual amounts of RNA that were being analyzed, the blot was also rehybridized with a human β-actin cDNA sequence (*panel C*).

CCRF-CEM cells (*lane 1*) demonstrated a single 3.1-kilobase RFC1 transcript, which is also present in CEM/MTX cells (*lane 4*), albeit at a 5-fold lower level. In CEM/MTX-LF cells (*lane 3*), like in CEM-7A cells (*lane 2*), this 3.1-kilobase principal transcript was markedly overexpressed (20–50 fold) as compared with CCRF/MTX and CCRF-CEM cells, respectively. The phenotype of increased RFC1 mRNA expression in CEM/MTX-LF cells was found to be genetically stable, since repeated transfers of these cells for 1 month in medium containing 2.3 μM folic acid retained the RFC1 mRNA overexpression (*lane 5*). As a control experiment (Fig. 3, *panel B*) it was demonstrated that CEM/MTX-LF cells did not express human membrane folate receptor, which is highly expressed in KB-3-1 nasopharyngeal carcinoma cells (8, 62).

To investigate the molecular basis for this RFC1 mRNA overexpression, RFC1 gene copy numbers were examined by Southern blot analysis. Digestion of genomic DNA from CCRF-

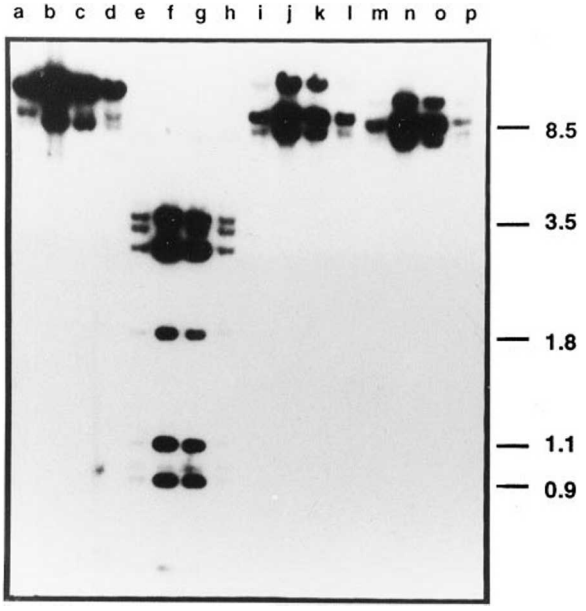

FIG. 4. **Autoradiogram of a Southern blot performed with genomic DNA isolated from parental CCRF-CEM cells and their various sublines.** High molecular weight DNA was first extracted from parental CCRF-CEM cells (*lanes a*, *e*, *i*, and *m*), MTX transport up-regulated CEM-7A cells (*lanes b*, *f*, *j*, and *n*), CEM/MTX-LF cells maintained in medium containing 2 nM folic acid (*lanes c*, *g*, *k*, and *o*) and CEM/MTX cells (*lanes d*, *h*, *l*, and *p*). DNA samples (10 μg/lane) were then digested with *Eco*RI (*lanes a–d*), *Pst*I (*lanes e–h*), *Bam*HI (*lanes i–l*), and *Hin*dIII (*lanes m–p*), fractionated on a 0.8% agarose gel, transferred to Zetabind GT filter membrane (Bio-Rad), and probed with a ^{32}P-oligo-labeled RFC1 cDNA clone, hRFC25. The blot was hybridized and washed under high stringency conditions according to the manufacturer's instructions (Bio-Rad). Shown on the *right* are size markers given in kilobase pairs.

CEM, CEM/MTX, CEM-7A, and CEM/MTX-LF cells revealed a 10-fold RFC1 gene amplification in both CEM-7A cells and CEM/MTX-LF cells as compared with CCRF-CEM and CEM/MTX cells (Fig. 4). Reprobing this Southern blot with a multidrug resistance (MDR1) cDNA probe confirmed that similar amounts of genomic DNA were being analyzed (not shown).

Characterization of (Anti)folate Transport Kinetic Parameters in CEM/MTX-LF Versus CEM Cells—The transport kinetic parameters (K_m and V_{max}) for influx of [^{3}H]MTX, [^{3}H]leucovorin, and [^{3}H]folic acid were determined for CEM and CEM/MTX-LF cells. Table II shows a 3–5-fold increased V_{max} value for influx of leucovorin, folic acid, and MTX in CEM/MTX-LF cells as compared with CCRF-CEM cells. Interestingly, a major shift to lower K_m values was noted for the influx of [^{3}H]folic acid and [^{3}H]leucovorin in CEM/MTX-LF cells; 31- and 9-fold lower K_m than in CEM cells, respectively. In contrast, the K_m value for [^{3}H]MTX influx in CEM/MTX-LF cells was similar to that in CEM cells.

Anion Dependence of MTX Transport in CEM/MTX-LF Cells—A characteristic feature of (anti)folate transport via wild type RFC is a differential inhibitory effect by various (in)organic anions (3, 4, 10, 20). We measured [^{3}H]MTX influx in CEM-7A cells (wild type RFC) and CEM/MTX-LF cells as a function of increasing concentrations of chloride in an isotonic extracellular buffer (Fig. 5). [^{3}H]MTX influx in CEM-7A cells was optimal in the anion-deficient buffer and gradually dropped to 40% of maximum in the presence of 140 mM chloride. Results for parental CEM cells were superimposable to that of CEM-7A cells (results not shown). Conversely, in CEM/MTX-LF cells, [^{3}H]MTX influx is lowest in the anion-deficient buffer and stimulated 10-fold following gradual increase in the extracellular chloride concentration.

TABLE II
Kinetic parameters of (anti)folate transport in CCRF-CEM and CEM/MTX-LF cells

Results are the mean of two separate experiments performed in duplicate. V_{max} and K_m values were calculated from double reciprocal plots of influx rate *versus* extracellular substrate concentration. Transport kinetic parameters for CEM/MTX cells could not be determined because influx rates were too low (<0.05 pmol/min/10^7 cells) to provide a reliable assessment.

Compound	V_{max}		K_m		Affinity increase
	CCRF-CEM	CEM/MTX-LF	CCRF-CEM	CEM/MTX-LF	
	pmol/min/10^7 cells		μM		*-fold*
[^{3}H]Folic acid	3.6	18.5	175	5.7	31
[^{3}H]Leucovorin	5.1	15.2	1.4	0.16	9
[^{3}H]MTX	4.0	20.1	5.1	4.0	1.3

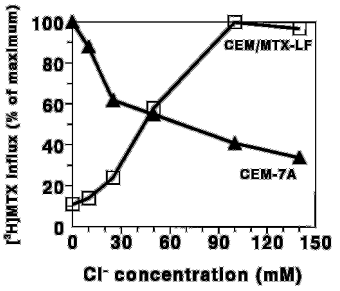

FIG. 5. **Chloride dependence of [^{3}H]MTX influx in CEM-7A and CEM/MTX-LF cells.** CEM-7A cells (4×10^6/assay) and CEM/MTX-LF cells (10×10^6/assay) were harvested by centrifugation and washed twice with an anion-deficient buffer (20 mM HEPES, 225 mM sucrose, pH 7.4, with KOH). Subsequently, cells were resuspended in the anion-deficient buffer or in a buffer in which this buffer was mixed isotonically with HEPES buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 7 mM D-glucose, pH 7.4, with NaOH) to achieve final extracellular chloride concentrations of 0, 10, 25, 50, 100, and 140 mM. Linear rates of [^{3}H]MTX influx were measured for 1.5 min (*CEM-7A*) and 3 min (*CEM/MTX-LF*) at an extracellular concentration of 2 μM as described under "Experimental Procedures."

Identification of Mutations in the RFC1 Coding Region—In order to determine whether the marked increase in the affinity of these folates for RFC1 in CEM/MTX-LF was associated with genetic alterations (*e.g.* mutation) in the RFC1 gene, we have undertaken, prior to sequencing, various methodologies at both the genomic and cDNA levels of parental cells, CEM/MTX, and CEM/MTX-LF including (see details under "Experimental Procedures"): (*a*) construction of a cDNA library from CEM/MTX cells and screening of RFC cDNA clones, (*b*) reverse transcriptase-PCR of the entire RFC coding region, (*c*) genomic PCR-single strand conformational polymorphism and isolation of multiple clones, and (*d*) genomic PCR and direct sequencing. Table III summarizes the sequence analyses using the various methodologies in both parental CEM/MTX cells and their CEM/MTX-LF subline. We have identified at both the genomic and RFC1 cDNA levels of both cell lines an identical single G to A mutation at nucleotide position 227 (based on the nucleotide numbering of Prasad *et al.* (15), GenBank™ accession no. UI15939). This single purine nucleotide change occurring in both CEM/MTX and CEM/MTX-LF cells resulted in the substitution of a lysine for a glutamate at amino acid residue 45 of the human RFC. This mutation appeared to be heterozygous as both the mutant and the wild type alleles were detected in CEM/MTX and CEM/MTX-LF. Furthermore, an additional C to T mutation at nucleotide position 352 was identified in CEM/MTX. This mutation, however, was a silent one thus retaining the wild type leucine at amino acid residue 86.

Antifolate Growth Inhibition in CEM/MTX-LF Cells Versus CCRF-CEM and CEM/MTX Cells—The impact of differences in transport kinetic parameters between CCRF-CEM and

TABLE III
Summary of sequencing analyses using the various methodologies at both the genomic and cDNA levels in CEM/MTX and CEM/MTX-LF cells

A. CEM/MTX

Method[a]	Mutation[b]					Remarks
	No. of clones sequenced	Nucleotide	Position	Amino acid	position	
cDNA library full length RFC clone	1	G → A	227	Glu → Lys	45	
RT-PCR clones	6	G → A (2 clones)	227	Glu → Lys	45	Heterozygous
		C → T (2 clones, the rest WT)	352	Leu → Leu	86	
Genomic PCR-SSCP clones of:						
(a) Exon 2	7	G → A (2 clones, the rest WT)	227	Glu → Lys	45	
(b) Exon 3	15	C → T (4 clones, the rest WT)	352	Leu → Leu	86	
Genomic PCR direct sequencing	Direct sequence[c]	G → A	227	Glu → Lys	45	Heterozygous

B. CEM/MTX-LF

Method	No. of clones sequenced	Mutation			
		Nucleotide	position	Amino acid	position
RT-PCR clones	2	G → A	227	Glu→Lys	45
Genomic PCR-SSCP clones of:					
(a) Exon 2	4	G → A (3 clones, 1 clone WT)	227	Glu→Lys	45
(b) Exon 3	9	C → T (1 clone, the rest WT)	352		
Genomic PCR direct sequencing	Direct sequence[c]	G → A	227	Glu→Lys	45

[a] RT, reverse transcriptase; SSCP, single strand conformational polymorphism.
[b] WT, wild type.
[c] Independent genomic PCR-direct sequencing of exon 2 revealed both the mutant G → A allele and the wild type alleles.

TABLE IV
Growth inhibitory effects of folate analogues against CCRF-CEM, CEM/MTX, and CEM/MTX-LF cells in folate-deplete medium (containing 2 nM folic acid (FA)) and folate-replete medium (containing 2.3 μM FA)

Folate Analogue	Target enzyme[a]	Polyglutamylation	IC_{50} (nM)[b]			
			CCRF-CEM (10% FCS/2.3 μM FA)	CEM/MTX (10% FCS/2.3 μM FA)	CEM/MTX-LF (10% dFCS/2 nM FA)	CEM/MTX-LF (10% FCS/2.3 μM FA)
MTX	DHFR	+	8.1 ± 2.2	1,950 ± 220	66 ± 9	367 ± 69
EDX	DHFR	+	1.3 ± 0.3	533 ± 139	16 ± 6	360 ± 27
TMQ	DHFR	−	7.5 ± 2.1	2.2 ± 0.5	16 ± 11	341 ± 111
PT523	DHFR	−	1.1 ± 0.3	33 ± 5	4.5 ± 0.4	18 ± 8
ZD1694	TS	+	3.5 ± 0.6	444 ± 30	5.9 ± 1.5	3,025 ± 1,840
BW1843	TS	+	2.4 ± 0.4	520 ± 65	5.1 ± 2.5	17 ± 5
ZD9331	TS	−	16 ± 1	286 ± 41	3.6 ± 0.5	34 ± 20
DDATHF	GARTFase	+	11 ± 4	80 ± 13	3.0 ± 1.4	2,960 ± 640
AG2034	GARTFase	+	30 ± 10	122 ± 20	4.1 ± 0.2	3,325 ± 675

[a] TS, thymidylate synthase; GARTFase, glycinamide ribonucleotide transformylase; DHFR, dihydrofolate reductase.
[b] IC_{50}, drug concentration that inhibits growth of cells by 50% following a 72-h drug exposure. Results are the mean ± S.D. of 4–10 experiments.

CEM/MTX-LF cells on the biological activity (growth inhibitory effects) of a selected group of (non)polyglutamatable and lipophilic folate analogues (5, 66) are presented in Table IV. Except for the lipophilic DHFR inhibitors, all of these folate analogues were reported to be efficiently transported via the wild type RFC (5). Table IV shows the growth inhibitory effects of the panel of folate analogues, following a 72-h continuous drug exposure, against CCRF-CEM cells, CEM/MTX cells, and CEM/MTX-LF cells in folate-deplete and folate-replete medium. CEM/MTX cells exhibited a high degree of cross-resistance to EDX (410-fold), ZD1694 (127-fold), and GW1843 (220-fold). An intermediate level of cross-resistance was also noted for the nonpolyglutamatable folate analogues PT523 (30-fold) and ZD9331 (18-fold), whereas only a modest (7- and 4-fold) cross-resistance was found for the two glycinamide ribonucleotide transformylase inhibitors DDATHF and AG2034, respectively. In contrast, CEM/MTX cells displayed collateral sensitivity to the lipophilic DHFR inhibitor TMQ. In folate-deplete medium, CEM/MTX-LF cells displayed a complete wild type CEM cell sensitivity to ZD1694 and GW1843 and even greater drug sensitivity than CEM cells for ZD9331, DDATHF, and AG2034. CEM/MTX-LF cells in folate-deplete medium also exhibited an increased sensitivity for MTX, EDX, and PT523, but the drug sensitivity of wild type CEM cells was not reached. Remarkably, a dramatic drug resistance was observed for ZD-1694 (513-fold), DDATHF (822-fold), and AG2034 (811-fold) when CEM/MTX-LF cells were transferred to folate-replete medium. Likewise, growth of CEM/MTX-LF cells in folate-replete medium provoked a resistance to the lipophilic antifolates TMQ (21-fold) and pyrimethamine (154-fold; IC_{50} values, 64 μM *versus* 0.4 μM in folate-replete *versus* folate-deplete medium, respectively), as well as EDX (23-fold) and MTX (6-fold). It is noteworthy that in folate-replete medium CEM/MTX-LF cells retained appreciable sensitivity to PT523, ZD-9331, and GW1843 as compared with CEM/MTX-LF cells in folate-deplete medium.

Enzyme Levels in CEM, CEM/MTX, and CEM/MTX-LF Cells—The levels of target enzymes (DHFR and thymidylate synthase) and (anti)folate metabolizing enzymes (folylpolyglutamate synthetase and folylpolyglutamate hydrolase) were de-

TABLE V
Activity of folylpolyglutamate synthetase (FPGS), folylpolyglutamate hydrolase (FPGH), thymidylate synthase (TS), and dihydrofolate reductase (DHFR) levels in CCRF-CEM, CEM/MTX, and CEM/MTX-LF cells

Cell line	DHFR[a]	TS[b]	TS[c]	FPGS[d]	FPGH[e]
CCRF-CEM	2.8 ± 0.5[f]	16.7 ± 4.0	6.1 ± 1.2	1295 ± 502	62.4 ± 11.1
CEM/MTX	3.1 ± 0.9	12.8 ± 2.9	4.3 ± 0.2	774 ± 217	38.4 ± 3.1
CEM/MTX-LF	12.0 ± 3.7	7.8 ± 2.4	3.4 ± 0.4	1320 ± 162	97.7 ± 32.4

[a] DHFR level is expressed as picomoles of [^{3}H]MTX binding/mg of protein.
[b] TS catalytic activity is expressed as nanomoles of [^3H_2O]-release/h/mg protein.
[c] TS protein levels obtained by the FdUMP binding assay (expressed as picomoles of FdUMP bound/mg of protein).
[d] FPGS activity is expressed as picomoles of [^{3}H]glutamate incorporated/h/mg of protein using 250 μM MTX as a substrate.
[e] FPGH activity is expressed as nanomoles of MTX formed/h/mg protein using 100 μM MTX-Glu_2 as a substrate.
[f] Results presented are the mean ± S.D. of three to six experiments. Further experimental details are described under "Experimental Procedures."

termined in CEM, CEM/MTX, and CEM/MTX-LF cells. Table V shows that DHFR levels were similar in CCRF-CEM and CEM/MTX cells, but were 4-fold increased in CEM/MTX-LF cells, most likely due to the use of MTX during the selection procedure of these cells (see "Experimental Procedures"). No major differences in levels of thymidylate synthase catalytic activity and FdUMP binding sites were observed among the three cell lines. Folylpolyglutamate synthetase activity in CEM/MTX cells was 1.7-fold lower than in CEM cells. In CEM/MTX-LF cells, folylpolyglutamate synthetase activity increased to the level of wild type CEM cells. Finally, the activity of folylpolyglutamate hydrolase was lowest in CEM/MTX cells and 1.6- and 2.5-fold higher in CEM and CEM/MTX-LF cells, respectively.

Folate Pools in CEM, CEM/MTX, and CEM/MTX-LF Cells—Analysis of reduced folate cofactor pools (Table VI) showed that in folate-replete medium CEM/MTX-LF cells had a total intracellular folate pool (mainly 10-CHO-THF, CH_2THF, and THF) of 500 pmol/mg of protein, which is almost 500-fold higher than for CEM/MTX-LF cells maintained in folate-deplete medium. In addition, under folate-replete growth conditions, the intracellular folate pools in CEM/MTX-LF cells were markedly higher than in CEM cells (7-fold) and CEM/MTX cells (24-fold).

Polyglutamylation of MTX and EDX in CCRF-CEM and CEM/MTX-LF Cells—The accumulation and formation of polyglutamates of [^{3}H]MTX and [^{3}H]EDX was analyzed in CEM and CEM/MTX-LF cells in folate-deplete and folate-replete medium (Fig. 6). Following a 24-h exposure to 1 μM [^{3}H]MTX or 0.5 μM [^{3}H]EDX, the total accumulation of radiolabeled drug varied approximately 2-fold between the cell lines; however, their polyglutamates distribution profile was substantially different. In CEM cells 88% of [^{3}H]MTX and >95% of [^{3}H]EDX was metabolized to Glu_{2-5} polyglutamate forms. Likewise, for CEM/MTX-LF cells under folate-deplete growth conditions, >86% of [^{3}H]MTX and >95% of [^{3}H]EDX was converted to Glu_{2-5} polyglutamate forms. In contrast, in CEM/MTX-LF cells grown in folate-replete medium, polyglutamylation of [^{3}H]MTX and [^{3}H]EDX to long chain polyglutamates was markedly impaired. Under folate-replete conditions, 64% of total accumulated [^{3}H]MTX was unmetabolized in CEM/MTX-LF cells while only 12% was metabolized to no further than the Glu_3 metabolite. [^{3}H]EDX polyglutamylation showed a similar picture as for MTX, 47% of EDX was unmetabolized, and the Glu_3, Glu_4, and Glu_5 metabolites comprised only 8, 6, and 5% of the total accumulated drug, respectively.

DISCUSSION

An increased K_m and/or decreased V_{max} of RFC-mediated transport has been recognized as a common mechanistic basis of transport-related resistance to antifolates (4, 6, 7, 9, 12, 22–30, 43). In contrast, this study describes a first case for human cells in which a decreased K_m and increased V_{max} for transport of folic acid and reduced folates provides a phenotype that confers resistance to various antifolates. This novel mechanism of antifolate resistance is associated with the overexpression in human CEM/MTX-LF leukemia cells of a structurally altered RFC that is characterized by a markedly increased affinity (decreased K_m) for folic acid (31-fold) and leucovorin (9-fold) transport as compared with the wild type RFC. In folate-replete medium, the preferential folic acid transport results in a markedly expanded intracellular folate pool. Consequently, polyglutamylation of antifolate compounds is markedly impaired, thereby abolishing their cytotoxic activity. With respect to lipophilic antifolates, the increased intracellular folate pool leads to circumvention of target enzyme inhibition and consequent antifolate resistance.

Previous studies from our laboratory (58) and Matherly *et al.* (59) had provided preliminary evidence that CEM/MTX cells expressed a folate transporter with altered kinetic properties as compared with the wild type RFC. This evidence was based on observations that CEM/MTX cells displayed a differential drug sensitivity for the tetrahydrofolate-based antifolate DDATHF as compared with 2,4-diaminopteridine or quinazoline-based antifolates such as MTX and ZD-1694 (5, 59). However, since the expression level of this putative folate transporter in CEM/MTX cells was very low (12, 43), a detailed functional characterization of this transporter was not feasible. In the present study we were able to increase the expression of this folate transporter by adapting CEM/MTX cells to a gradual deprivation of folic acid in the cell culture medium of CEM/MTX cells. Such a strategy has previously been proven successful in up-regulating the expression of the wild type RFC or membrane folate receptors in human and murine leukemia cells (20, 42). Indeed, CEM/MTX-LF cells had undergone 10-fold RFC gene amplification and subsequent overexpression of the principal 3.1-kilobase RFC transcript. Consequently, affinity labeling studies (Table I) revealed that the folate-transporter in CEM/MTX-LF cells was overexpressed from below the limit of detection to a level 17-fold higher than that present in wild type CEM cells and comparable to the RFC overexpressing CEM-7A cells (20).

The transport kinetic data with CEM/MTX-LF cells suggested that the marked decrease in the transport K_m for folic acid and leucovorin was a result of a structural alteration in the RFC. Various genomic and cDNA analyses followed by sequencing of the RFC gene from CEM/MTX cells consistently revealed both the mutant allele (*i.e.* G → A at nucleotide position 227, see Table III) and the wild type allele. This was in good agreement with the full-length RFC cDNA clones isolated and sequenced in which both the mutant and wild type RFC alleles were present in CEM/MTX cells. These consistent findings suggested that this glutamate to lysine substitution at amino acid 45 in the human RFC in CEM/MTX cells was heterozygous. In contrast, sequencing of the RFC PCR products

TABLE VI

Effect of media folic acid (2 nM or 2.3 μM) on the intracellular concentration of reduced folate pools in CEM, CEM-7A, CEM/MTX, and CEM/MTX-LF cells

Values represent the mean ± S.E. from at least 10 separate determinations. Assays for LV/5-CHO-THF were conducted but were found to be below the detection limits (0.3 pmol/mg) in all samples. The presence of small amounts of DHF in CEM/MTX cells may be the result of residual MTX that is left over intracellularly from the 1 μM MTX that routinely added to the cell culture medium of CEM/MTX cells.

Sample	Medium folate[a]	Reduced folates				
		CH_2THF + THF	5-CH_3THF	DHF	10-CHO-THF	Total
		picomoles/mg of protein				
CCRF-CEM	2.3 μM FA	24.1 ± 1.1	9.9 ± 3.1	<0.3	36.6 ± 2.1	69.7 ± 2.8
CEM-7A	0.2 nM LV	0.9 ± 0.2	<0.3	<0.3	0.3 ± 0.1	1.2 ± 0.2
CEM/MTX	2.3 μM FA	13.9 ± 0.5	<0.3	6.0 ± 0.8	<0.3	21.0 ± 1.1
CEM/MTX-LF	2 nM FA	<0.3	<0.3	1.1 ± 0.1	<0.3	1.1 ± 0.1
CEM/MTX-LF	2.3 μM FA	184.8 ± 9	<0.3	<0.3	315.4 ± 19	500.2 ± 25

[a] FA, folic acid; LV, leucovorin.

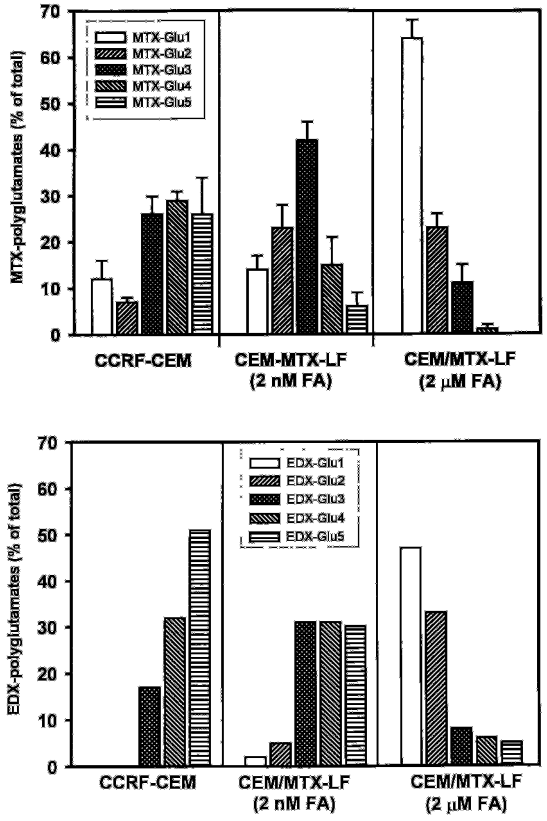

FIG. 6. **Distribution of [^{3}H]MTX and [^{3}H]EDX polyglutamates in CCRF-CEM and CEM/MTX-LF cells in folate-deplete (2 nM folic acid) and folate-replete (2 μM folic acid) medium.** Polyglutamate distribution was determined in CCRF-CEM cells and CEM/MTX-LF cells in folate-deplete and folate-replete medium following a 24-h exposure to 1 μM [^{3}H]MTX and 0.5 μM [^{3}H]EDX. Total levels of [^{3}H]MTX accumulation after 24 h were: 91 ± 16, 87 ± 27, and 58 ± 29 pmol/10^7 cells for CCRF-CEM, CEM/MTX-LF folate-deplete, and CEM/MTX-LF folate-replete cells, respectively. Total levels of [^{3}H]EDX accumulation after 24 h were: 162, 263, and 94 pmol/10^7 cells for CCRF-CEM, CEM/MTX-LF folate-deplete, and CEM/MTX-LF folate-replete cells, respectively. Results are expressed as the mean ± S.D. of three to four separate experiments (for [^{3}H]MTX polyglutamate formation) and the mean of 2 separate experiments for [^{3}H]EDX polyglutamate formation.

of the subline CEM/MTX-LF revealed primarily the mutant allele. When combined with the finding of the 10-fold RFC1 gene amplification in CEM/MTX-LF cells (Fig. 4), it appears that the mutant RFC allele but not the wild type allele has undergone gene amplification. According to this scenario of selective mutant allele amplification, the resultant overexpression of the mutant RFC with glutamate 45 to lysine may equip CEM/MTX-LF cells with the ability to preferably transport folic acid thereby enabling their survival under folate deplete conditions. Further evidence for a structural alteration in RFC in CEM/MTX-LF cells was also revealed by the striking anion (Cl^-) dependence for [^{3}H]MTX influx (Fig. 5). Unlike wild type RFC (with the negatively charged Glu-45 residue), functional activity of RFC in CEM/MTX-LF cells is stimulated by chloride, conceivably by Cl^- neutralization of the positively charged Lys-45 residue (67).

These findings stand in perfect agreement with recent results obtained by the group of Goldman (67) which characterized a MTX-resistant murine L1210 cell line with a severe transport impairment that was derived using a chemical mutagen; the same lysine for glutamate 45 (E45K) substitution was observed due to a G → A in this mutated murine RFC1 gene. Strikingly, this murine E45K mutant displayed the very same chloride-dependence for MTX influx. At the transport kinetic level, these L1210 mutant cells displayed a 7-fold decreased K_m for folic acid transport, whereas the K_m for MTX increased by 7-fold. In contrast, here we noted a 31-fold decrease in the K_m for transport of folic acid, whereas the K_m for MTX, as well as for the antifolates EDX, PT523, GW1843, ZD9331, and ZD1694,[2] was largely unchanged in CEM/MTX-LF cells. Thus, there seems to be a disparity between the transport kinetic data of the murine E45K mutant (67) and those observed for human CEM/MTX-LF cells. Although the molecular basis for this disparity in kinetic data between the human and murine E45K mutants is yet unclear, several findings suggest that the rodent and human RFC bear some structural and kinetic differences that are likely to translate into some differences in folate transport properties: 1) It should be recognized that kinetic differences in antifolate transport between murine and human RFC are not unusual. The best example is a 15–20-fold higher K_m of murine RFC for transport of the antifolate GW1843 as compared with human RFC (5, 68). Hence, since such prominent kinetic differences distinguish between the *wild type* murine RFC from its human counterpart, it is likely that identical mutations occurring in these carriers may show kinetic disparities. 2) While the rodent and human RFC proteins bear a significant amino acid sequence homology especially at predicted transmembrane domains, the C-terminal end of the murine RFC is shorter by 77 amino acids than its human counterpart (15–19). 3) In transfection experiments in which the human RFC was transfected into rodent cells, hybrid kinetic properties were observed, thus supporting the notion of differences in folate transport function between the two carriers (13). 4) Whereas murine RFC overexpression in cDNA-transfected mouse leukemia L1210 cells shows little MTX concentrative ability (69), transfection of the human RFC cDNA into transport-defective ZR-75–1/MTX carcinoma cells, shows a prominent MTX concentrative ability (70). 5) The

[2] S. Drori, G. Jansen, and Y. G. Assaraf, manuscript in preparation.

human but not the mouse RFC1 gene undergoes a substantial alternative splicing that results in truncation of ~100 amino acids (71). 6) Finally, the human RFC undergoes an extensive glycosylation, whereas the rodent RFC does not (11, 12).

Collectively, recent studies with rodent cells (26–28, 67) and the present one with human cells indicate that single amino acid substitutions in the RFC can ultimately influence folate homeostasis by altering the transport efficiency of natural reduced folate cofactors. The impact of this on the growth inhibitory potential of antifolates can vary substantially. Tables IV and VI illustrate that a relatively small increase in the intracellular folate pools (*e.g.* 7-fold between CEM and CEM/MTX-LF cells in folate-replete medium) can result in a disproportional level of 1,000-fold resistance to some antifolates. Among the most sensitive for changes in intracellular folate pools, due to competition at the level of folylpolyglutamate synthetase and consequent abolishment of polyglutamylation, were antifolates that are dependent on polyglutamylation (ZD1694, AG2034, and DDATHF) (72–74). Furthermore, a marked expansion of the intracellular folate pool provoked resistance to lipophilic antifolate inhibitors of DHFR. In this case, the increased reduced folate pool may bypass a decreasing folate pool that arises from the inhibition of DHFR by TMQ/pyrimethamine. Moreover, TMQ/pyrimethamine inhibition of DHFR brings about a rapid increase in the level of dihydrofolate, which upon very efficient polyglutamylation can readily displace TMQ/pyrimethamine from DHFR (75, 76). In this way, DHFR enzyme activity can be restored, at least in part, thereby resulting in resistance to these DHFR inhibitors. Expanded folate pools had a less dramatic effect on the growth inhibitory potential of antifolates for which polyglutamylation is of lower importance (*e.g.* MTX, EDX, and GW1843) since monoglutamate forms of these compounds are already potent inhibitors of their target enzymes (5, 34). Consistently, it is of importance to note that the growth inhibitory effect of the nonpolyglutamatable antifolates PT523 and ZD-9331 was retained even under conditions of high intracellular folate levels.

Taken altogether, these results point to the key role of RFC-mediated folate transport in controlling intracellular folate pool size. Along with the recently identified alterations in folate efflux, which can also provoke an increased intracellular folate pool (77, 78), this will provide novel mechanistic insights into transport-related resistance to antifolates which may occur in the clinical setting (79).

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