Methotrexate Transport in Variant Human CCRF-CEM Leukemia Cells with Elevated Levels of the Reduced Folate Carrier

SELECTIVE EFFECT ON CARRIER-MEDIATED TRANSPORT OF PHYSIOLOGICAL CONCENTRATIONS OF REDUCED FOLATES*

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Gerrit Jansen, G. Robbin Westerhoff, Marc J. A. Jarmuszewski, Ietje Kathmann, Gert Rijken, and Jan H. Schornagel

From the Department of Internal Medicine, Oncology Unit and the Laboratory of Medical Enzymology, Department of Haematology, University Hospital Utrecht, Utrecht, The Netherlands

This study reports the isolation and characterization of a variant of the human CCRF-CEM leukemia cell line that overproduces the carrier protein responsible for the uptake of reduced folates and the folate analogue methotrexate. The variant was obtained by adapting CCRF-CEM cells for prolonged times to stepwise decreasing concentrations of 5-formyltetrahydrofolate as the sole folate source in the cell culture medium. From cells that were grown on <1 nM 5-formyltetrahydrofolate, a variant (CEM-7A) was isolated exhibiting a 95-fold increased Vmax for [3H]methotrexate influx compared to parental CCRF-CEM cells. The values for influx Km, efflux Keff, and Kf, for inhibition by other folate (analogue) compounds were unchanged. Affinity labeling of the carrier with an N-hydroxysuccinimide ester of [3H]methotrexate demonstrated an approximately 30-fold increased incorporation of [3H]methotrexate in CEM-7A cells. This suggests that the up-regulation of [3H]methotrexate influx is not only due to an increased amount of carrier protein, but also to an increased rate of carrier translocation or an improved cooperativity between carrier protein molecules.

Incubation for 1 h at 37 °C of CEM-7A cells with a concentration of 5-formyltetrahydrofolate or 5-methyltetrahydrofolate in the physiological range (25 nM) resulted in a 7-fold decline in [3H]methotrexate influx. This down-regulation during incubations with 5-formyltetrahydrofolate or 5-methyltetrahydrofolate could be prevented by either the addition of 10-25 nM of the lipophilic antifolate trimethopterin, aminopterin, or folic acid, or a mixture of purines and thymidine, had no effect on [3H]methotrexate influx. Similarly, these down-regulatory effects on [3H]methotrexate transport by 5-formyltetrahydrofolate, and its reversal by trimethopterin or methotrexate, were also observed, though to a lower extent, for parental CCRF-CEM cells grown in folate-depleted medium rather than in standard medium containing high folate concentrations.

These results indicate that mediation of reduced folate/methotrexate transport can occur at reduced folate concentrations in the physiological range, and suggest that the intracellular folate content may be a critical determinant in the regulation of methotrexate transport.

It is generally accepted that, next to dihydrofolate reductase levels and rate of polyglutamylation, membrane transport of MTX is one of the most important factors in effective chemotherapy with this antifolate compound (1-13). At present, two different membrane transport systems, a carrier system and a membrane-associated folate-binding protein, are believed to be involved in reduced folate/MTX transport. Membrane-associated folate-binding proteins have recently been identified in several types of cultured human cells (8, 9, 14, 15-17) and in an established monkey kidney MA104 cell line (18, 19). The major route, however, for reduced folate/MTX transport is a carrier-mediated process (2-4, 7, 13, 20, 21). MTX, as well as 5-methyl-THF, are transported by this carrier with similar Km values for influx (ranging from 1-20 μM) (2). The driving force in the uptake of MTX in carrier-mediated transport is probably provided by anion gradients in which anions can act as exchange substrates for MTX (1, 3, 4, 6). Despite the relatively high affinity of the carrier, it is to be expected that, at physiological concentrations of reduced folates (5-50 nM) (22), transport proceeds far below its maximal capacity. Nevertheless, studies with murine and human leukemia cells have demonstrated that, in these conditions, sufficient folates can be provided for cell growth (4, 23, 24). It has not yet been established whether this is merely the result of the transport kinetic properties of the carrier protein (Km and Vmax for influx) or involves some other, unknown, regulatory effect in the uptake of 5-methyl-THF, the preponderant folate in plasma and other biological fluids. A putative regulatory effect in reduced folate transport could be difficult to identify in cell cultures which are maintained in tissue culture media containing high levels of folate. In particular, commercial culture...
media are supplemented with supraphysiological concentrations (2-10 μM) of folic acid, which, unlike reduced folates, may use an alternative route of entry (2, 7, 25). Therefore, we have grown CCRF-CEM cells in media devoid of folic acid and adapted the cells for prolonged periods to stepwise decreasing concentrations of 5-formyl-THF as the sole folate source. This resulted in the isolation of a variant exhibiting a 95-fold increased Vₘₐₓ, for influx of [¹³C]MTX as compared to CCRF-CEM cells grown at "standard" conditions (3, 26, 27). The increased [¹³C]MTX influx is assumed to be based on an approximately 30-fold elevated level of carrier protein, and a more efficient carrier function. Moreover, we observed that [¹³C]MTX transport in variant CEM cells can be influenced by the addition of nanomolar concentrations of 5-formyl-THF or 5-methyl-THF to the cell culture medium.

**EXPERIMENTAL PROCEDURES**

**Materials**—[¹³C]Methotrexate (10-20 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA, and purified prior to use by thin layer chromatography as described previously (3, 8, 9). RPMI 1640 medium, with or without folic acid, and dialyzed fetal calf serum were purchased from Gibco. Triamterene (glucuronate salt) was a gift from Warner-Lambert/Parke Davis, Ann Arbor, MI. Methotrexate was kindly provided by Pharmacia, Haarlem, The Netherlands. 1-Ethyl-10-deazapurinopterin was a gift from Ciba-Geigy, Basel, Switzerland. 2-Desamino-2-methyl-N⁵-propargyl-5,8,9-dideazafolic acid was kindly provided by Dr. A. L. Jackson, Institute of Cancer Research, Sutton, United Kingdom. d⁵-Methyltetrahydrofolate, d⁵-formyltetrahydrofolic acid, and folic acid were obtained from Sigma. Unless otherwise indicated, concentrations of 5-formyl-THF and 5-methyl-THF are expressed as the concentration of the natural diastereoisomer in the racemic mixture employed. All other chemicals were reagent grade.

**Cell Cultures**—Human CCRF-CEM leukemia cells were grown as a suspension culture by standard RPMI 1640 medium (with 2.2 μM folic acid) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere. For the selection procedure, CCRF-CEM cells were transferred to folate-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum, 2 mM glutamine, antibiotics, and 5-formyl-THF as the sole folate source. Cells were transferred in serial passages in stepwise decreasing concentrations of 5-formyl-THF, starting with 10 nM 5-formyl-THF and ending up with 0.25 nM 5-formyl-THF. At this concentration, cells were maintained for 6 months, after which they were cloned twice by plating in folate-free medium containing 0.25 nM 5-formyl-THF. After 10-13 days of incubation at 37 °C, colonies were picked, grown in folate-free medium supplemented with 10% dialyzed fetal calf serum and 0.25 nM 5-formyl-THF, and screened for [¹³C]MTX transport as described below. One of the clones, CEM-7A, exhibiting the highest increase in rates of [¹³C]MTX transport, was selected for further studies and maintained in the medium described above (unless otherwise indicated). During the selection procedure, cells were repeatedly checked and found negative for mycoplasma contamination. Cell doubling times for CCRF-CEM cells and CEM-7A cells were 23 and 32 h, respectively.

**Dihydrofolate reductase activity** was determined as described by Miti et al. (26).

**Growth Inhibition Studies**—Cells in the mid-log phase of growth were plated at a density of 1 x 10⁶ cells/ml in folate-free medium supplemented with 10% dialyzed fetal calf serum and 1 nM 5-formyl-THF as the sole folate source. After 96 h of continuous exposure to appropriate concentrations of MTX, 10-EdA, or TMQ, cells were counted by a Sysmex micro-cell counter CC-110 and cell viability was counted by a trypan blue exclusion test.

**[¹³C]MTX Transport**—CCRF-CEM and CEM-7A cells were harvested by centrifugation, washed, and resuspended in the desired transport buffer. Cell suspensions (15 x 10⁶/ml of CCRF-CEM and 5 x 10⁶/ml of CEM-7A) were prepared either in a Hepes balanced saline solution (HBSS) (10) containing 107 mM NaCl, 20 mM Hepes, 26.2 mM NaHCO₃, 2 mM CaCl₂, 1 mM MgCl₂, and 7.0 mM d-glucose, pH 7.4 with NaOH, or in an anion-deficient magnesium/Hepes/sucrose buffer (MHS) containing 20 mM Hepes, 225 mM sucrose, pH 7.4 with MgO (3, 28). Uptake studies of [¹³C]MTX (specific activity, 0.2 Ci/mmol) were carried out at 37 °C at an extracellular concentration of 2 μM. At selected time intervals, uptake was terminated by the addition of 9 volumes of ice-cold transport buffer, followed by centrifugation at 800 x g for 5 min and another wash with 10 ml of ice-cold transport buffer. The pellet was resuspended in 0.5 ml of water and analyzed for radioactivity in Optifluor scintillation fluid (United Technologies Packard, Brussels, Belgium) using an Isocap/300 (Searle, Nuclear Chicago) scintillation counter with a counting efficiency for [¹³C]MTX uptake of extracellular concentrations of 1-10 μM. Efflux of [¹³C]MTX was determined by incubating cells (15 x 10⁶/ml in HBSS, pH 7.4) with 3 μM [¹³C]MTX for 20 min at 37 °C. Cells were then washed twice in drug-free buffer at 4 °C and finally resuspended in drug-free buffer at 37 °C. At selected time points, 1 ml samples were diluted with 10 ml of ice-cold transport buffer and centrifuged for 5 min at 800 x g. The pellet was analyzed for radioactivity as described above.

**Affinity Labeling**—Affinity labeling of CEM and variant CEM cells was carried out with an N-hydroxysuccinimide ester of [¹³C]MTX (NHS-[¹³C]MTX) as previously described (6, 9, 11, 29, 32). In short, CEM cells (4 x 10⁶/ml in HBSS, pH 7.0, were incubated with 100 nM NHS-[¹³C]MTX (20 Ci/mmol) for 5 min at 37 °C, unless otherwise indicated. Labeling in the presence of 1 mM unlabeled MTX served as control for specificity. After labeling, cells were centrifuged and washed twice with 10 ml of HBSS pH 7.4 buffer. Cells were then incubated for 10 min at 25 °C in 1 ml of HBSS pH 7.4 buffer containing 5% (v/v) Triton X-100. The extract was centrifuged for 30 min at 15,000 x g. The supernatant was retained and an equal volume of methanol was added to the supernatant. After 1 h at 20 °C, the precipitated protein was recovered by centrifugation (10 min, 1000 x g), dissolved in 10 mM sodium phosphate buffer, pH 7.5, containing 1% sodium dodecyl sulfate, and analyzed for [¹³C]MTX radioactivity as described above.

In order to prevent underestimation of the amount of transport protein due to an imbalance in the amount of cells and amount of label added, the conditions for labeling of CEM-7A cells were slightly different. 1 x 10⁶ CEM-7A cells were suspended in 1 ml of HBSS pH 7.0 and either treated once or repeatedly with 100 nM NHS-[¹³C]MTX. Labeling CEM-7A cells twice with 100 nM NHS-[¹³C]MTX resulted in a 20% further increase in incorporated [¹³C]MTX, but no further labeling was observed after a third treatment with NHS-[¹³C]MTX. Alternatively, the volume of the reaction mixture was increased to 2 ml and cells were labeled at a concentration of 100 mM NHS-[¹³C]MTX, unless otherwise indicated. This resulted in the same amount of incorporated [¹³C]MTX as 2-fold labeling with 100 nM NHS-[¹³C]MTX in the 1 ml reaction volume described above. For the experiments described in the last paragraph, the latter procedure for labeling of CEM-7A cells was used. Processing of labeled CEM-7A cells was again identical with that of CCRF-CEM cells, as described above.

**RESULTS AND DISCUSSION**

**Characteristics of Parental and Variant-CEM Cells**—From CCRF-CEM cells adapted for almost 2 years to low concentrations of 5-formyl-THF (i.e. 0.25 nM), we isolated a variant clone (CEM-7A) exhibiting an increased [¹³C]MTX uptake, compared to parental CEM cells (Fig. 1). Further characteristics of CEM and CEM-7A cells are summarized in Table I. With respect to transport kinetic parameters, there were no significant differences in values of Kᵣ for [¹³C]MTX influx, Kᵣ for [¹³C]MTX efflux, and structural specificity of folate compounds. The Vₘₐₓ for [¹³C]MTX influx of CEM-7A cells, however, showed a 95-fold increase. Furthermore, in growth inhibition experiments (Table II), CEM-7A cells were found to be 5-fold more sensitive to MTX or 10-EdA, and conversely, 11-fold less sensitive to TMQ. The specific activity of dihydrofolate reductase (Table II) is similar in CEM and CEM-7A cells. Thus, a more efficient uptake of MTX/10-EdA on one hand, and a more efficient uptake of 5-formyl-THF as protective agent for TMQ cytotoxicity on the other (30, 33, 34), accounts for an increased sensitivity of CEM-7A cells to MTX/10-EdA and a decreased sensitivity for TMQ, respectively.

**Effects of Reduced Folates and Antifolates on [¹³C]MTX**
Transport in CEM-7A Cells—A significant reduction in \[^{[H]}\]MTX uptake was observed when CEM-7A cells, grown on 0.25 nM 5-formyl-THF, were incubated for 1 h at 37 °C with 25 nM 5-formyl-THF (Fig. 2). After washing of the cells with transport buffer and analysis of \[^{[H]}\]MTX uptake, a 6–7-fold decrease in \[^{[H]}\]MTX influx and steady state levels was noticed, both in HBSS buffer and in the anion-deficient MHS buffer. This down-regulatory effect in \[^{[H]}\]MTX transport was also observed when CEM-7A cells were incubated with 25 nM 5-methyl-THF (results not shown).

Fig. 3A demonstrates a time course for the effect of 25 nM 5-formyl-THF or 5-methyl-THF on \[^{[H]}\]MTX transport in CEM-7A cells. Incubations for up to 1 h with 25 nM of either folic acid, MTX, aminopterin, or lo-EdAM, or with a mixture of thymidine (1.25 μM), adenine (25 μM), and hypoxanthine (2.5 μM) had no effect on \[^{[H]}\]MTX uptake (not shown). The fact that folic acid does not down-regulate \[^{[H]}\]MTX transport in CEM-7A cells might be explained by its poor transport buffer and analysis of \[^{[H]}\]MTX transport in CEM-7A cells, grown on 0.25 nM of either folic acid, MTX, aminopterin, or lo-EdAM, or with a mixture of thymidine, adenine, and hypoxanthine. This down-regulatory effect in \[^{[H]}\]MTX transport was also observed when CEM-7A cells were incubated with 25 nM 5-methyl-THF (results not shown).

An important question arising from the down-regulation of \[^{[H]}\]MTX transport in CEM-7A cells after pretreatment with reduced folate compounds is whether it can be observed in parental CCRF-CEM cells as well. Incubation of CCRF-CEM cells, grown in standard medium (containing 2 μM folic acid and 10% non-dialyzed fetal calf serum), with 5-formyl-THF concentrations up to 25 nM as described above, did not lead to a down-regulation in \[^{[H]}\]MTX transport (Fig. 4D). However, when CCRF-CEM cells were maintained for 1 week in folate-free medium supplemented with 10% dialyzed fetal calf serum and 0.25 nM 5-formyl-THF as the sole folate source, a 39% down-regulation in \[^{[H]}\]MTX transport was observed after a 1-h preincubation with 25 nM 5-formyl-THF (Fig. 4D).

At the same time, it should be noted that folate depletion in parental CCRF-CEM cells resulted in a 2.5-fold increased uptake of \[^{[H]}\]MTX, compared to CCRF-CEM cells grown under standard folate conditions. Hence, although the extent of down-regulation of \[^{[H]}\]MTX transport after incubation with reduced folates is more pronounced in CEM-7A cells, it can also be observed in CCRF-CEM cells that are depleted for folates.

Trimetrexate is a potent inhibitor of intracellular dihydrofolate reductase, but unlike MTX, it does not require the reduced folate/MTX carrier system for cell entry (27, 35, 36). Fig. 5 shows that the down-regulation of \[^{[H]}\]MTX transport in CEM-7A cells by 5-formyl-THF could be prevented either by the simultaneous addition of the lipophilic folate analogue TMQ or by preincubation with MTX. The protection against down-regulation of \[^{[H]}\]MTX influx by 25 nM 5-formyl-THF was intermediate at 10 nM TMQ and almost complete at TMQ concentrations above 25 nM. Complete protection by MTX was noticed at concentrations above 10 nM. Both TMQ and MTX only partially protected against the inhibition of \[^{[H]}\]MTX influx when added after 1 h of incubation with 25 nM 5-formyl-THF. In similar experiments, we also observed that an antifolate compound with a target enzyme other than DHFR (i.e., 2,3-diamino-2-methyl-N\(^\text{N}^{\text{pp}}\)-propargyl-5,8-dideazafolic acid, a folate-based inhibitor of thymidylate synthase that is transported by the reduced folate/MTX carrier system for cell entry (27, 35, 36)), could not provide a protective effect similar to that of MTX or TMQ (results not shown). These results suggest that inhibition of DHFR could play a role in mediating down-regulation.

Table I

<table>
<thead>
<tr>
<th>Kinetic properties of mediated transport of [^{[H]}]MTX in parental and variant CCRF-CEM cells</th>
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<tbody>
<tr>
<td>The methodology is described in the text and under &quot;Experimental Procedures.&quot; Values are mean ± S.D.; ( n ), number of determinations.</td>
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<tr>
<td>CCRF-CEM</td>
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<tr>
<td>---</td>
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<tr>
<td>( K_w ) (nM)</td>
</tr>
<tr>
<td>( V_{\text{max}} ) (pmol/min/10(^6) cells)</td>
</tr>
<tr>
<td>( t_{\text{1/2}} ) (min)</td>
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<tr>
<td>Structural specificity (( K_{\text{app}} ) (μM))(^a)</td>
</tr>
<tr>
<td>Aminopterin</td>
</tr>
<tr>
<td>10-EdAM</td>
</tr>
<tr>
<td>5 Formyl-THF</td>
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<tr>
<td>Folic acid</td>
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\( K_{\text{app}} \) is defined as the concentration of folate (analogue) compound providing 50% inhibition of \[^{[H]}\]MTX influx using MHS, pH 7.4, as influx buffer and an extracellular \[^{[H]}\]MTX concentration of 2 μM.
Methotrexate Transport in a CCRF-CEM Cell Variant

TABLE II
Growth inhibitory effects of folate analogues against parental CCRF-CEM cells and variant CEM-7A cells

Methodology is described under "Experimental Procedures." Values are mean ± S.D. n, number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>CCRF-CEM</th>
<th>CEM-7A</th>
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<tbody>
<tr>
<td>IC50 (nM)</td>
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<tr>
<td>MTX</td>
<td>7.2 ± 2.2 (n = 6)</td>
<td>1.4 ± 0.3 (n = 4)</td>
</tr>
<tr>
<td>10-EdAM</td>
<td>1.3 ± 0.3 (n = 4)</td>
<td>0.3 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>TMQ</td>
<td>7.5 ± 2.1 (n = 5)</td>
<td>82 ± 23 (n = 3)</td>
</tr>
<tr>
<td>DHFR specific activity</td>
<td>0.13 ± 0.04 (n = 3)</td>
<td>0.19 ± 0.07 (n = 3)</td>
</tr>
</tbody>
</table>

*IC50 is defined as the concentration of drug required to inhibit cell growth by 50%.

FIG. 2. Effect of 5-formyl-THF on [3H]MTX transport in CEM-7A cells. A CEM-7A cell suspension (1 x 10⁶/ml) in folate-free RPMI medium containing 0.25 nM 5-formyl-THF was incubated for 1 h at 37 °C in the presence (closed symbols) or absence (open symbols) of 25 nM 5-formyl-THF. Control cells and 5-formyl-THF incubated cells were washed in either anion-deficient MHS buffer (triangles) or HBSS buffer (circles). [3H]MTX accumulation in CEM-7A cells was determined in the same buffers (HBSS pH 7.4 or MHS pH 7.4) as transport buffer at an extracellular concentration of [3H]MTX of 2 μM.

FIG. 3. Time and concentration dependence of the effect of reduced folates on [3H]MTX transport in CEM-7A cells. A, CEM-7A cells (1 x 10⁶/ml) in folate-free RPMI medium supplemented with 0.25 nM 5-formyl-THF were incubated at 37 °C for 15 min in the presence of the indicated concentrations of the reduced folate. B, CEM-7A cells (1 x 10⁶/ml) in folate-free RPMI medium were preincubated with 0.25 nM 5-formyl-THF for 1 h, washed, and analyzed for [3H]MTX influx at an extracellular concentration of [3H]MTX of 2 μM.

FIG. 4. Effects of 5-formyl-THF and/or antifolate compounds on [3H]MTX transport in CCRF-CEM cells. □, CCRF-CEM cells grown in standard RPMI medium containing 2 μM folic acid supplemented with 10% nondialyzed fetal calf serum; □, CCRF-CEM cells grown for 1 week in folate-free RPMI medium supplemented with 10% dialyzed fetal calf serum and 0.25 nM 5-formyl-THF as folate source (CEM-D cells). Cells (1 x 10⁶/ml) in 10-ml medium were preincubated under the following conditions before analysis of [3H]MTX uptake: A, control cells; B, 1-h preincubation with 25 nM MTX; C, 1-h preincubation with 100 nM TMQ; D, 1-h preincubation with 25 nM 5-formyl-THF; E, 1-h preincubation with 25 nM MTX followed by 1-h preincubation with 25 nM 5-formyl-THF; and F, 1-h preincubation with 100 nM TMQ and 25 nM 5-formyl-THF. After incubation, cells were washed in HBSS pH 7.4 and analyzed for [3H]MTX influx in HBSS pH 7.4 buffer at a 2 μM [3H]MTX concentration for a period of 3 min at 37 °C.

FIG. 5. Effect of MTX and TMQ on [3H]MTX transport in CEM-7A cells. Open symbols, CEM-7A cells (1 x 10⁶/ml) in 10-ml folate-free RPMI medium were preincubated at 37 °C with 25 nM 5-formyl-THF in the presence of the indicated concentrations of TMQ (▲) or after 1-h preincubation at 37 °C with the indicated concentrations of MTX (●). After 1 h, cells were washed in HBSS pH 7.4 buffer and analyzed for [3H]MTX influx as described in the legend to Fig. 3. Closed symbols, CEM-7A cells were preincubated at 37 °C with 25 nM 5-formyl-THF. After 1 h, cells were centrifuged and resuspended in the original medium containing 0.25 nM 5-formyl-THF supplemented with the indicated concentrations of TMQ (▲) or MTX (●). After 1 h incubation at 37 °C, [3H]MTX influx was determined as described above.

The down-regulation of [3H]MTX influx by 5-formyl-THF. Importantly, Fig. 4, E and F, shows that protection from down-regulation of [3H]MTX influx by TMQ or MTX was also observed in parental CCRF-CEM cells (grown for 1 week in folate-free medium and dialyzed fetal calf serum) incubated for 1 h with 25 nM 5-formyl-THF. Finally, incubation with 100 nM TMQ up-regulated the influx of [3H]MTX by 51% in CCRF-CEM cells grown in standard folate medium (Fig. 4, C and F).

Affinity Labeling with NHS-[3H]MTX of CEM-7A Cells before and after Incubation with 5-Formyl-THF/TMQ—The amount of folate transporter was determined by affinity labeling with NHS-[3H]MTX (8, 11, 29, 31, 32). Labeling of CCRF-CEM and CEM-7A cells as a function of NHS-[3H]MTX concentration.
MTX transport by the high affinity/low capacity reduced folate/MTX carrier system in a subline (CEM-7A) of human CCRF-CEM leukemia cells, which were adapted to nanomolar concentrations of 5-formyl THF as the sole folate source. The variant cells exhibited a 95-fold increased V_{max} for [3H]MTX influx, compared to parental cells grown at standard high folate concentrations (Table I). By a slightly different selection procedure, Sirotnak and co-workers (10, 39) have obtained variants of L1210 cells that were up to 40-fold up-regulated in [3H]MTX influx. In these L1210 cell variants, the up-regulation in [3H]MTX influx was accounted for completely by a concomitant increase in the amount of carrier protein (10, 39). However, in CEM-7A cells, and also in parental CCRF-CEM cells grown in folate-depleted medium (Fig. 4), an increased amount of carrier protein (Table III) contributed only 30% in the increase in V_{max} for [3H]MTX influx (Table I). The discrepancy between V_{max} for [3H]MTX influx and affinity labeling with NHS-[3H]MTX suggests that a significant part of the increased [3H]MTX uptake was due to a stimulus other than the amount of carrier protein. This might involve an increased cooperativity between carrier protein molecules or an increased rate of carrier translocation (11, 32).

This study also shows that [3H]MTX transport in CEM-7A cells can be down-regulated by the addition of natural reduced folates and reversed by two inhibitors of DHFR: MTX and TMQ (Fig. 5). These effects were also observed for parental cells grown in folate-depleted medium, though at a lower extent, but not for CCRF-CEM cells grown in standard high folate medium. This suggests that, due to their prolonged exposure to folates present in the medium, are normally in a down-regulated state with respect to MTX transport. As shown for CEM-7A cells (Fig. 3B), a 50% down-regulation of [3H]MTX transport can already be achieved by incubating cells for 24 h at a reduced folate concentration of 1 nM. It is of interest to note that the effects of 5-methyl-THF and 5-formyl-THF on [3H]MTX transport in CEM-7A cells are observed at concentrations that are near physiological and at least 100-fold below the K_s value for transport by the carrier system (2). Moreover, since all the effects of 5-methyl-THF and 5-formyl-THF on [3H]MTX transport in CEM-7A cells were obtained under transport-permissive conditions at 37 °C (Figs. 2-5, Table III), it is conceivable that changes in the intracellular levels of reduced folates, their metabolites, or polyglutamate forms, whether or not induced by inhibition of DHFR with TMQ or MTX, play a direct or indirect role in the regulation of reduced folate/MTX transport. Alternatively, the differential effects of reduced folates...
were incubated with 25 nM 5-formyl-THF at 37 °C for 1 h, subsequently centrifuged and resuspended to a density of 3 x 10^6/ml in the original medium supplemented with 0.25 nM 5-formyl-THF. [3H]MTX influx was monitored for 6 days, including a refreshment of the original medium supplemented with 0.25 nM 5-formyl-THF. [3H]MTX transport in CEM-7A cells would be consistent with a different intracellular processing of these compounds after translocation by the carrier system. The present study suggests the folate transport by a membrane-associated folate binding-protein could be mediated at folate levels approximating those for transport by the reduced folate/MTX carrier.

and folate analogues on [3H]MTX transport in CEM-7A cells would be consistent with a different intracellular processing of these compounds after translocation by the carrier system. Price et al. (20, 21) recently identified in L1210 cells a protein loosely associated with the cytoplasmic surface of the membrane that is possibly involved in mediating the intracellular trafficking of reduced folate compounds rather than that of folate analogues such as MTX which bind to DHFR. With the availability of variants such as CEM-7A, further studies on the role of multiple proteins in reduced folate/MTX transport and its regulation will now be feasible and can be of great physiological and pharmacological importance. In concert with this, Kamen et al. (18, 19) demonstrated recently that folate transport by a membrane-associated folate binding-protein could be mediated at folate levels approximating physiological concentrations. The present study suggests the same for transport by the reduced folate/MTX carrier.

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Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. Selective effect on carrier-mediated transport of physiological concentrations of reduced folates.

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