Loss of Multidrug Resistance Protein 1 Expression and Folate Efflux Activity Results in a Highly Concentrative Folate Transport in Human Leukemia Cells

Yehuda G. Assaraf‡‡, Lilah Rothem‡, Jan Hendrik Hooijberg‡, Michal Stark‡, Ilan Ifergan‡, Ietje Kathmann†, Ben A. C. Dijkmans‡, Godefridus J. Peters†, and Gerrit Jansen‡

From the ‡Department of Biology, The Technion, Haifa 32000, Israel and the Departments of †Rheumatology, Vrije Universiteit Medical Center, 1081 HV Amsterdam, The Netherlands

We studied the molecular basis of the up to 46-fold increased accumulation of folates and methotrexate (MTX) in human leukemia CEM-7A cells established by gradual deprivation of leucovorin (LCV). CEM-7A cells consequently exhibited 10- and 68-fold decreased LCV and folic acid growth requirements and 23–25-fold hypersensitivity to MTX and edatrexate. Although CEM-7A cells displayed a 74–86-fold increase in the reduced folate carrier (RFC)-mediated influx of LCV and MTX, RFC overexpression per se cannot induce a prominently increased folate/MTX accumulation because RFC functions as a nonconcentrative anion exchanger. We therefore explored the possibility that folate efflux activity mediated by members of the multidrug resistance protein (MRP) family was impaired in CEM-7A cells. Parental CEM cells expressed substantial levels of MRP1, MRP4, poor MRP5 levels, whereas MRP2, MRP3 and breast cancer resistance protein were undetectable. In contrast, CEM-7A cells lost 95% of MRP1 levels while retaining parental expression of MRP4 and MRP5. Consequently, CEM-7A cells displayed a 5-fold decrease in the [3H]folic acid efflux rate constant, which was identical to that obtained with parental CEM cells, when their folic acid efflux was blocked (78%) with probenecid. Furthermore, when compared with parental CEM, CEM-7A cells accumulated 2-fold more calcein fluorescence. Treatment of parental cells with the MRP1 efflux inhibitors MK571 and probenecid resulted in a 60–100% increase in calcein fluorescence. In contrast, these inhibitors failed to alter the calcein fluorescence in CEM-7A cells, which markedly lost MRP1 expression. Replenishment of LCV in the growth medium of CEM-7A cells resulted in resumption of normal MRP1 expression. These results establish for the first time that MRP1 is the primary folate efflux route in CEM leukemia cells and that the loss of folate efflux activity is an efficient means of markedly augmenting cellular folate pools. These findings suggest a functional role for MRP1 in the maintenance of cellular folate homeostasis.

Folate cofactors serve as one-carbon donors in the de novo biosynthesis of purines and thymidylate (1). As such, normal and neoplastic dividing cells have an absolute folate requirement for DNA replication (1). Disruption of folate biosynthesis with folic acid antagonists (i.e. antifolates) is the pharmacological basis for the antitumor activity of methotrexate (MTX) and various antifolates (2). Because mammalian cells are devoid of folate biosynthesis, they rely on folate vitamin uptake from exogenous sources. Membrane transport of folates and MTX is mediated by several systems (3, 4): (a) the reduced folate carrier (RFC) is the major uptake route that functions as a bi-directional anion exchanger (5, 6) taking up folates through an antiport exchange mechanism with intracellular organic phosphates (7); (b) folate receptors mediate the unidirectional uptake of folate cofactors into mammalian cells via an endocytotic process (8); and (c) an apparently independent transport system with optimal folate uptake activity at low pH (9–11).

Apart from RFC, efflux of folates and MTX (12, 13) is mediated by multidrug resistance proteins (MRP) MRP1–4 (14–19), which belong to the ATP-binding cassette superfamily (20, 21). Members of the MRP family, currently comprising nine genes (i.e. MRP1–9), function as ATP-driven efflux transporters of various natural product anions and acidic charged drug conjugates (14, 15). Mammalian cells transfected with MRP1–4 accumulate decreased levels of MTX and consequently display resistance to this drug, particularly upon short term drug exposure (16–19). Membrane vesicles isolated from MRP1- and MRP2-transfected cells exhibit ATP-dependent transport of MTX (16). Detailed kinetic analysis of folic acid, leucovorin (LCV; 5-formyl-tetrahydrofolate) and MTX transport into MRP1- and MRP3-rich membrane vesicles reveals Km values in the low millimolar range (22). Hence, the free intracellular level of folates and antifolates including MTX is determined by the net activities of these influx (i.e. RFC) and efflux (RFC and MRP) transport pathways.

CEM-7A is a human leukemia CCRF-CEM subline previously established by gradual deprivation of LCV from the growth medium (23), resulting in RFC gene amplification (24) and carrier overexpression (23, 24). Consequently, CEM-7A cells displayed a marked increase in the influx of MTX and LCV accompanied by a comparable increase in the steady-state transmembrane gradient of MTX (23). Surprisingly, however, there was no increase in the efflux rate constant for MTX (23). This is in contrast with previous studies (25) demonstrating
that upon transfection of RFC cDNA into murine leukemia cells, there is a marked increase (5–10-fold) in the bi-directional fluxes (i.e. influx and efflux) of MTX with only a small increase in the transmembrane gradient. This discrepancy raised the possibility that the lack of increase in the MTX efflux rate in CEM-7A cells may be due to a second alteration, such as a decrease in MRP-mediated folate efflux activity. To explore this further, studies were undertaken to characterize the bi-directional fluxes and net transport of folic acid in CEM-7A cells. Folic acid has a very low affinity for the RFC (26). Thus, folic acid efflux should be largely mediated by RFC-independent pathways such as MRPs (14–19). Accordingly, alterations in folic acid efflux (27) should primarily reflect changes in MRP expression and efflux activity. We report here that CEM-7A cells exhibit a high influx and transmembrane gradient for folic acid, LCV, and MTX and that this is accompanied by a 5-fold decrease in the folic acid efflux rate constant. This markedly defective folate export appears to be due to a 95% loss of MRP1 expression, thereby resulting in high transmembrane folate gradients. These results establish for the first time that MRP1 is the primary folate efflux transporter in CEM leukemia cells and that the loss of this major folate efflux route is an efficient means of markedly augmenting cellular folate pools. These findings suggest a functional role for MRP1 in folate homeostasis in mammalian cells.

**MATERIALS AND METHODS**

**Chemicals**—Folic acid, LCV (sodium salt), MTX, probenecid, and N-hydroxysuccinimide were obtained from Sigma. Tris(2-carboxyethyl)phosphine (TCEP) was kindly provided by Dr. J. H. Schornagel (Netherlands Cancer Institute, Amsterdam, NL), and GW184343(U9) was kindly provided by Dr. G. K. Smith (GlaxoWellcome Research Laboratories). [3H,5',7'-(H)folinic acid (25 Ci/mmol), [3H]MTX (15 Ci/mmol), and [3H]leucovorin (50 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA) and purified prior to use as specified above (28). Calcein AM was from Molecular Probes (Eugene, OR), and MK571 was from Cayman Chemicals (Ann Arbor, MI).

**Tissue Culture**—Parental CCRF-CEM leukemia cells were grown in RPMI 1640 medium (Invitrogen) containing 2.3 μM folic acid and 10% fetal calf serum (Invitrogen) supplemented with 2 mM glutamine and 100 μg/ml penicillin/streptomycin. CEM-7A cells, originally isolated by gradual deprivation of LCV from the growth medium (23), were cultured in folate-free RPMI 1640 medium (Biological Industries, Beth-Haemek, Israel) supplemented with 10% dialyzed fetal calf serum (Invitrogen), antibiotics, and 0.25 mM leucovorin as the sole folate source. CEM/MTX (29) and CEM/GW70 cells (30) were originally obtained by stepwise selection in gradually increasing concentrations of MTX and GW184343, respectively. CEM/MTX (26) and CEM/GW70 (30) displayed defective MTX transport because of mutations in the RFC gene. CEM/MTX and CEM/GW70 were grown in RPMI 1640 medium containing 2.3 μM folic acid and 10% fetal calf serum as well as 1 mM MTX and 70 mM GW184343, respectively. These cell lines were further selected by gradual folic acid deprivation, thereby establishing the sublines CEM/MTX-LF (26) and CEM/GW70-LF (30). These cell lines were cultured in folate-free RPMI medium containing 10% dialyzed fetal calf serum, 2 mM glutamine, antibiotics as well as 2 and 5 mM folate acid, respectively, as the sole folate source. The human ovarian carcinoma cell line, COX-2, and its sublines stably transduced with MRP1, 2, 3, and 5 CDNA were kindly provided by Prof. P. Borst and Dr. M. Kool (The Netherlands Cancer Institute, Amsterdam, The Netherlands), whereas HEK293 cells were used as a control for MRP4 overexpression. These cell lines were cultivated in RPMI 1640 medium containing 2.3 μM folic acid, 10% fetal calf serum, 2 mM glutamine, and antibiotics.

**Folic Acid and Leucovorin Growth Requirement**—Exponentially growing CEM and CEM-7A cells were washed three times with phosphate-buffered saline and transferred to folate-free acid RP 1640 medium (Biological Industries) supplemented with 10% dialyzed fetal calf serum. Following 4–6 days of growth in folate-deficient medium, cellular growth was arrested. The cells were then seeded (3 × 10^5/well) in medium (0.15 ml) containing increasing concentrations of either folic acid or LCV ranging from 1 μM to 3 mM in 96-well tissue culture plates. Following 3 days of incubation at 37°C, viable cell numbers were determined by hemocytometer count using Trypan blue exclusion. EC_{50} is defined as the folate acid concentration necessary to produce 50% of maximal cell growth.

**Antifolate Growth Inhibition**—Parental CEM cells and their CEM-7A subline from the mid-logarithmic phase were seeded in 96-well plates (3 × 10^3/well) in growth medium (0.15 ml/well) containing various concentrations of the antifolates MTX or edatrexate. After 3 days of incubation at 37°C, cell numbers were determined by hemocytometer count using Trypan blue exclusion. The 50% inhibitory concentration (IC_{50}) is defined as the drug concentration at which cell growth is inhibited by 50% relative to untreated controls.

**Transport of Radiolabeled Folate and MTX**—(Cells (2 × 10^5 CEM and 3 × 10^5 CEM-7A cells) at the mid-log phase of growth were washed three times in an HBS transport buffer containing 20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM MgCl_2, and 5 mM d-glucose, pH 7.4, with NaOH (29). The cells were then incubated at 37°C for 0–45 min in HBS (1-mM suspensions containing 2 μM [3H]MTX, [3H]LCV, or [3H]folic acid. TMQ was included (5 μM) to block [3H]folic acid and dihydrofolate acid reduction by dihydrofolate reductase (27). Transport controls contained a 500-fold excess of unlabeled MTX (1 mM). Transport was stopped by the addition of 10 μl of ice-cold HBS. Then the cell suspension was centrifuged at 500 × g for 5 min at 4°C, and the cell pellet was washed twice with 10 μl of ice-cold transport buffer. The final cell pellet was suspended in 0.2 ml of water, and the radioactivity was determined on an Ultima Gold (Packard) liquid scintillation spectrometer.

**Folic Acid Efflux**—The cells in exponential growth phase were harvested by centrifugation (500 × g for 5 min), washed twice with HBS, and adjusted to a density of 5 × 10^6/ml (CEM-7A cells) or 2 × 10^6 cells/ml (CEM, CEM/MTX-LF, and CEM/GW70-LF cells) in 10 mM HBS (pH 7.4 at 37°C) and then transferred to 50-mI capped tubes and placed in a shaking 37°C water bath. Ten min before the addition of [3H]folic acid, 5 μM was added at 5 μM and was present during the entire period of loading and efflux. Following a 20-min loading period, the cells were centrifuged (750 × g for 5 min), the supernatant was aspirated, and the cell pellet was resuspended in 11 ml of prewarmed (37°C) HBS and placed in a shaking water bath at 37°C. Then 1-mI samples were drawn and transferred to a centrifuge tube containing 10 ml ice-cold HBS. The cells were centrifuged, and the cell pellet was processed for radioactivity. To distinguish between the RFC-mediated and MRP-mediated folic acid efflux routes, the irreversible RFC inhibitor N-hydroxysuccinimide ester of MTX (NHS-MTX, 7.5 μM) (31) and probenecid (1 mM), which inhibits MRP1 activity (32), were added to the transport buffer. Because the influx capacities among the leukemia cell lines differed to a large extent, CEM/MTX-LF, CEM/GW70-LF, CEM-7A, and parental CEM cells were loaded to comparable levels with the following [3H]folic acid concentrations: 2, 2.5, 10, and 5 μM, respectively. Folic acid efflux kinetic data were expressed as the log of the ratio of exchangeable intracellular folate at a given transport time to the initial exchangeable intracellular level (27).

**Western Blot Analysis of MRPs and RFC Expression**—To examine the expression of RFC and the various MRPs in parental CEM cells and its various sublines (2 × 10^5 cells), total cellular proteins were extracted in a buffer (250 μl) containing 50 mM Tris, pH 7.5, 50 mM β-mercaptoethanol, 0.5% Triton X-100, and the protease inhibitors aprothin (60 μg/ml), leupeptin (5 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), and EDTA (1 mM). Following 1 h of incubation on ice, the extract, the cell pellet was centrifuged at 15,000 × g for 30 min at 4°C, and the supernatant containing the fraction of detergent-soluble proteins was collected. The proteins (10–50 μg) were resolved by electrophoresis on 7.5% (for MRP) or 10% (for RFC) polyacrylamide gels containing SDS and electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked for 1 h at room temperature in Tris-buffered saline (150 mM NaCl, 0.5% Tween 20, 10 mM Tris, pH 8.0) containing 1% skim milk. The blots were then reacted with the following anti-human MRP monoclonal antibodies (kindly provided by Prof. R. J. Schepers, VU Medical Center, Amsterdam, The Netherlands) at a 1:500 dilution: rat anti-MRP1 (MRP-r1) (5), mouse anti-MRP2, anti-MRP3, anti-MRP7 (Dr. G. D. Krub), anti-MRP5, anti-BCRP, and anti-Pgp. To determine RFC expression, the blots were reacted with a polyclonal antiserum (1:700) prepared in mice against a C-terminal hRFC peptide. The blots were then rinsed in the same buffer for 10 min at room temperature and reacted with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. Following three 10-min washes
Folate growth requirement and antifolate growth inhibition of parental CEM and CEM-7A cells

| Folate      | EC_{50} (nM) CEM | EC_{50} (nM) CEM-7A | CEM-7A/CEM | Fold |
|-------------|------------------|---------------------|------------|------|
| Folic acid  | 54.0 ± 8.0       | 0.79 ± 0.08         | 68         |      |
| Leucovorin  | 0.2 ± 0.05       | 0.02 ± 0.01         | 10         |      |

| Antifolate  | IC_{50} (nM)     | CEM/CEM-7A          | Fold       |
|-------------|------------------|---------------------|------------|
| Methotrexate| 1.6 ± 0.5        | 0.07 ± 0.02         | 23         |
| Edatrexate  | 0.5 ± 0.1        | 0.02 ± 0.01         | 25         |

EC_{50} is the folate concentration necessary to produce 50% maximal growth following a 72-h incubation at 37 °C. IC_{50} antifolate concentration that inhibits cell growth by 50% following a 72-h drug exposure.

The results depicted are the means ± S.D. of 3–6 experiments.

RESULTS

Folate Growth Requirement and Folate/antifolate Accumulation—Folic acid and LCV growth requirements in parental CCRF-CEM cells were compared with those of CEM-7A cells adapted to grow in a medium containing 0.25 mM LCV as the sole folate source (23). The growth requirements of CEM-7A cells for folic acid and LCV were markedly decreased when compared with parental CEM cells. The LCV and folic acid concentrations necessary to produce 50% maximal growth (EC_{50}) of CEM-7A cells were 10- and 68-fold lower than those obtained with parental CEM cells (Table I). Furthermore, the growth requirements of CEM-7A cells were 46- and 15-fold higher than in parental CEM and CEM-7A cells, respectively (Table II). Consistently, LCV accumulated 490 pmol of folic acid/10^7 cells, as compared with 19 pmol of folic acid/10^7 cells, of incubation at 37 °C.

The markedly increased levels of folate and MTX accumulation in CEM-7A cells cannot result from RFC overexpression per se because the latter functions as an anion exchanger (5, 6) generating very little concentrative gradients (25). Rather, the high level accumulation of folates and MTX was suggestive of the loss of some RFC-independent folate efflux function as previously shown in pyrimethamine-resistant Chinese hamster ovary cells (27). Hence, folic acid efflux activity in CEM and CEM-7A cells was determined. Whereas LCV and MTX are good RFC transport substrates (K_m = 1.4 and 5 μM, respectively) (23), folic acid is a very poor transport substrate (K_m = 175 μM) (26). To minimize the RFC-mediated component of efflux and thereby maximize the ATP-driven folate efflux route(s), [3H]folic acid rather than [3H]MTX was used in all efflux studies. [3H]Folic acid efflux was rapid in parental CEM cells with an average T_1/2 of 3 ± 1 min (Fig. 2A), whereas the T_1/2 in CEM-7A was 5-fold higher (15 ± 4 min) (Fig. 2B). This was consistent with a 5-fold decrease in the folate efflux rate constant in CEM-7A cells relative to parental CEM cells (Table III). To distinguish between the relative contributions of RFC and MRPs to the overall efflux of [3H]folic acid, the N-hydroxysuccinimide ester of MTX (NHS-MTX, 7.5 μM), and probenecid (1 mM), were used as transport inhibitors of RFC...
In the presence of 1 mM probenecid (solid squares), in CEM-7A cells had a 30-fold RFC protein overexpression, as revealed by the reprobing of the MRP Western blot with anti-RFC antibodies (Fig. 3A). β-Actin expression confirmed that equal amounts of proteins were being analyzed in the various gels (Fig. 3B).

Flow Cytometric Analysis of Calcein AM Accumulation—Calcein AM, a membrane-permeable chromoporic ester of calcine, is rapidly converted by intracellular esterases to its impermeable anionic form, calcine. The latter was shown to be a good efflux substrate of MRP1 (33). Consistent with the loss of MRP efflux function, CEM-7A cells had a 30-fold RFC protein overexpression, as revealed by the reprobing of the MRP Western blot with anti-RFC antibodies (Fig. 3A). β-Actin expression confirmed that equal amounts of proteins were being analyzed in the various gels (Fig. 3B).

### Table II

| Parameter                  | CEM     | CEM-7A  | CEM-7A/CEM |
|----------------------------|---------|---------|------------|
| Folic acid influx          | 0.2     | 5       | 25         |
| Leucovorin influx          | 1.9 ± 0.4 | 140 ± 20 | 74         |
| MTX influx                 | 1.4 ± 0.3 | 120 ± 30 | 86         |
| Steady-state folic acid accumulation | 2      | 30      | 15         |
| Steady-state leucovorin accumulation | 19 ± 4 | 490 ± 50 | 26         |
| Steady-state MTX accumulation | 16 ± 3 | 510 ± 130 | 32        |
| Bound MTX                 | 5       | 5       | 1          |
| Net MTX accumulation       | 11      | 510     | 46         |

* Influx rates are given in pmol/10⁷ cells/min.
* Levels of accumulation of (anti)folate is given in pmol/10⁷ cells.
* Bound MTX levels (pmol/10⁷ cells) were obtained after 60 min of [³H]MTX efflux.

### Table III

| Treatment                   | Folic acid efflux rate constant | CEM     | CEM-7A  | CEM/CEM-7A |
|-----------------------------|---------------------------------|---------|---------|------------|
| None (control)              | 0.25 ± 0.05                      | 0.05 ± 0.01 | 5       |
| NHS-MTX (7.5 μM)            | 0.1 ± 0.05                       | 0.05 ± 0.01 | 2       |
| Probencend (1 mM)           | 0.05 ± 0.01                      | 0.03 ± 0.01 | 2       |
| NHS-MTX + probencend        | 0.04 ± 0.01                      | 0.01     | 4       |

\(^a\) Exponentially growing cells were washed three times with HBS loaded with [³H]folic acid to a level of 30–40 pmol/10⁷ cells, and efflux rate constants were determined in the absence or the presence of RFC and MRP inhibitors as detailed under “Materials and Methods.”

\(^b\) The results presented were obtained from 3–5 independent [³H]folic acid efflux experiments.
Western blot analysis of RFC and MRP expression in CEM and CEM-7A cells. Exponentially growing CEM and CEM-7A cells were harvested by centrifugation and lysed in a buffer containing 0.5% Triton X-100 (36). Then detergent-soluble proteins (10–50 µg) were resolved by electrophoresis on 7.5% (for MRP) or 10% (for RFC) polyacrylamide gels containing SDS and electroblotted onto Nytran membranes. The blots were then reacted with polyclonal antibodies to hRFC (A, lower panel) or monoclonal antibodies to MRP1 (A and B, upper panel), MRP2, MRP3, MRP4, MRP5, Pgp, and BCRP (B). To confirm the uniformity of protein loading the blots were also reacted with monoclonal antibodies to β-actin (B, lowest panel). A, lane 1, parental CEM cells; lane 2, CEM-7A cells grown in 0.25 nM LCV (LF); lane 3, CEM-7A cells grown in 5 nM LCV for 1 month (LF → HF). The molecular masses (kDa) of MRP1 and RFC are given on the bottom of the panel. Reprobing with antibodies to β-actin was performed to confirm that equal amounts of proteins were being analyzed (bottom).

Flow cytometric analysis of calcine accumulation in CEM and CEM-7A cells. A, exponentially growing CEM (dark line) and CEM-7A cells (gray line) were incubated for 20 min in growth medium at 37 °C in the absence (dashed line), or presence of 3 nM calcine AM (dark and gray lines), washed, and analyzed by flow cytometry for mean fluorescence per cell. B, CEM and CEM-7A cells were preincubated for 10 min at 37 °C in the absence (dark symbol), or presence of the calcine efflux inhibitors MK571 (20 µM; light gray shading) or probenecid (1 mM; dark gray shading), after which the cells were incubated for an additional 20 min in the presence of 3 nM calcine AM + MK571 and probenecid. Then cells were washed and analyzed for mean fluorescence per cell.

DISCUSSION

In an initial report (23), CCRF-CEM-7A leukemia cells were shown to have a markedly augmented concentrative transport of MTX relative to their parental CCRF-CEM cells. This was associated with RFC gene amplification (24) and carrier overexpression (23, 24). Because RFC functions as a bi-directional anion exchanger (5, 6), one would expect that the markedly augmented influx of MTX in CEM-7A cells should be accompanied with a symmetrical increase in RFC-mediated efflux of MTX. However, the highly concentrative transport of folic acid in CEM-7A cells was correlated with a marked increase in folate and MTX influx but, surprisingly, without any change in efflux (23). We therefore undertook the present study to further explore the molecular basis of this unexpected phe-
Decreased MRP1 Efflux and Increased Folate Accumulation

Fig. 5. Time course of \(^{3}H\) folic acid efflux in parental CEM cells and their sublines CEM/MTX-LF and CEM/GW70-LF harboring RFC mutations. After 30 min of loading of CEM (solid squares), CEM/MTX-LF (open squares), and CEM/GW70-LF cells (circles) with 10, 2, and 2.5 \(\mu\)M \(^{3}H\) folic acid, respectively, in a transport buffer containing 5 \(\mu\)M TMQ at 37 \(^\circ\)C, the cells were rapidly centrifuged and resuspended in TMQ-containing buffer lacking folic acid. Then efflux of \(^{3}H\) folic acid was followed for up to 20 min. To determine the folic acid efflux rate constants, the log value of the percentage of initial free folic acid obtained at each time point was plotted as a function of time as previously described (27).

Fig. 6. Western blot analysis of MRP1 expression in parental CEM cells and their CEM/MTX-LF and CEM/GW70-LF sublines grown under low or high folate concentrations. Triton X-100-soluble proteins (10–50 \(\mu\)g) from parental CEM (A and B), CEM/MTX-LF (A), and CEM/GW70-LF cells (B) were resolved by electrophoresis on 7.5% polyacrylamide gels containing SDS, electroblotted onto Nytran membranes, and reacted with monoclonal antibodies to MRP1 as detailed in Fig. 3 legend. LF refers to growth of CEM/MTX-LF and CEM/GW70-LF under low folate conditions (2 and 5 nM folic acid, respectively), whereas LF -> HF refers to cellular growth at the regular medium concentration of folic acid (2.3 \(\mu\)M). Reprobing with antibodies to \(\beta\)-actin was performed to confirm that equal amounts of proteins were being analyzed (bottom panels).

Table IV

| Cell line          | Folic acid efflux (\% of control) | Parental/variant fold |
|-------------------|----------------------------------|-----------------------|
| CEM               | 10 ± 2                           | 2                     |
| CEM/MTX-LF        | 6 ± 1                            | 1                     |

nomenon. The present data confirm a high level accumulation of MTX and, in addition, of LCV and folic acid in CEM-7A cells under conditions in which the reduction and polyglutamylation of folic acid were suppressed. To explore the role of alterations in efflux kinetics as the basis for this difference, folic acid was utilized to exploit its poor affinity for RFC (26), making it a much better indicator than MTX of changes in MRP-mediated folate exporter activity.

The following findings are consistent with a marked loss of folate exporter function in CEM-7A cells: (a) CEM-7A cells had a 5-fold fall in the folic acid efflux rate constant relative to parental CEM cells; (b) incubation of parental CEM cells with probenecid, an organic anion transport inhibitor that also blocks MRP1 efflux activity (32), blocked 78% of folic acid efflux and yielded a folic acid efflux rate constant \((k = 0.05 \text{ min}^{-1})\) that was identical to that obtained with untreated CEM-7A cells; (c) despite the 86-fold overexpression of RFC-dependent MTX influx in CEM-7A cells, treatment of CEM-7A and parental CEM cells with a combination of NHS-MTX (an irreversible inhibitor of RFC) and probenecid resulted in only a marginal additional inhibition of folic acid efflux when compared with probenecid alone; (d) when compared with parental CEM, CEM-7A cells accumulated 2-fold more calcine, a chromophoric anionic substrate also exported by MRP1 (33, 35); furthermore, treatment of parental CEM cells with probenecid and the MRP1-specific efflux inhibitor, MK571, resulted in resumption of a calcine fluorescence that was nearly identical to that obtained with CEM-7A cells; and (e) finally, determination of MRP protein levels revealed that the markedly decreased folate efflux in CEM-7A cells was associated with a dramatic loss of MRP1 expression in CEM-7A cells. These results establish that MRP1 is the predominant folate efflux route in human leukemia CEM cells. However, one cannot rule out the possibility that alternative routes exist that may also contribute, at least to some extent, to the energy-driven efflux of folates and antifolates (e.g. MTX) in parental CEM cells. It was recently shown that the human MRP4 functions not only as an ATP-driven exporter of nucleotide analogues (37) but also as a high capacity \((V_{\text{max}} = 0.2–2 \text{ nmol/mg/min})\), low affinity \((K_{\text{m}} = 0.2–0.6 \text{ mM})\) efflux transporter of MTX, folic acid, and LCV (38). Thus, because MRP4 is equally expressed at substantial levels in both parental CEM and CEM-7A cells, one cannot exclude the possibility that under physiological conditions MRP4 may also contribute to cellular efflux of folates. Furthermore, it is also possible that some of the most recently discovered MRPs (but yet insufficiently characterized) including MRP6–9 may potentially contribute to folate/MTX efflux, at least to some extent.

Overexpression of murine RFC following cDNA transfection into MTX transport null mouse leukemia cells resulted in a marked increase in both MTX influx (10-fold) and efflux (5-fold) (25). This consequently brought about only a small (2-fold) increase in the concentrative transport of MTX (25). Hence, the major impact of the increase in RFC expression was a rapid cycling of the carrier with a much lesser change in the steady-state MTX level achieved. Because these cells were obtained by transfection without folate deprivation or antifolate selective pressure, there was no apparent requirement or stimulus for secondary alterations in other transport routes (i.e. such as decreased MRP1 expression) to sustain growth. On the other hand, during the establishment of CEM-7A cells, the selection of parental CEM cells was based upon a gradual LCV deprivation. Because the critical element regulated by the transport processes is the free cellular folate level achieved, there was an
apparent requirement for a secondary change in efflux with loss of MRPI expression aimed at augmenting the intracellular folate pool to a level sufficient to sustain DNA replication.

In the present paper we provide the first evidence that MRPI may play a functional role in the maintenance of cellular folate homeostasis. This is based on the fact that CEM leukemia cells have, on the one hand, the ability to down-regulate MRPI expression and folate efflux activity under conditions of folate deprivation. On the other hand, upon medium replenishment with normal folate levels (2.3 \( \mu \text{M} \) folate), CEM variants (adapted to grow in nM folic acid or LCV concentrations) resume MRPI expression. Thus, CEM cells can respond to extracellular folate status by decreasing MRPI expression upon folate deprivation and resume normal MRPI levels upon replenition of normal folate concentrations in the growth medium. This mechanism of adaptation becomes clear when analyzing the cellular folate pools in CEM-7A, CEM/MTX-LF, and CEM/GW70-LF cells as compared with their parental CEM cells. Whereas parental cells contain an average cellular folate pool of 65 pmol/mg protein (23, 26, 36), parental CEM-7A (26), CEM/MTX-LF (26), and CEM/GW70-LF cells (30) possess miniscule folate pools of 1.2, 1.1, and 7.7 pmol/mg protein, respectively. Because these dramatically shrunken folate pools represent the lower folate limit sustaining DNA replication and cell proliferation (36), the cells must acquire mechanisms to ensure the maintenance of this minimal cellular folate pools. Indeed, like CEM-7A, CEM/MTX-LF and CEM/GW70-LF cells had a simultaneous RFC overexpression (15–30-fold) because of gene amplification (26, 30) and 3-fold decreased MRPI expression and folate efflux activity. Furthermore, replenishment of 2.3 \( \mu \text{M} \) folic acid to CEM/GW70-LF (30) and CEM/MTX-LF cells (26) resulted in total folate pools of 303 and 500 pmol/mg protein, respectively. Consistently, CEM-7A cells accumulated 352 pmol of LCV/mg of protein upon 20 min of exposure to 2 \( \mu \text{M} \) LCV (Fig. I A and Table II). Thus, replenishment of folates to CEM/GW70-LF, CEM-7A, and CEM/MTX-LF resulted in 39-, 293-, and 456-fold expansions in the cellular folate pools when compared with the cellular folate content obtained with these cells under low folate status. These folate pools represented 5–8-fold increases when compared with the total folate content in parental CEM cells grown under the same conditions. This dramatic expansion in the cellular folate pools may explain the resumption of MRPI expression in these CEM variants upon folate replenishment. However, the exact molecular mechanism by which mammalian cells up- or down-regulate MRPI expression and folate efflux must await further characterization.

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