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

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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 prevents the addition of 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 calcine fluorescence. Treatment of parental cells with the MRP1 efflux inhibitors MK571 and probenecid resulted in a 60–100% increase in calcine fluorescence. In contrast, these inhibitors failed to alter the calcine 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 endocytic 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 K_{m} 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

1 The abbreviations used are: MTX, methotrexate; MRP, multidrug resistance protein; RFC, reduced folate carrier; LCV, Leucovorin (5-formyl-tetrahydrofolate); TMQ, trimetrexate; BCRP, breast cancer resistance protein; Pgp, P-glycoprotein; NHS-MTX, N-hydroxysuccinimide ester of MTX; AM, acetoxymethyl ester; HHS, Hepes-buffered saline.

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¶ From the Departments of Rheumatology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands.
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 folate 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. Trinitratexate (TMQ) glucuronate was a gift from Warner-Lambert/Parke-Davis (Ann Arbor, MI), edatrexate was kindly provided by Dr. J. H. Schornagel (Netherlands Cancer Institute, Amsterdam, NL), and GW1843UST (5-S)-(1,2-dihydro-3-methyl-1-oxobenzo[f]quinazolin-9-yl)methyl-1-oxo-2-isindolinyl-gluaric acid) was provided by Dr. G. K. Smith (Glaxo-Wellcome Research Laboratories). [3′,5′,7′,9′H]Folic acid (25 Ci/mmol), [3H]MTX, [3H]LCV, or [3H]folic acid. TMQ was included (5 μCi) to block [3H]folic acid and dihydrofolate reduction by dihydrofolate reductase (27). Transport controls contained a 500-fold excess of unlabeled MTX (1 μM). Transport was stopped by the addition of 10 ml 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 ml 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.

**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 × 10^6 cells), total cellular proteins were extracted in a buffer (250 μl) containing 50 mM Tris, pH 7.5, 50 mM β-mercapto-ethanol, 0.5% Triton X-100, and the protease inhibitors aprotonin (60 μg/ml), leupeptin (5 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), and EGTa (1 ml/l). Following 1 h of incubation on ice, the extract 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 electroblotted 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) (G. D. Kruh), anti-MRP5, anti-BCRP, and anti-Pgp. To determine RFC expression, the blots were reacted with a polyclonal antisera (1:700) prepared in mice against a C-terminal hRFC peptide.2 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-rat (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. Following three 10-min washes

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Decreased MRP1 Efflux and Increased Folate Accumulation

**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 nM 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 \( \text{EC}_{50} \) of CEM-7A cells were 10- and 68-fold lower than those observed with parental CEM cells (Table I). Furthermore, relative to parental CEM cells, CEM-7A cells were 23- and 25-fold more sensitive to the dihydrofolate reductase inhibitors MTX and edatrexate (Table I). Because these results were consistent with an increased capacity of CEM-7A cells to accumulate folates and antifolates, the transport of \(^{3}H\)LCV (Fig. 1A), \(^{3}H\)MTX (Fig. 1B), and \(^{3}H\)Folic acid (Fig. 1C) was determined in parental CEM and CEM-7A cells. The accumulation of 2 μM tritiated folates and MTX was markedly increased in CEM-7A cells following 20 min of uptake (Fig. 1; CEM-7A cells accumulated 490 ± 50 pmol LCV, 510 ± 130 pmol of MTX, and 30 pmol of folic acid/10^7 cells, as compared with 19 ± 4 pmol of LCV, 16 ± 3 pmol of MTX, and 2 pmol of folic acid/10^7 cells, respectively, in parental CEM cells (Table II). Based on the level of MTX tightly bound to dihydrofolate reductase (determined by efflux studies), the net MTX and folic acid pools in CEM-7A cells were 46- and 15-fold higher than in parental CEM cells, respectively (Table II). Consistently, LCV accumulation in CEM-7A cells was 26-fold higher than that in parental CEM cells (Table II). This marked accumulation of folates and MTX was not associated with increased activities of folate-dependent enzymes including dihydrofolate reductase, thymidylate synthase, and folympoly-γ-glutamate synthetase in CEM-7A cells (23).

**TABLE I**

<table>
<thead>
<tr>
<th>Folate</th>
<th>( \text{EC}_{50} ) (nM)</th>
<th>CEM/CEM-7A</th>
<th>Fold</th>
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<tbody>
<tr>
<td>Folic acid</td>
<td>54.0 ± 8.0</td>
<td>0.79 ± 0.08</td>
<td>68.0</td>
</tr>
<tr>
<td>Leucovorin</td>
<td>0.2 ± 0.05</td>
<td>0.02 ± 0.01</td>
<td>10.0</td>
</tr>
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</table>

**Antifolate      | \( \text{IC}_{50} \) (nM) | CEM/CEM-7A | Fold |
<table>
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<th></th>
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<tbody>
<tr>
<td>Methotrexate</td>
<td>1.6 ± 0.5</td>
<td>0.07 ± 0.02</td>
<td>23.0</td>
</tr>
<tr>
<td>Edatrexate</td>
<td>0.5 ± 0.1</td>
<td>0.02 ± 0.01</td>
<td>25.0</td>
</tr>
</tbody>
</table>

* EC_{50} is the folate concentration necessary to produce 50% of maximal cell 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.

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 \( ^{3}H\)Folic acid, 5 μM TMQ was present in the transport buffer to block folic acid reduction to tetrahydrofolate (27). Following various times of incubation at 37 °C, the transport of the radiolabeled folic acid or MTX was determined as detailed under “Materials and Methods.”

**\(^{3}H\)Folic Acid Efflux**—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 \( ^{3}H\)Folic acid, 5 μM TMQ was present in the transport buffer to block folic acid reduction to tetrahydrofolate (27). Following various times of incubation at 37 °C, the transport of the radiolabeled folic acid or MTX was determined as detailed under “Materials and Methods.”
suggested that the major folic acid efflux route present in
by probenecid alone in both cell lines (Table III). These results
ditional inhibition of folic acid efflux beyond the effect achieved
nant MRP1 and MRP3 in purified membrane vesicles from Sf9
shown to inhibit 98% of folic acid efflux mediated by recombi-
(

solid circles
open squares
).

To

( ), or both probenecid and NHS-MTX ( ), or both probenecid and NHS-MTX ( ), or both probenecid and NHS-MTX ( ).

Remarkably, the folic acid efflux rate constants obtained
fall in the folic acid efflux rate constant in CEM cells (Table

(31) and MRP1 (32), respectively. Probenecid induced a 5-fold
fall in the folic acid efflux rate constant in CEM cells (Table

Fig. 2. Time course of [3H]folic acid efflux in parental CEM
cells and their CEM-7A subline. After 30 min of loading (to the same
cellular level) of CEM (A) and CEM-7A cells (B) with 10 and 2.5 µM
[3H]folic acid, respectively, in a transport buffer containing 5 µM TMQ
at 37 °C, the cells were rapidly centrifuged and resuspended in TMQ-
containing buffer lacking folic acid. Then efflux of [3H]folic acid was
followed for up to 40 min in the absence of inhibitors (solid squares),
in the presence of 1 mM probenecid (open triangles), 7.5 µM NHS-MTX
(open squares), or both probenecid and NHS-MTX (solid circles). 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).

Expression of Various MRPs—Because probenecid has been
shown to inhibit 98% of folic acid efflux mediated by recombi-
nant MRP1 and MRP3 in purified membrane vesicles from Sf9
insect cells (22), the expression of various MRPs and other
members of the ATP-binding cassette superfamily was deter-
mined in CEM and CEM-7A cells. Parental CEM cells ex-
pressed substantial levels of MRP1 (Fig. 3) but had no decrease in the
levels of MRP4 and MRP5 (Fig. 3). Consistent with the
marked increase in LCV and MTX influx (74

levels of MRP4 and MRP5 (Fig. 3)

parental MRP1 expression (Fig. 3) but had no decrease in the
expression of BCRP, and the multidrug resistance transporter Pgp
pressed substantial levels of MRP1 (Fig. 3) and MRP4 (Fig. 3

mined in CEM and CEM-7A cells. Parental CEM cells ex-
pressed substantial levels of MRP1 (Fig. 3) in the absence or
the presence of RFC and MRP transport inhibitors as detailed under
"Materials and Methods." The results presented were obtained from 3–5 independent [3H]folic
acid efflux experiments.

Table II

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

* Influx rates are given in pmol/10^7 cells/min.
* Levels of accumulation of (anti)folate is given in pmol/10^7 cells.
* Bound MTX levels (pmol/10^7 cells) were obtained after 60 min of [3H]MTX efflux.

Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Folic acid efflux rate constant</th>
<th>CEM</th>
<th>CEM-7A</th>
<th>CEM/CEM-7A</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min^-1)</td>
<td></td>
<td>CEM</td>
<td>CEM-7A</td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>0.25 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>NHS-MTX (7.5 µM)</td>
<td>0.1 ± 0.04</td>
<td>0.05 ± 0.01</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Probenecid (1 mM)</td>
<td>0.05 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>NHS-MTX + probenecid</td>
<td>0.04 ± 0.01</td>
<td>0.01</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

* Exponentially growing cells were washed three times with HBS loaded with [3H]folate to a level of 30–40 pmol/10^7 cells, and efflux rate constants were determined in the absence or the presence of RFC and MRP inhibitors as detailed under "Materials and Methods."
* The flow cytometric analysis of calcine AM accumulation—Calcine AM, a membrane-permeable chromophoric 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 accumulated 2-fold more calcine (Fig. 4). Furthermore, inhibition of calcine efflux by the amphipathic anion, probenecid (1 mM), resulted in a 2-fold increase in the intracellular calcine fluorescence in CEM cells with no change in the fluorescence of probenecid-treated CEM-7A cells (Fig. 4B). Similarly, treatment of CEM cells with 20 µM MK571, a specific inhibitor of MRP1 efflux activity (34, 35), resulted in a 60% increase in the calcine fluorescence (Fig. 4B). In contrast, MK571 failed to alter the calcine fluorescence in CEM-7A cells, which markedly lost MRP1 expression (Fig. 4B). This further indicates that CEM-7A cells were deficient in the MRP1-mediated anion efflux function.

[3H]Folic Acid Efflux and MRP1 Expression in CEM Sublines—Because probenecid has been shown to inhibit 98% of folic acid efflux mediated by recombinant MRP1 and MRP3 in purified membrane vesicles from Sf9
To confirm the uniformity of protein loading the blots were also reacted with monoclonal antibodies to
B grown at 0.25 mM LCV; lowest panel (B), resp., because MRP-mediated efflux is sensitive to probenecid (1 mM; 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 mM LCV; lane 3, CEM-7A cells grown in 5 mM LCV for 1 month (LF → HF). The molecular masses (kDa) of MRP1 and RFC are given on the left (A). Following the acquisition of RFC mutations, these sublines were subjected to a gradual deprivation of folic acid, resulting in growth at 2.5 mM folic acid in the medium. Hence, the RFC-mediated component of folic acid efflux in these cells should be minimal, thereby rendering the MRP-mediated efflux of folates more amenable for quantitation. Fig. 5 shows that whereas parental CEM cells displayed a rapid [3H]folic acid efflux (T½ = 3 ± 1), CEM/MTX-LF and CEM/GW70-LF cells grown in low folate medium containing only 2 and 5 mM folic acid, respectively, had substantially increased efflux T½ values of 10 ± 2 and 6 ± 1, respectively (Table IV). These translated into 3- and 2-fold decreased folic acid efflux rates in CEM/MTX-LF (k = 0.07 min⁻¹) and CEM/GW70-LF cells (k = 0.12 min⁻¹), respectively. Because these results were suggestive of decreased MRP1-dependent folate efflux activity, MRP1 expression was determined in CEM/MTX-LF and CEM/GW70-LF cells (Fig. 6). Relative to parental CEM cells, MRP1 expression was decreased by 3-fold in CEM/MTX-LF cells (Fig. 6A). Remarkably, CEM/MTX-LF cells grown for 1 month in normal folic acid concentration (i.e. 2.3 µM; cells termed CEM/MTX-LF → HF) resumed parental MRP1 expression (Fig. 6A). Similarly, CEM/GW70-LF cells selected to grow in medium containing 5 mM folic acid also expressed 3-fold less MRP1 compared with parental CEM and CEM/MTX cells growing in 2.3 µM folic acid (Fig. 6B). CEM/GW70-LF cells grown for 1 month in normal folate concentration (i.e. 2.3 µM; cells termed CEM/GW70-LF → HF) resumed parental MRP1 expression (Fig. 6B). Consistently, CEM-7A cells replenished with 5 mM LCV in the growth medium for 1 month (termed CEM-7A LF → HF) resumed parental MRP1 expression (Fig. 3A). These results establish that MRP1 plays a functional role in folate efflux and suggest a function for MRP1 in cellular folate homeostasis.

**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 folates and MTX 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-
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 \( k \) 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 calcein, 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 calcein 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 leukemic 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 nmol/mg/min), low affinity \( K_m \) (0.2–0.6 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 may also 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 repletion with normal folate levels (2.3 μM folic acid), CEM variants (adapted to grow in nontoxic folic acid or LC5 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 repletion 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), CEM variants (adapted to grow in nontoxic folic acid or LC5 concentrations) are unable to sustain 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 assay of Yaffa Both is gratefully acknowledged.

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REFERENCES