

Impact of Overexpression of the Reduced Folate Carrier (RFC1), an Anion Exchanger, on Concentrative Transport in Murine L1210 Leukemia Cells*

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Transport of reduced folates in murine leukemia cells is mediated by the bidirectional reduced folate carrier (RFC1) and independent unidirectional exit pumps. RFC1 has been proposed to be intrinsically equilibrating, generating transmembrane gradients by exchange with inorganic and organic anions. This paper defines the role of high level carrier expression, through transfection with RFC1 cDNA, on concentrative transport of the folate analog, methotrexate (MTX) in murine L1210 leukemia cells. RFC1 was expressed in the MTX^rA line, which lacks a functional endogenous carrier to obtain the MTX^rA-R16 clonal derivative. Influx was increased ~9-fold in MTX^rA-R16 cells without a change in K_m . The efflux rate constant was increased by a factor of 5.1 relative to L1210 cells, and this resulted in only a 2.1-fold increase in the steady-state level of free intracellular MTX, $[MTX]_i$, when $[MTX]_e$ was 1 μ M. The concentrative advantage for RFC1 (the ratio of $[MTX]_i$ in MTX^rA-R16 to L1210 cells) increased from 1.8 at 0.1 μ M MTX to 3.8 at an $[MTX]_e$ level of 30 μ M. Augmented transport in MTX^rA-R16 cells was accompanied by a 2-fold increase in accumulation of MTX polyglutamate derivatives and a ~50% decrease in the EC_{50} for 5-formyltetrahydrofolate and folic acid and the MTX IC_{50} relative to L1210 cells. These alterations paralleled changes in $[MTX]_i$ and not the much larger change in influx at low $[MTX]_e$ levels, consistent with the critical role that free intracellular folates and drug play in meeting cellular needs for folates and as a determinant of antifolate activity, respectively. The data indicate that RFC1 produces a large and near symmetrical increase in the bidirectional fluxes of MTX resulting in only a small increase in the transmembrane chemical gradient at low extracellular folate levels. Hence, increased expression of RFC1, alone, may not be an efficient adaptive response to folate deprivation, and other factors may come into play to account for the marked increases in concentrative folate transport which occur when cells are subjected to low folate-selective pressure.

The membrane transport of folates and antifolates in L1210 murine leukemia cells is mediated by several distinct processes. The reduced folate carrier mediates the bidirectional fluxes of reduced folates (1–4). Under conditions of low folate-selective pressure, folate receptors can be expressed which are anchored to the cell membrane through a glycosylphosphatidylinositol moiety and translocate folates into L1210 cells via an endocytotic process (5, 6). Finally, there are two independent pumps that export folates (7–9).

The properties of the reduced folate carrier have been characterized in detail based largely upon studies with the folate analog methotrexate (MTX).¹ This carrier is not directly linked to energy metabolism and has a high affinity for reduced folates and 4-aminoantifolates but a low affinity for oxidized folates such as folic acid (1, 10, 11). Of particular interest, but least well understood, is the impact of the carrier on transmembrane folate gradients. Uphill folate transport is generated through an anion exchange mechanism linked to the transmembrane organic phosphate gradient (10, 12, 13). Under steady-state conditions an electrochemical potential difference for free MTX (a bivalent anion) across the cell membrane is observed ($[MTX]_i > [MTX]_e$) which decreases as the extracellular drug concentration increases and the carrier saturates (1). The extent of the transmembrane folate gradient achieved is modulated further by the energy status of the cells through the activity of the independent exit pumps that are highly sensitive to the energy charge of the cell (7). Hence, glucose depresses and metabolic poisons increase the free intracellular MTX level through their respective stimulation and inhibition of the exit pumps (7, 10, 14, 15).

With the recent cloning of the putative reduced folate carrier RFC1 from several species (16–22), new opportunities have arisen to understand better the characteristics of this carrier and the contributions it makes to the transport of folates. This paper focuses, for the first time, on the role that RFC1 plays in concentrative transport within the context of a highly quantitative analysis of the transport properties of an L1210 murine leukemia cell line, MTX^rA-R16, which expresses a high level of RFC1. This line was derived from MTX^rA in which endogenous carrier is present at the cell surface but is not functional because of a point mutation in RFC1 (21, 23). MTX^rA-R16 is therefore a model for studying the properties and consequences of a quantifiable level of RFC1 distinct from the properties of the endogenous transporter.

The specific issues addressed are: (i) the impact of RFC1

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¹ The abbreviations used are: MTX, methotrexate; RFC, reduced folate carrier; GAT, glycine, adenosine, thymidine; HBS, HEPES-buffered saline; $[MTX]_e$ and $[MTX]_i$, extracellular and free intracellular MTX levels, respectively.

expression on the transmembrane fluxes of MTX and on MTX gradients achieved and (ii) how these parameters correlate with the level of polyglutamyl derivatives formed and changes in the cellular growth requirements for 5-formyltetrahydrofolate and folic acid and growth inhibition by MTX. Finally, the properties of MTX transport in RFC1-transfected cells are analyzed within the context of what has been reported in cell lines in which carrier-mediated transport is enhanced under conditions of low folate-selective pressure (24–26).

MATERIALS AND METHODS

Chemicals—[3',5',7-³H]MTX was obtained from Moravsek Biochemicals (Brea, CA) and purified by high performance liquid chromatography before use (27). The final specific radioactivity used in transport experiments was 170 dpm/pmol. [carboxyl-¹⁴C]Inulin (2.34 mCi/g) was purchased from NEN Life Science Products. Authentic polyglutamate standards were a gift from Dr. J. Bertino (Memorial Sloan-Kettering Cancer Center). All other reagents were obtained in the highest purity available from various commercial companies.

Derivation of Cell Lines and Culture Conditions—Cells were grown in RPMI 1640 medium containing 2.3 μ M folic acid, supplemented with 10% bovine calf serum (HyClone), 2 mM glutamine, 20 μ M 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μ g/ml). For some experiments, cells were grown in folate-free RPMI 1640 medium supplemented as above and with 10% dialyzed bovine calf serum, 200 μ M glycine, 100 μ M adenosine, and 10 μ M thymidine (GAT).

MTX^rA-R16 was obtained by transfection of MTX^rA with a cDNA encoding the murine reduced folate carrier as reported previously (21). Transfection of L1210 cells with RFC1 cDNA produced the L1210-T2 clone that was used in some experiments. This was achieved with the same cDNA and experimental strategy. Briefly, $\sim 1 \times 10^7$ L1210 cells were electroporated (250 V, 200 microfarads) with BglII-linearized pPGK-RFC1(21). Transfected cells were selected in RPMI 1640 medium containing G418 (750 μ g/ml). The L1210-T2 line was isolated by dilution cloning.

Northern Analyses—Total RNA was isolated using TRIzol (Life Technologies, Inc.), and 30 μ g of RNA was fractionated by electrophoresis on 1.0% formaldehyde-agarose gels. Transfer and hybridization were performed as described previously (21) except that the transferred RNA was fixed with a Stratalinker UV cross-linker, (Stratagene, La Jolla, CA).

Growth Studies—Cells grown in 96-well plates (1×10^5 cells/ml) were exposed continuously to the appropriate concentrations of MTX, folic acid, or 5-formyltetrahydrofolate for 72 h after which cell numbers were determined by hemocytometer count and viability assessed by trypan blue exclusion. Cells were grown in folate-free medium containing GAT for 2 weeks before the assessment of folic acid and 5-formyltetrahydrofolate growth requirements.

MTX Transport Studies—Influx measurements were performed by methods described previously (21) with minor modifications. Briefly, cells were harvested, washed twice with HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose, pH 7.4) and resuspended in HBS to 1.5×10^7 cells/ml. Cell suspensions were incubated at 37 °C for 20 min after which uptake was initiated by the addition of [³H]MTX and samples taken at the indicated times. Uptake was terminated by injection of 1 ml of the cell suspension into 10 ml of ice-cold HBS. Cells were collected by centrifugation, washed twice with ice-cold HBS, and processed for determination of intracellular tritium (21). For most influx determinations uptake intervals were adjusted so that cell MTX did not exceed the dihydrofolate reductase binding capacity assuring that unidirectional uptake conditions were sustained. At high [MTX]_e concentrations in transfected cells uptake was so rapid that intracellular levels sometimes exceeded the dihydrofolate reductase binding capacity, but under these conditions uptake was always shown to represent initial rates. For influx determinations in L1210 and MTX^rA-R16 cells, intracellular tritium was 250–1,000 dpm. Under steady-state conditions intracellular tritium was 1,800 and 2,600 dpm in L1210 and MTX^rA-R16 cells, respectively. Intracellular water was determined as reported previously using [carboxyl-¹⁴C]inulin as the extracellular marker (15).

For efflux measurements cells were loaded with [³H]MTX, a small portion was taken for measurement of intracellular MTX, and the remaining cells were separated by centrifugation and resuspended into a large volume of MTX-free buffer. Samples of L1210 and RFC1-transfected cells were obtained every 10 or 60 s, respectively, injected into 10 ml of ice-cold HBS, separated by centrifugation, washed twice, and then

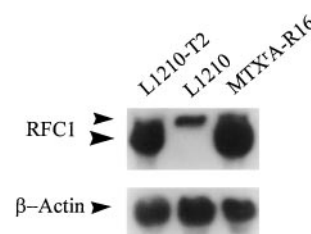


FIG. 1. Representative Northern blot analysis of total RNA from L1210-T2, L1210, and MTX^rA-R16 cells. RNA was probed successively with the full-length RFC1 and β -actin cDNA. The *small* and *large* arrows designate, respectively, the endogenous RFC1 transcript and the transcript produced from the expression vector. Transcripts were quantitated by PhosphorImager analysis of the radioactive blots and normalized to β -actin. Values for MTX^rA-R16 and L1210-T2 are normalized to an L1210 level of 1.

processed for intracellular tritium. The dihydrofolate reductase binding capacity was the level of MTX remaining in cells after efflux for 30–45 min, an interval sufficient to allow for the exit of all free drug.

Formation and Analysis of MTX Polyglutamates—Cells grown for 3–7 days in folate-free RPMI medium containing GAT were harvested and resuspended at a density of 5×10^5 cells/ml in fresh medium containing 0.1 μ M [³H]MTX. After a 14-h incubation, cells were collected by centrifugation and washed twice with ice-cold HBS. One portion of the cells was assayed for total antifolate. The larger portion was extracted with 0.5 ml of 20% trichloroacetic acid for 10 min at 4 °C, then neutralized by the addition of 0.25 ml of 1 M NaH₂PO₄ and 0.15 ml of 6 M NaOH. After separating the precipitate by centrifugation, the supernatant was analyzed by high performance liquid chromatography as described previously (28). Authentic MTX-Glu_(1–6) standards were included in the samples and the fractions monitored by UV absorbance (280 nm).

RESULTS

Impact of RFC1 Transfection on the Net Transport and Bidirectional Fluxes of MTX in MTX^rA-R16 Cells—Fig. 1 is an analysis of the level of endogenous and transfected RFC1 mRNA in the cell lines probed with the full-length murine RFC1 cDNA (21). Based upon Northern blot determinations of four separate RNA preparations, the mRNA levels were comparable in MTX^rA-R16 and L1210-T2 cells and exceeded the level in L1210 cells by factors of 6.8 ± 1.3 and 6.7 ± 0.4 , respectively. As illustrated in Fig. 2A, when the extracellular MTX level is 1 μ M the rate and extent of MTX uptake in the MTX^rA-R16 transfectant are increased. Steady-state levels of MTX are reached rapidly in the MTX^rA-R16 cells (~ 10 min) with about twice that duration required to achieve steady state in L1210 cells. The free intracellular MTX level (indicated by the *double-headed arrows*) is increased by a factor of about 2 in the MTX^rA-R16 line based upon the difference between total drug and tightly bound MTX determined when cells are separated by centrifugation and resuspended in MTX-free buffer. As indicated in Fig. 2B, the initial uptake rate in MTX^rA-R16 cells is increased markedly.

Efflux of MTX is enhanced in MTX^rA-R16 cells as well. When cells are loaded with MTX and then cellular levels are determined upon resuspension into MTX-free buffer, free MTX is cleared from MTX^rA-R16 cells within 2 min. Efflux is much slower in L1210 cells (Fig. 3A). When the efflux of free drug is analyzed by subtracting the bound fraction from total MTX, differences in the efflux rate constants can be derived from the slope of the semilogarithmic plot as shown in Fig. 3B. The efflux rate constant for MTX in the MTX^rA-R16 line is increased markedly.

Table I summarizes transport parameters along with other cellular properties in MTX^rA-R16 and L1210 cells from four separate experiments in which the extracellular MTX level was 1 μ M. The tightly bound fractions are comparable; the ratio of the intracellular water to dry weight is slightly increased in

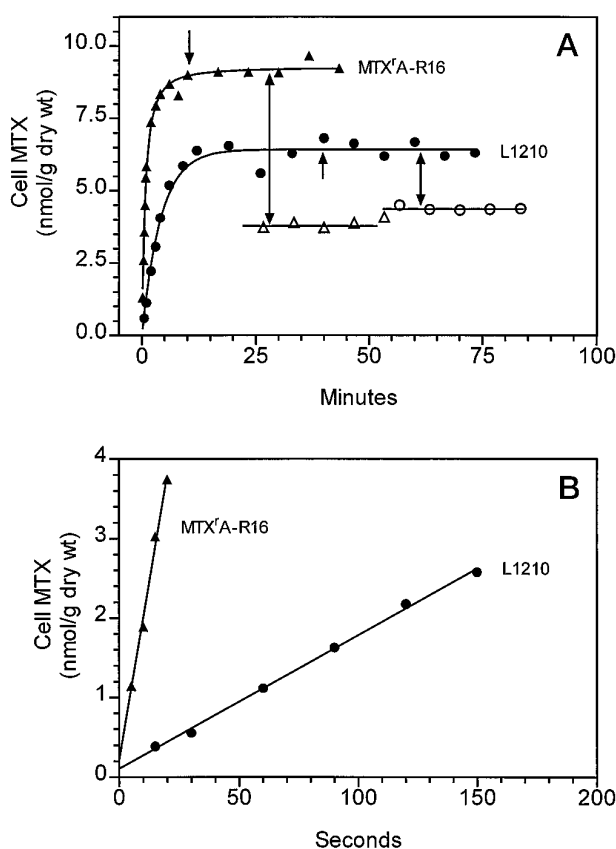


FIG. 2. *Panel A*, representative time course of the net uptake of MTX in L1210 and MTX^rA-R16 cells. Cells were exposed to 1 μ M [³H-MTX] at time zero and the time course of uptake monitored (closed symbols). At the single-headed arrows portions of the cell suspension were separated by centrifugation and resuspended into large volumes of drug-free buffer for determination of the loss of free drug (indicated by the double-headed arrows) and the tightly bound component (open symbols). *Panel B*, analysis of MTX influx. Cells were exposed to [³H-MTX] at time zero, and samples of the suspension were taken for cell MTX determinations.

MTX^rA-R16 cells. Overall, at this extracellular MTX level, MTX influx is increased by a factor of 9.1, the efflux rate constant is increased by a factor of 5.1, and steady-state [MTX]_i is increased by a factor of only 2.1 in MTX^rA-R16 cells. Hence, the major impact of the increased expression of RFC1 is a marked increase in the bidirectional fluxes of MTX (influx > efflux) with a much smaller change in the free drug level achieved.

The extent to which transport properties associated with the overexpression of RFC1 in MTX^rA-R16 cells might be influenced by the characteristics of the MTX^rA line from which it was derived was assessed. Parent L1210 cells were transfected with RFC1 cDNA and a clone obtained, L1210-T2, with a comparable increase in carrier expression (Fig. 1). Changes in initial uptake rate and steady-state levels achieved in L1210-T2 cells were similar to what was observed in MTX^rA-R16 cells (data not shown). Hence, the uptake characteristics produced by overexpression of RFC1 were not unique to the MTX^rA-R16 line.

Effect of RFC1 on Influx Kinetics in MTX^rA-R16 Cells—MTX influx kinetic parameters determined by the methods of Line-weaver-Burk and Eadie-Hofstee were identical. Based upon three separate experiments, the influx K_m for L1210 and MTX^rA-R16 cells was identical (5.7 ± 1.0 and 5.8 ± 1.8 μ M, respectively). The V_{max} was increased by a factor of 8.4 in MTX^rA-R16 relative to L1210 cells (69.0 ± 9.1 and 8.2 ± 0.78 nmol/g dry weight/min, respectively), comparable to the in-

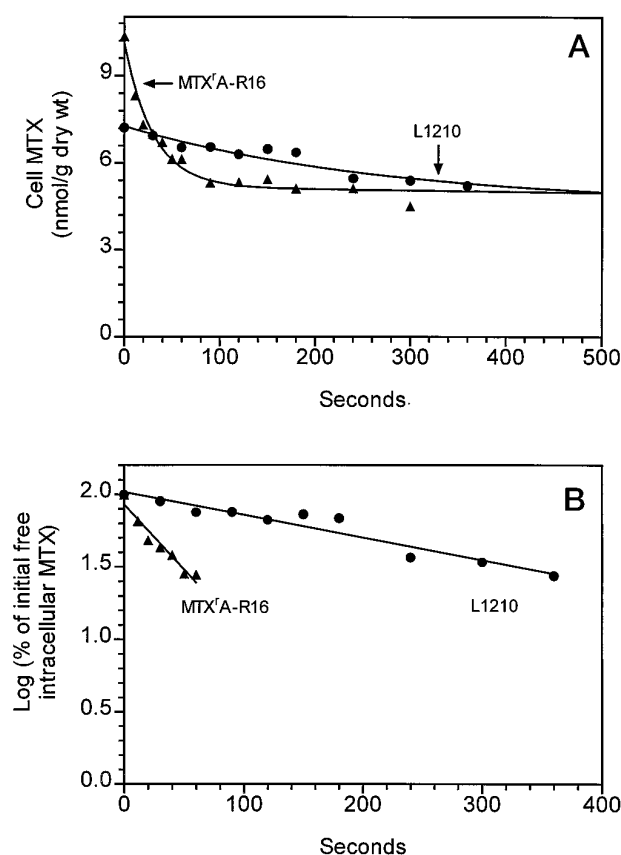


FIG. 3. *Panel A*, representative time course of MTX efflux. Cells loaded with 1 μ M MTX to achieve steady-state conditions were separated by centrifugation, resuspended into a large volume of drug-free buffer, and intracellular MTX levels measured every 10–30 s. *Panel B*, a corresponding semilogarithmic analysis of the data which illustrates the log of the percentage of the initial free intracellular MTX level as a function of time. The slope of the line is the efflux rate constant.

TABLE I
Comparison of MTX transport between L1210 and MTX^rA-R16 cells

Transport parameters were measured at [MTX]_e = 1 μ M. Free MTX levels measured under steady-state conditions represent total drug less drug bound to cells after efflux into MTX-free buffer. Data are the mean \pm S.E. of four experiments.

	L1210	MTX ^r A-R16	Δ
			fold
Initial uptake rate (nmol/min/g dry wt)	1.03 ± 0.07	9.35 ± 0.68	9.1
Efflux rate constant (min ⁻¹)	0.13 ± 0.05	0.64 ± 0.10	5.1
Total cell MTX (nmol/g dry wt)	7.07 ± 0.33	10.15 ± 0.23	1.4
Bound MTX (nmol/g dry wt)	4.50 ± 0.09	4.35 ± 0.07	1.0
Free MTX (nmol/g dry wt)	2.56 ± 0.43	5.82 ± 0.18	2.3
[H ₂ O]/dry wt (μ l/mg)	3.52 ± 0.06	3.86 ± 0.17	1.1
[MTX] _i (μ M)	0.73 ± 0.12	1.51 ± 0.05	2.1

crease in influx as indicated in Table I.

Effect of the Extracellular MTX Level on the Transmembrane Gradients Achieved in L1210 and MTX^rA-R16 cells—As reported previously for L1210 cells (1), when [MTX]_e is increased the steady-state intracellular MTX concentration approaches maximum values of 8.8 and 41.4 μ M, for L1210 and MTX^rA-R16 cells, respectively (Fig. 4A). Further, the concentrating capacity of MTX^rA-R16 cells is increased relative to that of L1210 cells over this 300-fold concentration range (Fig. 4B). Hence, the concentrating advantage, i.e. the ratio of the steady-state [MTX]_i levels in MTX^rA-R16 to L1210 lines, increased from 1.8 at 0.1 μ M to 3.8 at 30 μ M MTX. The maximum concentrating advantage for MTX^rA-R16 cells based upon the predicted max-

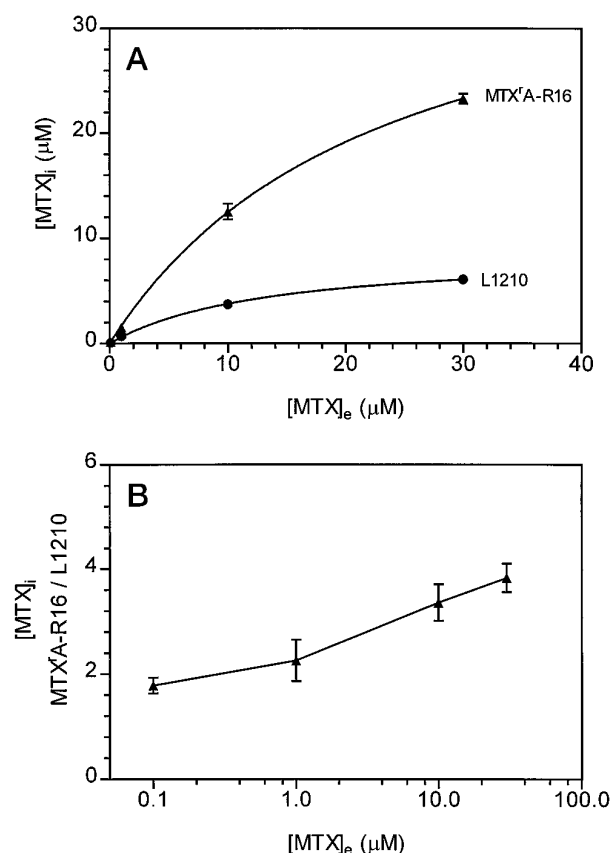


FIG. 4. *Panel A*, free intracellular MTX concentrations as a function of the extracellular drug levels under steady-state conditions. *Panel B*, ratio of the free drug concentration in the MTX^A-R16 transfectant to L1210 cells as a function of the extracellular drug level (the latter plotted logarithmically). The data are the average \pm S.E. of three experiments.

imum intracellular levels indicated above is 4.8.

Polyglutamylation of MTX—The monoglutamyl MTX substrate level, controlled by the membrane transport system, is one key determinant of the rate of formation of its polyglutamate derivatives (27, 29). The impact of increased carrier expression on MTX polyglutamate formation was evaluated in MTX^A-R16 cells at the lowest feasible extracellular drug level to approximate those conditions at which cytotoxicity is determined (see below). When cells are incubated with 0.1 μ M MTX over 14 h, there is a \sim 20% increase in the total antifolate level in MTX^A-R16 cells (representing free, bound, mono-, and polyglutamyl forms of drug). There is, however, a nearly 2-fold increase in the total polyglutamate level in the transfectant (Fig. 5). The major derivative in L1210 cells is the triglutamate, whereas the major derivatives in the MTX^A-R16 line are the tri- and tetraglutamates with appreciable levels of the pentaglutamate as well. The monoglutamate level in the MTX^A-R16 (1.2 nmol/g dry weight) is only a small fraction of the dihydrofolate reductase binding capacity (4.5 nmol/g dry weight), indicating that the drug is replaced on the enzyme by the larger levels of polyglutamyl congeners within these cells. Monoglutamyl MTX bound to dihydrofolate reductase in L1210 cells was also replaced by the polyglutamyl forms but less so than in MTX^A-R16 cells.

Folate Growth Requirements and MTX Sensitivity in the L1210 and MTX^A-R16 Lines—Of particular interest was the impact that increased carrier expression might have on MTX growth inhibition in MTX^A-R16 cells. Would the pharmacologic effect of this agent parallel the large changes in influx or the much smaller change in the free MTX level achieved? As

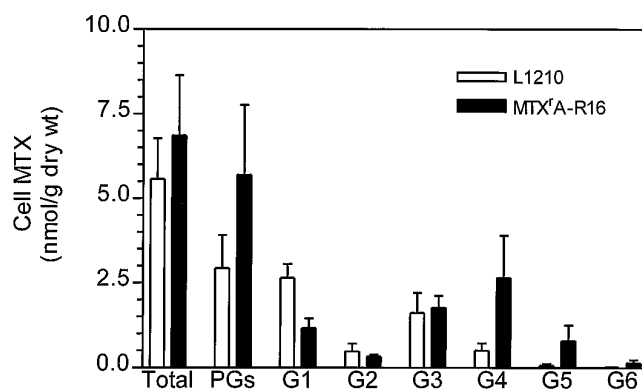


FIG. 5. **Formation of MTX polyglutamyl derivatives in L1210 and MTX^A-R16 cells.** Cells were exposed to 0.1 μ M [³H]MTX for 14 h under usual growth conditions but in the presence of GAT to circumvent MTX cytotoxicity. After this, mono- and polyglutamyl derivatives were measured as described under "Materials and Methods." Data are the average of three experiments performed on different days; G1 is MTX, G2 the dihydroglutamate, etc.

indicated in Fig. 6A, the MTX IC₅₀ decreased by a factor of less than 2 in MTX^A-R16 cells, comparable to the increase in [MTX]_i at low levels of [MTX]_e (Table I and Fig. 4) and much less than the increase in influx of the drug. Similar to this finding, the EC₅₀ for both 5-formyltetrahydrofolate (Fig. 6B) and folic acid (Fig. 6C) also decreased by a factor of less than 2.

DISCUSSION

The membrane transport of physiological substrates and antineoplastic agents is often considered within the context of how rapidly drugs enter cells. This, alone, can be an important determinant of the rate of biosynthetic processes and drug activity if, at the extracellular levels achieved, transport is rate-limiting to a critical metabolic event within the intracellular compartment such as, for instance, the metabolism of the substrate or agent to an impermeant derivative. For MTX and related antifolates, influx is a major determinant of the free drug level achieved, the critical determinant of cytotoxicity which is controlled by the membrane transport systems (30, 31). In this paper, we have quantitated and compared, for the first time, the impact of overexpression of high levels of RFC1 in L1210 leukemia cell lines on the bidirectional fluxes and net transport of MTX. The major focus of these studies is on the MTX^A-R16 transfectant derived from the MTX^A line. The latter is a derivative of L1210 cells resistant to MTX by virtue of an alanine to proline substitution within a predicted transmembrane domain which results in the immobilization of the reduced folate carrier (21). This model cell line provided the opportunity for evaluating the transport capacity and properties of RFC1 distinct from the properties of the endogenous transporter.

As expected, increased expression of RFC1 produced a large change in the rate of MTX transport into cells. In fact, the 8–9-fold increase in influx in the MTX^A-R16 line approximated the increase in the RFC1 mRNA level as determined by Northern blot analysis. This increase in influx did not, however, translate into a comparable increase in the steady-state free intracellular MTX level achieved. Indeed, at low extracellular MTX levels, the major change that RFC1 produces is a large, near symmetrical, increase in the bidirectional fluxes of MTX. Based upon the increase in the influx V_{\max} for MTX, the net cycling of carrier is more than 8-fold faster in MTX^A-R16 than L1210 cells. There was no change in the influx K_m . The small (2-fold) difference between the increase in the influx V_{\max} and the efflux rate constants is consistent with the small but comparable \sim 2-fold increase in the net accumulation of MTX in

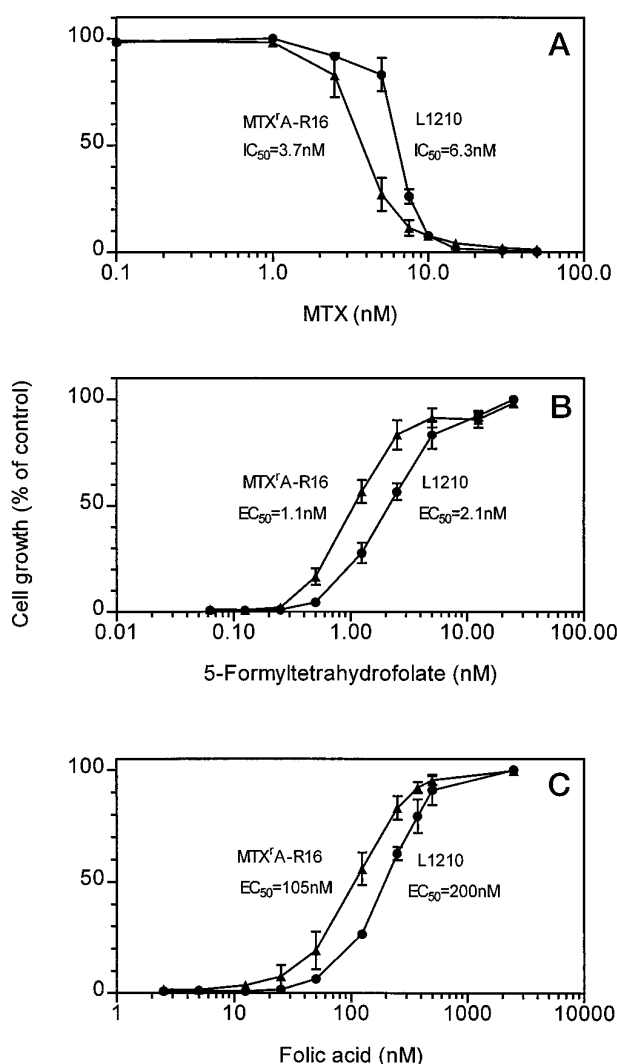


FIG. 6. Growth inhibition by MTX (panel A) and growth requirements for 5-formyltetrahydrofolate (panel B) and folic acid (panel C) in L1210 and MTX^R-A-R16 cells. Cells were grown in GAT-containing medium with dialyzed calf serum and without added folates for 2 weeks before analysis of growth requirements for folates. The data represent the average \pm S.E. of three experiments.

MTX^R-A-R16 cells. However, as the extracellular MTX level was increased, the concentrating capacity of the transfected cells relative to L1210 cells increased with a maximum achievable gradient difference of nearly 5. This is a level still \sim half the increase in the influx V_{max} .

Changes in MTX cytotoxicity mirror the changes in accumulation of the drug and not the rate that MTX enters cells in that the IC_{50} for MTX dropped by a factor of less than 2 in the MTX^R-A-R16 line. This is consistent with the concept that transport of MTX into cells at the low concentrations utilized in these studies must be much faster than the rate of polyglutamylation to its less permeable derivatives, a phenomenon that was recently shown to be the case at MTX levels at least as low as 0.1 μ M (28). Further, if drug entry was rate-limiting, then the marked difference in MTX influx between L1210 and MTX^R-A-R16 cells would result in a similar difference in the rate and extent of formation of polyglutamates. Instead, differences in the accumulation of polyglutamyl derivatives mirror differences in the free monoglutamyl drug levels achieved. Likewise, the 5-formyltetrahydrofolate and folic acid growth requirements, as reflected in the EC_{50} , were also decreased by a factor of only 2. Hence, influx must not be rate-limiting to the utili-

zation of these folates under physiological conditions, and free folate levels must be sustained during growth. It is of considerable interest that the change in folic acid growth requirement is exactly the same as that observed for 5-formyltetrahydrofolate, suggesting that despite the low affinity of folic acid for the RFC, transport by this route can be an important mechanism of transport for this oxidized folate when expression of RFC1 is sufficiently high. The data also indicate that when cells are deprived of reduced folates, overexpression of RFC1, alone, is not an efficient mechanism of adaptation because this has only a small salutary effect on the free folate level achieved.

RFC1 has the characteristics of a large family of membrane-spanning proteins that transport a variety of substrates across cell membranes and do not contain an ATP binding region so that transport is not linked directly to ATP hydrolysis (32–35). Some of these carriers are equilibrating; they facilitate translocation across the cell membrane but do not produce uphill transport (32). Others are coupled to some other transmembrane gradients and produce uphill transport such as the sodium-dependent iodide and nucleoside transporters (33, 34). RFC-mediated transport is not sodium-dependent (10). Rather, MTX transport is highly sensitive to the anionic composition of its environment, and virtually any negatively charged species inhibits influx of this agent when present at a high enough concentration. The reduced folate carrier demonstrates a high degree of structural specificity for folates, and there is a spectrum of inhibitory activity observed with structurally unrelated anionic compounds as well. In general, the greater the negative charge, the greater the inhibitory effect. Organic anions are more inhibitory than inorganic anions; the most potent inhibitors are the organic phosphates (10, 12, 13).

Studies from this laboratory suggested a model in which the organic phosphate gradient across the cell membrane, through its interaction with carrier, acts as a battery that drives MTX uphill into cells (10). Subsequent studies further supported this concept (36–38). According to this paradigm organic phosphates that accumulate within cells inhibit efflux of folates much more than influx is inhibited by extracellular inorganic anions. This asymmetrical inhibition of the bidirectional fluxes results in uphill transport of folates into cells (10). This interaction would not be immediately perturbed by metabolic poisons that decrease the cellular energy charge but not the net organic anion level as phosphate and ADP, both inhibitors of the carrier (10, 13), are released in the hydrolysis of ATP. This would account for the maintenance, indeed, enhancement of uphill MTX transport under conditions in which cells are energy-depleted (7, 10, 14, 15).

In L1210 cells then, the free MTX level achieved will be determined by the mobility and number of carriers, the composite anion gradient across the cell membrane, and the relative potency of the independent exit pumps (see the Introduction). As carrier cycling increases relative to efflux via the exit pumps, as occurs with increased carrier expression in transfected cells and with increasing extracellular MTX levels, the relative contribution to the net transport of MTX by the exit pumps decreases, and the free intracellular MTX level rises. Ultimately, when efflux via the pumps becomes negligible compared with efflux mediated by the carrier, the transmembrane MTX gradient increases to a level determined solely by the interaction among inorganic and organic anions, MTX and the reduced folate carrier.

Of particular interest is the comparison between these observations and the data obtained when carrier expression is enhanced under low 5-formyltetrahydrofolate-selective pressure. Studies from three laboratories have demonstrated that under these conditions there are marked increases in MTX

influx because of an increase in the influx V_{\max} with little or no change in the efflux rate constant, thereby resulting in a marked increase in the free drug level achieved and the generation of large transmembrane gradients for MTX (24–26). As indicated above, the most efficient adaptation to low 5-formyltetrahydrofolate-selective pressure would be one that results in a large increase in the free folate level. The *asymmetrical* change in the bidirectional fluxes of MTX obtained with low folate-selective pressure is different from the more *symmetrical* changes observed with the transfection of RFC1 into L1210 cells. The basis for this difference is not clear. It is possible that another protein element is expressed, or RFC1 is modified, such that uphill folate transport is increased through the suppression of carrier-mediated efflux, when cells are exposed to low folate-selective pressure. Alternatively, folate deprivation, although increasing carrier expression, might produce a secondary depression in the exit pumps (39) holding the net kinetics of the efflux processes essentially constant. The near symmetrical changes in the bidirectional fluxes in MTX^rA-R16 cells with only a small increase in net transport at low [MTX]_e levels cannot be attributed to the unique properties of the MTX^rA line from which the RFC1-transfected subline was derived since a similar phenomenon is observed in the L1210 derivative, L1210-T2, expressing a comparable high level of carrier.

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