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Comparison of Methotrexate Polyglutamylation in L1210 Leukemia Cells When Influx Is Mediated by the Reduced Folate Carrier or the Folate Receptor

LACK OF EVIDENCE FOR INFLUX ROUTE-SPECIFIC EFFECTS

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ABSTRACT. We previously described a methotrexate-resistant L1210 cell line (MTX^{r}A) that lacks a functional reduced folate carrier and does not appreciably express the folate receptor. In the present study, we utilized MTX^{r}A cell lines stably transfected with cDNAs encoding either the folate receptor or the reduced folate carrier to investigate the influence of the route of folate influx on the rate and extent of methotrexate polyglutamylation. At an extracellular methotrexate concentration of 0.1 μM, influx in the folate receptor transfectant (MTX^{r}A-TF1) and in the reduced folate carrier transfectant (MTX^{r}A-R1) was equal and methotrexate polyglutamates accumulated at an identical rate, but the onset was delayed until dihydrofolate reductase was saturated with the monoglutamate (~3 hr). The onset of polyglutamate formation was immediate and identical among the lines in cells pretreated with the lipophilic dihydrofolate reductase inhibitor trimetrexate to block methotrexate binding to dihydrofolate reductase. The spectra of individual methotrexate polyglutamates that accumulated were similar, with the tetraglutamate present as the predominant form. A 100-fold higher methotrexate concentration was required to detect methotrexate uptake and polyglutamylation in the transport defective parent MTX^{r}A line, demonstrating that diffusion or an unidentified low affinity route also supports polyglutamylation. Since the folate receptor and the reduced folate carrier achieve nearly identical rates of polyglutamylation despite very different mechanisms of methotrexate delivery, the data suggest that transport-mediated substrate channeling to folylpolyglutamate synthetase is unlikely to play a role in tetrahydrofolate metabolism. This study supports the notion that it is the intracellular concentration of methotrexate achieved within the cell that drives polyglutamylation irrespective of its route of entry. BIOCHEM PHARMACOL 52;5:703–712, 1996.

KEY WORDS. folate receptor; reduced folate carrier; methotrexate; transport; polyglutamylation; L1210

In mammalian cells, reduced folates are essential for sustaining one-carbon transfer reactions that lead to the synthesis of thymidine, purines, and amino acids. Folate-dependent enzymes have been targeted in the treatment of neoplasia by virtue of inhibition of DNA, RNA, and protein synthesis. The classical antifolate MTX† has a long history as a chemotherapeutic agent due to potent inhibition of the enzyme DHFR. Newer classes of antifolates that directly inhibit enzymes involved in purine and pyrimidine biosynthesis are being evaluated currently (reviewed in Ref. 1).

Variations in membrane transport and intracellular metabolism of antifolates are important determinants of cytotoxicity and selectivity of these agents [2]. Defects in transport or metabolism are common mechanisms of antifolate drug resistance [3–8], along with amplification or alterations in the specific target enzyme [4, 9–11].

Folates and antifolates including MTX enter cells via two major transport systems. The sole or major component of the RFC system is a protein (RFC1) that in murine L1210 cells has a molecular mass of 57 kDa [12, 13] and is a member of a superfamily of proteins with twelve transmembrane domains that include P-glycoprotein and glucose transporters. This system is a typical carrier-mediated process [14–16], generates transmembrane gradients [14, 17, 18], and has a higher affinity for MTX and reduced folates (~1–5 μM) than for folic acid (100–200 μM) [17, 19, 20].

The second transport system is comprised of the glycosylphosphotidylinositol-anchored FR that internalizes folates via an endocytotic process (reviewed in Ref. 21; see also Refs. 22 and 23). In L1210 cells, two distinct folate receptor

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† *Abbreviations:* BSP, sulfobromophthalein; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; FR, folate receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAT, 200 μM glycine, 100 μM adenosine, and 10 μM thymidine; HBS, HEPES-buffered saline; MTX, methotrexate; RFC, reduced folate carrier; and TMQ, trimetrexate.

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isoforms (FR-α and FR-β) have been identified [24]. These heavily glycosylated proteins (38 kDa) have high affinity for folic acid (1 nM) and reduced folates (10–40 nM) and a lower affinity for MTX (150–170 nM for FR-α) [21, 22, 25]. Endocytosis mediated by the FR appears to be fundamentally different from transport mediated by the RFC in terms of energy, ion, and pH dependencies [23, 26] and in L1210 cells there is no evidence of a functional linkage between the two processes [23, 26]. In addition to these routes, diffusion can allow entry of folate compounds when the extracellular concentrations are high.

Upon entering cells, folates are metabolized to polyglutamate derivatives by the sequential addition of γ-glutamyl residues catalyzed by the enzyme FPGS. This derivitization is required for folate retention, optimal folate utilization, and survival of proliferating mammalian cells. Polyglutamylation has been shown to play a key role in the cytotoxic and selective action of MTX [5–8, 27–29].

Substrate channeling has been documented for several folate-dependent enzymes and likely plays a role in facilitating efficient tetrahydrofolate metabolism (reviewed in Ref. 30). Channeling involves multifunctional enzymes or enzyme–enzyme complexes in which the metabolic intermediate is passed directly to the next enzyme site without dissociation from the complex. Likewise, there is compartmentation of folates and folate-requiring enzymes, primarily between the cytoplasm and mitochondria [30, 31]. FPGS exists in both compartments [32, 33], and polyglutamylation in mitochondria plays a critical role in folate homeostasis of the cell [32–34].

We previously developed a transfection system that allowed separate study of the specific transport properties of the FR or the RFC [13, 23]. MTX^{r}A is an L1210 leukemia line that lacks a functional reduced folate carrier due to a missense mutation in the RFC1 protein [13, 35]. MTX^{r}A transfectants MTX^{r}A-TF1 and MTX^{r}A-R1 have been shown to transport MTX exclusively via the FR or the RFC due to the constitutive expression of each transporter cDNA [13, 23]. Since this is the sole genetic difference between the lines, any changes in folate metabolism can be attributed to transport route effects. In this study, we utilized these lines to assess the influence of the pathway of folate transport into the cell on MTX polyglutamylation. The results demonstrate that the rate and extent of MTX polyglutamylation are nearly identical when entry is mediated by the FR or the RFC. Furthermore, polyglutamylation was also detected in the absence of FR and RFC activity when the MTX concentration was sufficiently high. Since the FR and the RFC have very distinct mechanisms of MTX delivery, the data demonstrate that the intracellular concentration of MTX achieved drives polyglutamylation irrespective of its route of entry.

MATERIALS AND METHODS

Chemicals

[3′,5′,7′-^{3}H]MTX was obtained from Moravek Biochemicals (Brea, CA) and was purified by HPLC prior to use [36]. [3′,4′-^{3}H]Glutamic acid was purchased from ICN Radiochemicals (Irvine, CA) and was used without purification. All other reagents were obtained in the highest purity available from various commercial sources. MTX polyglutamate standards were the gift of Dr. C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile, AL). Purified thymidylate synthetase from *Lactobacillus casei* was prepared as previously described [37].

Cell Culture and Development of MTX^{r}A

Murine L1210 leukemia cells and sublines were grown in RPMI 1640 medium (containing 2.3 μM folic acid) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL). MTX^{r}A, an MTX-resistant L1210 leukemia cell line, was developed previously [35] from a single-step selection in 50 nM MTX and cloned in soft agar. The transport defect [35] in this cell line has been shown recently to result from a point mutation in the RFC gene [13]. MTX^{r}A was maintained in RPMI 1640 medium supplemented as above with the addition of 50 nM MTX. To deplete endogenous folates, cells were cultured in folate-free RPMI medium with 10% dialyzed bovine calf serum and GAT 4–7 days prior to use.

MTX^{r}A Transfectants

MTX^{r}A-TF1 was developed previously [23] by transfecting MTX^{r}A with an expression vector pKJ-FR-α, encoding the full-length murine FR-α cDNA previously isolated from an L1210 leukemia subline upregulated for the folate receptor [24]. MTX^{r}A-R1 was developed previously [35] by transfecting MTX^{r}A with an expression vector pKJ-RFC1 encoding the full-length murine RFC cDNA generated by reverse transcription-polymerase chain reaction utilizing RNA isolated from murine erythroleukemia cells with primers based on the sequence reported by Dixon *et al.* [12]. Several independent, stably transfected clones were generated in the course of the previous study (designated R1 to R16) [13], and MTX^{r}A-R1 was chosen since it regained RFC function to a level identical with wild-type L1210. The FR and RFC transcripts in the transfected cell lines are under the control of the constitutive murine phosphoglycerate kinase promoter [38]. Both the MTX^{r}A-TF1 and MTX^{r}A-R1 cell lines were isolated by dilution cloning.

Northern Analysis

Total RNA was isolated using RNAzol B (Biotecx), and Northern hybridizations were performed as previously described [24].

Probes

The probe for murine FPGS was generated by screening a λ phage cDNA library previously developed from MTX^{r}A cells [13] with a full-length human FPGS cDNA [33, 39]. We report the murine FPGS cDNA sequence elsewhere [40]. The DHFR and GAPDH probes are the full-length murine cDNAs as previously described [13, 23].

Transport Studies

Influx measurements were performed by methods described previously [41]. Cells grown for 3–7 days in folate-free RPMI medium supplemented with GAT were harvested and washed twice with folate-free RPMI medium with GAT but without serum (assay medium) and resuspended in this medium to 1×10^7 cells/mL. Cell suspensions were equilibrated at 37° with humidified 95% O_2/5% CO_2. Uptake was initiated by the addition of [^{3}H]MTX, and samples were taken over the indicated intervals. Influx was terminated by injecting 1.0 mL of the cell suspension into 10.0 mL of 0° acidic HBS (pH 4.5). Cells were collected by centrifugation, washed twice with 0° HBS (pH 7.4), and processed for determination of intracellular tritium as previously described [41]. Uptake intervals were adjusted so that intracellular MTX did not exceed the DHFR binding capacity, assuring that unidirectional uptake conditions were sustained. For some experiments, cells were first pretreated with 5 μM (TMQ) for 10 min to saturate DHFR. This allows for subsequently added [^{3}H]MTX to be immediately available for polyglutamylation. Portions of these cells were assayed for total MTX polyglutamates. For longer incubation intervals (12–24 hr), GAT and 10% bovine calf serum were added to the assay medium to maintain cell viability.

Analysis of Polyglutamates

A portion of the cell pellet was analyzed for polyglutamate metabolites of MTX. Immediately following the cold wash, the cell pellet was extracted with 0.25 mL trichloroacetic acid (10%) for 10 min at 0°. The acid extract was neutralized by the addition of 0.16 mL of 1 M KH_2HPO_4 and 0.040 mL of KOH. HPLC analysis was then performed as previously described [42]. MTX-$Glu_{(1-6)}$ standards were included in the samples and monitored by UV detection (254 nm).

Determination of Dihydrofolate Reductase Levels

Dihydrofolate reductase levels were determined as described previously [43]. Briefly, cells (1×10^7) were suspended in 0.5 mL of ice-cold buffer consisting of 0.05 M citrate, pH 6.0, 0.05 M mercaptoethanol, and 0.001 M EDTA. The cell suspension was disrupted with two 20-sec bursts from a model 300 probe sonifier (Artek, Farmingdale, NY), and the sonicate was centrifuged at 25,000 *g* for 30 min. The supernatant was collected, NADPH (final concentration 100 μM) and [^{3}H]MTX (1000 dpm/pmol; 1 μM) were added, and the mixture was incubated for 15 min at 37°. The supernatant was passed through a minicolumn of Bio-Gel P-6 (Bio-Rad, Richmond, CA) by centrifugation at 200 *g* for 10 min followed by 1300 *g* for 3 min. This permits separation of the enzyme–ligand complex in the centrifugate from the free ligand that remains on the column.

Determination of FPGS Activity

The FPGS microassay [37] was performed with minor modifications. Cells were homogenized in a buffer containing 250 mM sucrose, 50 mM Tris–Cl (pH 8.5), and 50 mM mercaptoethanol using the cup horn attachment of the cell sonicator (W385, Heat Systems, Ultrasonics, Inc). A 160,000 *g* supernatant was then prepared using a Beckman Airfuge at 30 psi for 20 min at 4°. The assay involves two consecutive reactions. In the first, which is carried out in 10 μL, the FPGS in the crude cytosol catalyzes the addition of L-[^{3}H]glutamic acid to tetrahydrofolate. In the second reaction, the newly formed L-[^{3}H]tetrahydropteroyldiglutamate is incorporated into a covalently bound complex in the presence of thymidylate synthetase, formaldehyde, and FdUMP. The reaction mixture is then passed through a Sephadex G-50 spin column to isolate the macromolecular product from unreacted [^{3}H]glutamic acid, and the radioactivity is determined in a liquid scintillation counter. Protein concentration was determined using the Bio-Rad Protein Assay.

RESULTS

Assessment of FPGS and DHFR Levels

MTX^{r}A is an L1210 leukemia cell subline resistant to MTX by virtue of a 100-fold decrease in MTX influx [23, 35] due to a defect in the function of the reduced folate carrier [35]. This defect has been shown recently to be the result of a point mutation in the RFC1 gene [13]. The cell line has no appreciable FR expression [23]. MTX^{r}A-TF1 and MTX^{r}A-R1 are MTX^{r}A sublines transfected with murine FR-α [23] or wild-type murine RFC1 [13], respectively. MTX^{r}A-TF1 mediates MTX uptake exclusively via the FR [23]. MTX^{r}A-R1 was chosen from a number of previously developed transfectants [13] based on its restoration of RFC-mediated transport to a level identical to that of L1210 cells. As shown in Fig. 1, FPGS and DHFR transcript levels were similar in all four cell lines. Compared with L1210 cells, FPGS activity did not vary among the MTX^{r}A, MTX^{r}A-

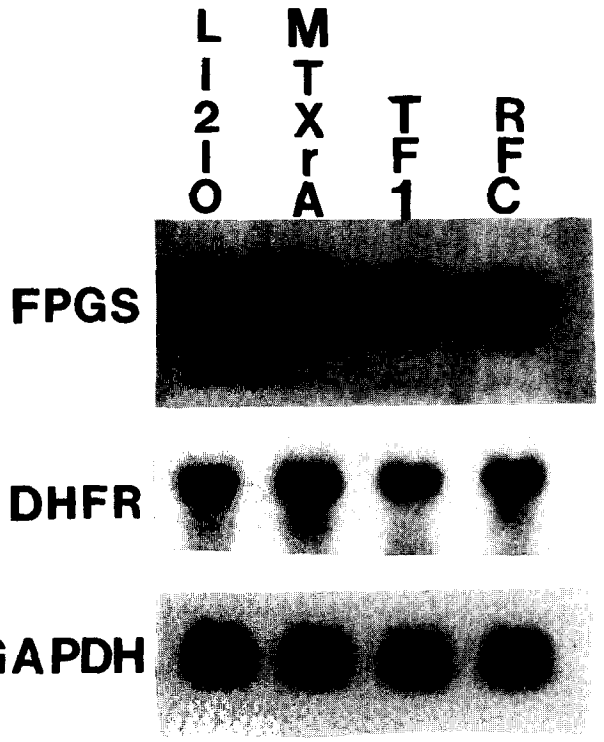

FIG. 1. Northern analyses of L1210, MTX^{r}A, and the MTX^{r}A transfectants MTX^{r}A-TF1 and MTX^{r}A-R1. Total RNA (10 μg) from L1210 (lane 1), MTX^{r}A (lane 2), MTX^{r}A-TF1 (lane 3), and MTX^{r}A-R1 (expressing RFC, lane 4) was hybridized with the cDNA for murine FPGS. The blot was then stripped and hybridized with probes for DHFR and GAPDH.

TF1, and MTX^{r}A-R1 cell lines, while DHFR binding levels were elevated slightly (1.2- to 1.4-fold) (Table 1).

Influx and Polyglutamylation of MTX

As shown previously [23] and in Fig. 2, the initial rate of MTX influx mediated by the RFC in L1210 cells was nearly identical to influx mediated exclusively by the FR in the MTX^{r}A-TF1 cell line when the extracellular MTX concentration was 0.1 μM. The MTX^{r}A-R1 line transported MTX at a rate comparable to L1210. At this MTX level, influx in MTX^{r}A is negligible. As shown in Fig. 2, MTX polyglutamates could not be detected until approximately 3 hr after the addition of 0.1 μM MTX, the time required to achieve intracellular MTX levels in excess of the level of DHFR in the various lines (3.5 to 5.0 nmol/g dry wt). Prior to saturation of the enzyme, influx of MTX was the same in all the lines. Following DHFR saturation, there was a fall in the net uptake rate to a constant slower velocity that was comparable in all the lines and paralleled the rate of accumulation of MTX polyglutamates. Over the 6 hr, the majority of MTX was in the monoglutamate form (determined as the difference between solid and dashed lines in Fig. 2). The slightly higher levels of total MTX that accumulated in MTX^{r}A-TF1 and MTX^{r}A-R1 compared with L1210 over the interval of 3–7 hr was due to the increase in DHFR-bound MTX monoglutamate consistent with the slightly higher levels of enzyme in these cell lines (Table 1).

TABLE 1. DHFR binding capacity and FPGS activity

	DHFR (nmol/g dry wt)	FPGS (nmol/mg protein/hr)
L1210	3.5 ± 0.4	1.5 ± 0.4
MTX^{r}A	4.3 ± 0.1	2.0 ± 0.9
MTX^{r}A-TF1	4.9 ± 0.2	1.6 ± 0.7
MTX^{r}A-R1	5.0 ± 0.1	1.6 ± 0.8

Values are means ± SD of 3 experiments.

Effect of TMQ on Influx and the Onset, Rate, and Extent of Polyglutamylation of MTX

To measure the initial rate of MTX polyglutamylation and to obviate the effects of slightly different levels of DHFR, cells were pretreated with the lipophilic DHFR inhibitor TMQ to saturate the enzyme prior to [^{3}H]MTX addition. TMQ is not transported via the RFC or the FR and is not a substrate for FPGS. Under these conditions, MTX is immediately available for polyglutamylation as it enters the cell. As shown in Fig. 3, MTX influx remained identical among the L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 cell lines (inset), but TMQ eliminated the differences in total net MTX accumulation over longer intervals. This reflects the loss of the DHFR-bound component of intracellular MTX monoglutamate. Loss of DHFR binding sites was also responsible for the overall 3-fold lower accumulation of total MTX in each cell line (note the difference in scale in Fig. 2 vs Fig. 3). The onset of polyglutamylation was essentially immediate; the line connecting polyglutamate levels as a function of time extrapolates through the origin. Moreover, under these conditions, the majority of accumulated MTX was in higher polyglutamate forms. Thus, pretreatment with TMQ resulted in a rapid initiation and hence more accurate measurement of the initial rate of MTX polyglutamylation.

The rates of accumulation of MTX polyglutamates were similar in L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 cells and appeared to be constant for at least 3 hr (Fig. 3). At 30 min, approximately 50% of the total intracellular MTX represented polyglutamate derivatives, and reached a level of 80% (~1.0 to 1.5 nmol/g dry wt) of the total intracellular MTX by 4 hr. There was essentially no accumulation of MTX mono- or polyglutamates in MTX^{r}A.

As shown in Fig. 4, the profile of the individual MTX polyglutamates for each cell line was similar. The level of the monoglutamate was relatively constant (~0.25 nmol/g dry wt) over the interval measured, and the predominant form present after 4 hr was MTX tetraglutamate. The diglutamate was always present at the lowest level.

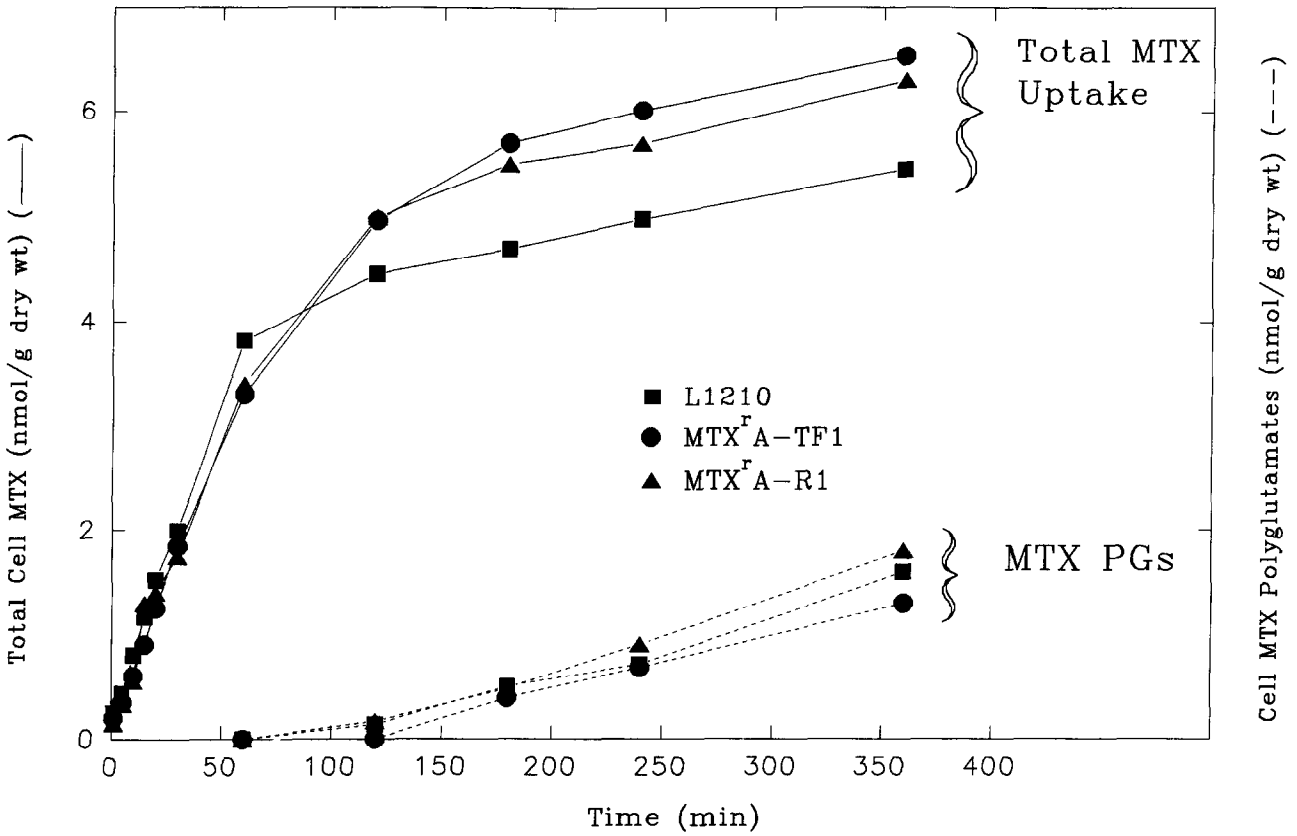

FIG. 2. Uptake and polyglutamylation of [^{3}H]MTX in L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 cells. Folate-depleted cells were washed, resuspended in folate-free RPMI medium (without serum) and, at time zero, exposed to 0.1 μM [^{3}H]MTX at 37°. Solid lines are total [^{3}H]antifolate and dashed lines are total [^{3}H]MTX polyglutamates as determined by HPLC. Data represent an average of 3 separate experiments.

Rate and Extent of Polyglutamylation over a 12-Hr Exposure to MTX

To assess the impact of the route of entry on polyglutamylation over a longer interval, MTX metabolites were measured in the absence of TMQ following a 12-hr exposure to either 0.1 to 1.0 μM [^{3}H]MTX. At 0.1 μM MTX, the MTX^{r}A-TF1 cell line accumulated intracellular MTX to a level comparable to that of L1210 and MTX^{r}A-R1 (Fig. 5). However, when the extracellular MTX level was increased to 1.0 μM, MTX accumulated to a lesser extent in the MTX^{r}A-TF1 line. This is consistent with the much lower influx K_m (20-fold) and V_{max} (20-fold) of FR-mediated transport in this line compared with RFC-mediated transport in L1210 cells [23]. MTX accumulation in the MTX^{r}A line at 0.1 μM was below, and at 1.0 μM barely in excess of, the DHFR binding capacity and, thus, very little free intracellular MTX was available for polyglutamylation (see below). Interestingly, there was no further increase in MTX accumulation when any of the cell lines were exposed to 0.1 μM MTX for 24 hr and only a slight (<5%) increase over 24 hr at 1.0 μM MTX (data not shown). Figure 6 depicts the profile of individual MTX polyglutamates after a 12-hr exposure to drug. At 0.1 μM MTX, MTX polyglutamates were 44% (2.9 nmol/g dry wt), 60% (4.1 nmol/g dry wt), and 33% (3.2 nmol/g dry wt) of the total antifolate for L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 cells, respectively (Fig. 6A). The major form was the triglutamate. In contrast, almost all MTX accumulation in the MTX^{r}A line was the monoglutamate (>92%). At 1.0 μM MTX, nearly all intracellular MTX was metabolized to polyglutamate forms (>94%) in L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 (Fig. 6B); the dominant form was MTX tetraglutamate. The least represented form was the diglutamate. Even at 1.0 μM MTX, polyglutamates in the MTX^{r}A line were only 12% of the total MTX accumulation or 0.62 nmol/g dry wt, primarily in the diglutamate form. However, after an incubation of 24 hr with 10.0 μM MTX, the total MTX level in MTX^{r}A increased to 8.5 nmol/g dry wt, 84% of which was polyglutamylated primarily to the tetraglutamate form (data not shown).

Polyglutamylation in the MTX^{r}A Line

To further study polyglutamylation in the MTX^{r}A line, transport and polyglutamylation were assessed after pretreatment with TMQ and exposure to 10 μM MTX. Under these conditions (Fig. 7A), the total free intracellular MTX level reached 3 nmol/g dry wt in 2 hr. MTX influx was not

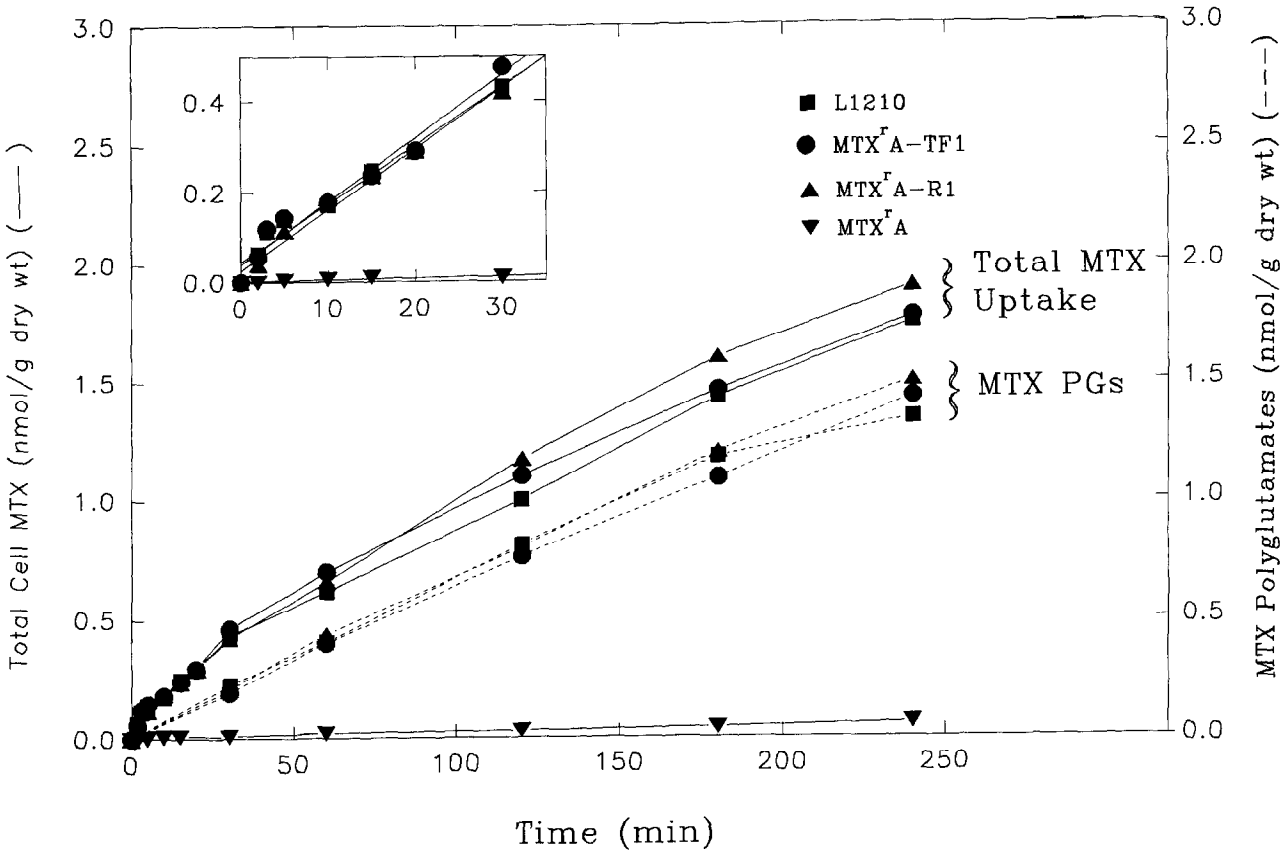

FIG. 3. Uptake and polyglutamylation of [^{3}H]MTX in cells pretreated with TMQ. Folate-depleted cells were incubated with 5 μM TMQ, washed, resuspended in folate-free RPMI, and at time zero, exposed to 0.1 μM [^{3}H]MTX at 37°. Solid lines are total [^{3}H]MTX and dashed lines are total [^{3}H]MTX polyglutamates as determined by HPLC. *Inset:* Expanded time scale of total MTX uptake within the first 30 min. Data are the average of 4 separate experiments.

sensitive to either 100 nM folic acid or 150 μM BSP, agents that under these conditions are potent inhibitors of FR- and RFC-mediated transport, respectively [23, 44–46]. Hence, residual uptake in this line proceeds either by passive diffusion or an unidentified route that is relatively insensitive to folic acid and BSP. Net accumulation of polyglutamates, which reached a level of about 50% of the total antifolate pool, was comparable under all conditions (Fig. 7B).

DISCUSSION

This study addresses whether folate transport is directly linked to subsequent folylpolyglutamylation by channeling between a given folate transport system and FPGS. Little is known concerning posttranslational modification, subcellular localization, or the regulation of FPGS activity. Since FPGS is very hydrophobic for a cytosolic protein, it may be associated with the plasma membrane where transport proteins reside. The data presented here demonstrate that cells which deliver MTX at the same rate via either FR-α or the RFC accumulate MTX polyglutamates at essentially identical velocities. These data thus support the notion that it is the concentration of MTX achieved within the cell that drives polyglutamylation, irrespective of its route of entry.

A transfection strategy was employed in order to obtain cell lines, derived from a common parental line, that exclusively express either the FR or the RFC. The parent cell line was a previously described L1210 subline, MTX^{r}A, which is 100-fold resistant to MTX by virtue of a missense mutation in the coding region of the RFC1 gene [13, 35]. This allowed comparison of the effects of FR and RFC, in cells of the same lineage, on polyglutamylation while avoiding secondary effects that occur during low folate selective pressure to increase expression of the membrane proteins. Thus, the transfected lines were shown to have similar DHFR and FPGS activities and are useful models to compare and contrast the effects of folate transport and delivery systems on other aspects of one-carbon metabolism.

Since this study was concerned with the efficiency of polyglutamylation and not interactions between MTX and DHFR prior to the accumulation of free monoglutamyl substrate, the lipophilic inhibitor TMQ was used in some experiments to block DHFR. Under these conditions, the onset of MTX polyglutamylation was immediate and identical between FR- and RFC-expressing cell lines (Fig. 3), and the initial uptake rates of the monoglutamates were the same. In the absence of TMQ, influx was also the same, but polyglutamylation was delayed (Fig. 2). Analysis of the time

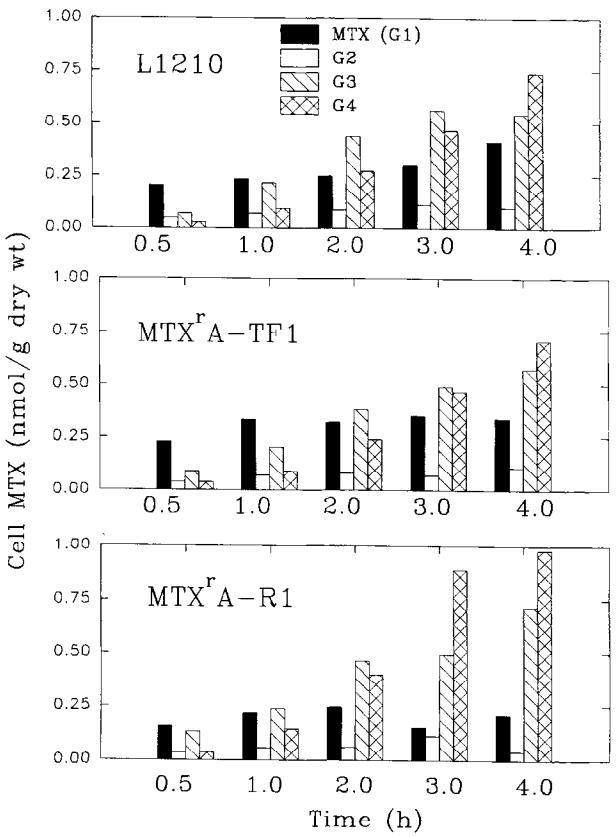

FIG. 4. Accumulation of [^{3}H]MTX polyglutamyl congeners in L1210, MTX^{r}A-TF1, and MTX^{r}A-R1 cells. Folate-depleted cells were pretreated with 5 μM TMQ, washed, resuspended in folate-free RPMI and, at time zero, exposed to 0.1 μM [^{3}H]MTX at 37°. [^{3}H]MTX polyglutamates were separated by HPLC. Upper panel, L1210 cells; middle panel, MTX^{r}A-TF1; lower panel, MTX^{r}A-R1. Solid bars, MTX monoglutamate; open bars, MTX diglutamate; hatched bars, MTX triglutamate; and crossed-hatched bars, MTX tetraglutamate. Data represent the average of 4 separate experiments.

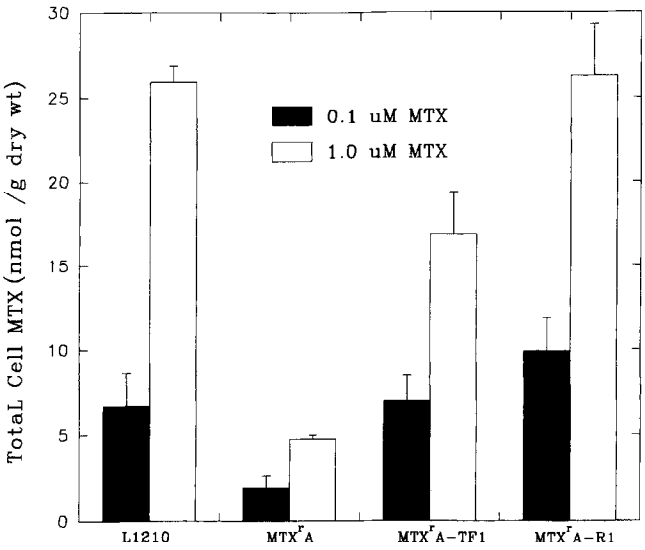

FIG. 5. Accumulation of [^{3}H]MTX in L1210, MTX^{r}A, MTX^{r}A-TF1, and MTX^{r}A-R1 following a 12-hr exposure to drug. Folate-depleted cells were washed, resuspended in folate-free RPMI medium, and exposed to 0.1 μM (solid bars) or 1.0 μM (open bars) [^{3}H]MTX for 12 hr. Data represent the means ± SEM of 3 separate experiments.

course of accumulation of each polyglutamate derivative revealed that the diglutamate was held to a constant low level in comparison to the monoglutamate and higher monoglutamate forms (Fig. 4). Assuming that diglutamate efflux is negligible over the interval of this study, the data suggest that diglutamate formation is rate limiting in MTX polyglutamylation.

The FR and the RFC employ diverse mechanisms of folate delivery. Previous studies using these cell lines established that the FR and the RFC exhibit distinct energy, ion, temperature, and pH dependence [23]. The fact that the endocytotic mechanism of the FR, which by definition involves vesicle trafficking, facilitated polyglutamylation as efficiently as that mediated by the RFC, a folate/anion exchanger, suggests that neither interacts directly with FPGS. However, in polarized cells, the FR may mediate folate uptake via a specialized endocytotic mechanism that is linked to an anion carrier [47, 48]. In this process, termed potocytosis, the FR appears to cluster in caveolin-coated pits termed caveolae [49]. The lack of caveolin expression as assessed by western and northern blots in L1210 cells suggests that a caveolar-based endocytotic mechanism is excluded in these cells (Spinella M and Goldman ID, unpublished results). Whether the caveolae-dependent endocytotic pathway would facilitate polyglutamylation with the same efficiency as the caveolae-independent route reported here is unclear.

It has not been established whether the FR, as compared with the RFC, represents a pharmacologically important route for uptake of antifolate drugs. The transfected cells in this study express FR or RFC at levels commonly seen for cell lines that endogenously express these proteins [13, 23]. We previously showed that the FR can be a significant transport route for folates at physiologic concentrations and for MTX at low blood levels (100–500 nM). However, FR becomes a very minor contributor to transport at higher levels of MTX (1–10 μM), when the RFC appears to dominate [23]. These effects are due to the slow net cycling rate of the FR as compared with the RFC. Slow receptor turnover is overcome partially by high levels of FR expression, and by the much higher affinity of folates for the FR than for the RFC. Using metabolism of drug as an endpoint, the present study demonstrated that the FR appears to deliver MTX to the same intracellular compartment as does the RFC.

Polyglutamylation in the transport-defective MTX^{r}A line was seen with high (10 μM) MTX concentrations and when DHFR binding sites were saturated with TMQ. This is in contrast to CEM, 3T6, and ZR-75 cell line variants defective in MTX transport and reported not to accumulate MTX polyglutamates despite high levels of intracellular MTX monoglutamate and unchanged levels of FPGS activity [50–52]. The reason for this discrepancy is unclear,

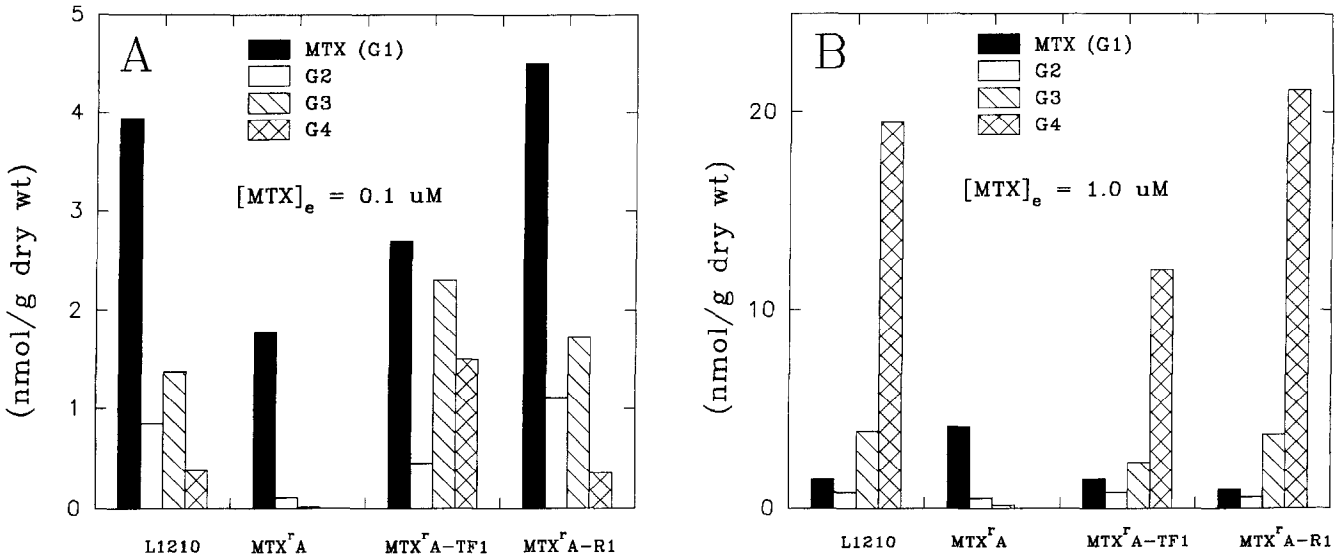

FIG. 6. Accumulation of [^{3}H]MTX polyglutamyl congeners in L1210, MTX^{r}A, MTX^{r}A-TF1, and MTX^{r}A-R1 cells following a 12-hr exposure to drug. (A) Folate-depleted cells were exposed to 0.1 μM [^{3}H]MTX at 37° for 12 hr after which [^{3}H]MTX polyglutamates were determined by HPLC. (B) Cells exposed to 1.0 μM [^{3}H]MTX at 37° for 12 hr. Data represent an average of 3 separate experiments.

but may be due to secondary changes during their selection such as increased expression of γ-glutamyl hydrolase. Residual transport of MTX in the MTX^{r}A line was not mediated by either the FR or the RFC based upon the lack of inhibition by BSP which suppresses both processes [23] and the lack of inhibition of folic acid which abolishes FR-mediated transport. Hence, the data suggest that a diffusional process achieves sufficient intracellular MTX levels to support polyglutamylation. This is an important consideration since "high dose" MTX chemotherapy, designed to circumvent drug resistance related to diminished transport, requires efficient polyglutamylation to achieve clinical efficacy [6–8, 27, 53].

Since there is substantial folate polyglutamylation in mitochondria, folates may be routed or targeted to this organelle. Lin and Shane [32] recently reported that reduced folates are transported to the mitochondria, polyglutamylated, and then efflux into the cytosolic pool. There is, however, evidence that MTX does not enter mitochondria [54, 55]. Thus, the lack of a detectable difference in the efficiency of MTX polyglutamylation in the present study does not preclude differences in mitochondrial metabolism that might be influenced by the route of entry into the cell. Further studies utilizing 5-formyltetrahydrofolate and dideazatetrahydrofolic acid (DDATHF), both substrates for mitochondrial transport, would address this issue.

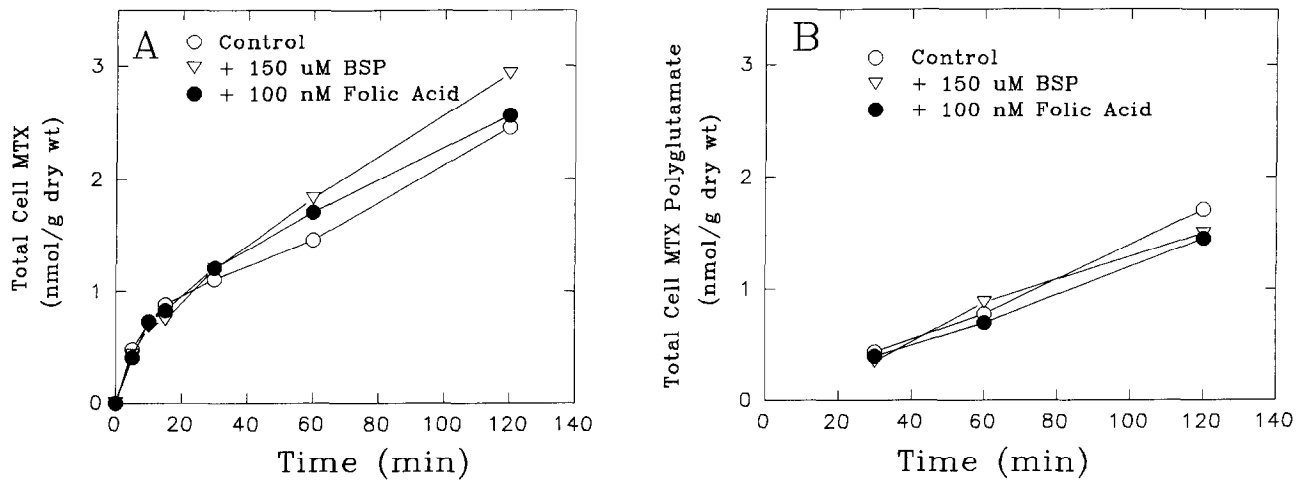

FIG. 7. Uptake and polyglutamylation of [^{3}H]MTX in MTX^{r}A at a high substrate level. (A) Folate-depleted MTX^{r}A cells were pretreated with 5 μM TMQ, washed, resuspended in folate-free RPMI medium and, at time zero, exposed to 10.0 μM [^{3}H]MTX at 37° in the presence or absence of indicated inhibitors. Total [^{3}H]MTX is plotted. (B) Portions of samples in panel A were analyzed for [^{3}]MTX polyglutamates. Data in both panels are the average of 2 separate experiments.

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