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COMPARISON OF TRANSPORT PROPERTIES OF THE REDUCED FOLATE CARRIER AND FOLATE RECEPTOR IN MURINE L1210 LEUKEMIA CELLS

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Abstract—This laboratory previously described an L1210 murine leukemia cell line with a functional defect in the reduced folate carrier and increased expression of folate receptor-β (F2-MTX^{r}A). This cell line was used to characterize methotrexate (MTX) influx mediated by folate receptor-β and to compare this with influx mediated by the reduced folate carrier in L1210 parental cells. Influx of 0.2 μM MTX in F2-MTX^{r}A cells was one-third that of L1210 cells and was abolished by very low concentrations of folic acid. Kinetic analysis revealed that MTX transport mediated by folate receptor-β exhibited an influx K_t one-third, and an influx V_{max} one-fourth, that of the reduced folate carrier. Metabolic inhibitors markedly suppressed influx in F2-MTX^{r}A cells but had no effect on MTX influx in L1210 cells. MTX influx in both cell lines was inhibited by the organic anions probenecid, sulfobromophthalein, and CI-920, but to a lesser extent in F2-MTX^{r}A cells. The inhibitory effects of these anions on transport in F2-MTX^{r}A cells could be attributed to their inhibition of MTX binding to the folate receptor. Although MTX influx in both cell lines was not sodium dependent, removal of extracellular chloride increased influx 2-fold in L1210 cells while markedly inhibiting influx in F2-MTX^{r}A cells. Substitution of Cl^- with isethionate or NO_3^- partially restored influx in the latter cells, whereas SO_4^{2-} was inhibitory. Anions enhanced MTX binding to folate receptor-β with isethionate > SO_4^{2-} > Cl^-. Decreasing the buffer pH to 6.2 produced a 69% reduction, and a 260% increase, in MTX influx in L1210 cells and F2-MTX^{r}A cells, respectively. The data indicate that folate receptor-β-mediated MTX influx has properties fundamentally different from transport mediated by the reduced folate carrier in terms of energy, ion, and pH dependence. There was no evidence indicating that these processes are functionally linked.

Key words: reduced folate carrier; folate receptor; methotrexate; transport; binding

The mechanism(s) by which tetrahydrofolate cofactors and antifolates traverse cell membranes has been an area of intense study since the relationship between transport and tumor sensitivity to MTX† was first recognized in the mid-1960s [1, 2]. A carrier mechanism for reduced folates that mediates transport of MTX in L1210 leukemia and a variety of other cell lines has been described in detail [3–6]. This RFC system preferentially transports 5-methyltetrahydrofolate and shows high affinity for 5-formyltetrahydrofolate and MTX, but has very low affinity for folic acid. Influx of folates by the RFC is sodium independent, inhibited by anions, and is either insensitive to, or increased by, sodium azide [4, 7, 8]. Recently, a cDNA encoding the RFC or one of its components was cloned and shown to restore function in cell lines that lack RFC transport activity [9, 10].

A second folate transport mechanism is mediated by FRs that are anchored to the cell membrane through a glycosylphosphatidylinositol moiety [11, 12]. Unlike the RFC, FRs exhibit very high affinity for folic acid and relatively low affinity for MTX. cDNAs encoding three distinct membrane FRs (α, β, and γ) have been cloned from a variety of cells and tissues in a number of laboratories [13–18]. It has been suggested that the folate-FR complex is internalized within a membrane vesicle, and, after acidification, the folate dissociates from the receptor and enters the cell by an organic anion carrier [19–21]. The ionic and energy dependencies of this system have not been characterized.

This laboratory has cloned two membrane FRs from L1210 murine leukemia cells [13]. FR-α, previously designated FBP1, is homologous to the receptor in KB cells [15]. FR-β, previously designated FBP2, has high homology to the isoform first identified in human placenta [17]. There is no sequence homology between the membrane-spanning RFC and the membrane-anchored FR that would suggest structural or functional similarities. The binding properties of the two receptor isoforms have been characterized in murine erythroleukemia cell lines in which the individual receptor proteins are overexpressed [22].

In other studies from this laboratory, an L1210 cell line (MTX^{r}A) selected for resistance to MTX was found to have impaired transport associated with loss of function of the RFC [23]. Further selection of this line for growth in low levels of folic acid resulted in a subline, F2-MTX^{r}A, which maintains MTX resistance and the RFC defect, but expresses high levels of FR-β [24]. The studies reported here exploited the carrier defect in the F2-MTX^{r}A line to characterize transport mediated by FR-β as distinct from the transport properties of the RFC. MTX was used as the transport substrate for influx determinations because it rapidly binds to dihydrofolate

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† Abbreviations: BSP, sulfobromophthalein; FR, folate receptor; MES, 2-[*N*-morpholino]ethanesulfonic acid; MTX, methotrexate; RFC, reduced folate carrier; and SITS, 4-acetamido-4′-isothiocyanatostilbene-2′,2′-disulfonic acid.

rereductase upon entering the cell, precluding back-flux components prior to saturation of this enzyme [3, 4]. The data indicated that FR-β-mediated MTX influx in F2-MTX^{r}A cells has properties of energy, ion, and pH dependencies distinct from, and functionally independent of, the RFC. There was no evidence to suggest that these processes are coupled.

MATERIALS AND METHODS

Chemicals

[3′,5′,7-^{3}H]MTX and [3′,5′,7′,9′-^{3}H]folic acid were obtained from Moravek Biochemicals (Brea, CA) and purified by HPLC prior to use [25]. CI-920 was a gift from Dr. David Fry (Warner-Lambert Co., Ann Arbor, MI). All other reagents were of the highest purity available.

Cell culture

L1210 cells were grown in RPMI 1640 medium (containing 2.2 μM folic acid) supplemented with 10% bovine calf serum (Hyclone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL). F2-MTX^{r}A is a clonal line that was selected for growth on low levels of folic acid [24]. This line was maintained in folate-free RPMI 1640 medium with 10% dialyzed bovine calf serum supplemented with 0.5 nM folic acid.

[^{3}H]MTX influx

[^{3}H]MTX influx was measured as previously described [26] with minor modifications. Briefly, cells were harvested and washed with 0° acid HEPES saline (20 mM HEPES, 137 mM NaCl, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, 7 mM glucose, pH 4.5) to release folates bound to the FR. This was followed by a wash with HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4) and resuspension into HBS at 1.5×10^7 cells/mL. After equilibration of the cell suspension at 37°, influx was initiated with the addition of the radiolabeled compound, and samples were taken over the intervals indicated on the figures. Uptake was terminated by injecting 1.0 mL of the cell suspension into 10 mL of 0° HBS. Cells were collected by centrifugation, washed in 0° acid HEPES saline followed by 0° HBS, and processed for determination of intracellular tritium as previously described [26]. All the studies were performed with 0.2 μM [^{3}H]MTX for 10 min unless stated otherwise. For determination of MTX influx kinetics, samples were taken over a broad spectrum of MTX concentrations with three different uptake points obtained over 1 min (1.5 to 5.0 μM) or 3 min (0.5 to 1.5 μM). During this interval [^{3}H]MTX uptake did not exceed the dihydrofolate reductase binding capacity, assuring that unidirectional uptake conditions were sustained. The dihydrofolate reductase binding capacities, determined by washout experiments, were 4.05 ± 0.35 and 3.77 ± 0.55 nmol/g dry weight for the L1210 and F2-MTX^{r}A lines, respectively. MTX influx kinetics mediated by the RFC in F2-MTX^{r}A cells were measured in the presence of 1 μM folic acid over 90–120 min. Experiments utilizing metabolic poisons were performed in HBS without glucose. For the ion substitution experiments, NaCl was replaced with the indicated salt and adjusted to 280–300 mOsmol/L. Experiments at pH 6.2 were performed in MBS (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose) [27]. MTX accumulation is expressed as nanomoles per gram dry weight. The dry weights of the L1210 and F2-MTX^{r}A lines per 10^7 cells were 0.96 ± 0.09 and 1.48 ± 0.05 mg, respectively (N = 6).

Analysis of specific MTX and folic acid binding to FR at the cell surface

Specific binding was determined using minor modifications of previously reported assays [22, 28]. The assay mixture contained 1×10^7 F2-MTX^{r}A cells in HBS buffer in the presence of either 0.2 μM [^{3}H]MTX or 0.3 μM [^{3}H]folic acid. Following incubation for 5 min at 0°, the samples were centrifuged (12,000 *g*, 0°, 2 min), the supernatant was aspirated, and residual fluid was removed by a second centrifugation and aspiration step. Cell pellets were processed as previously described [22], and radioactivity was counted. Specific binding was determined as the difference between total radiolabeled drug bound and binding in the presence of 100 μM unlabeled compound.

RESULTS

Analysis of MTX influx in cells with increased expression of FR-β

Selection of an RFC-defective L1210 variant (MTX^{r}A) for growth in low levels of folic acid resulted in the development of a subline (F2-MTX^{r}A) expressing high levels of FR-β [24]. In MTX^{r}A cells, transport of MTX is reduced markedly due to immobilization of the RFC. In these cells, there is a marked decrease is influx V_{max} without a change in influx K_m, and the affinity for MTX binding to the RFC is unchanged, as is the number of binding sites [23]. F2-MTX^{r}A cells retain the transport defect of the parental cell line [24]. Based on the cell surface folic acid binding capacity, F2-MTX^{r}A cells express 15.7 ± 1.8 nmol FR-β/g dry wt, whereas L1210 cells have no detectable FR message or membrane folic acid binding. Studies were undertaken to characterize and compare MTX influx in these two cell lines.

As shown in Fig. 1, influx of 0.2 μM [^{3}H]MTX in

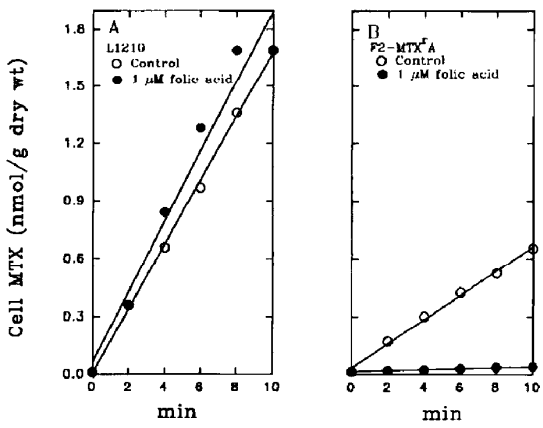

Fig. 1. Effects of folic acid on [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells. Cells were harvested and washed as described, resuspended in HBS, and at time zero exposed to 0.2 μM [^{3}H]MTX at 37° in the presence or absence of 1 μM unlabeled folic acid. The results are the averages of two (L1210) or five (F2-MTX^{r}A) experiments.

F2-MTX^{r}A cells (0.052 ± 0.018 nmol/g dry wt/min; N = 15) was approximately one-third the influx in L1210 cells (0.143 ± 0.029 nmol/g dry wt/min; N = 8) in the absence of folic acid. Since the affinity of folic acid for the RFC is two orders of magnitude lower than that of MTX [4, 28], and the affinity of folic acid for FR-β (K_b = 1.7 nM) is three orders of magnitude greater than MTX (K_i = 1.7 μM) [22], MTX influx mediated by the RFC can be distinguished from transport mediated by the FR on the basis of the transport component that is inhibited by relatively low levels of folic acid. The presence of folic acid at concentrations as high as 1 μM did not affect [^{3}H]MTX influx in L1210 cells but completely abolished influx in F2-MTX^{r}A cells (Fig. 1, A and B). In F2-MTX^{r}A cells, [^{3}H]MTX influx could be abolished completely by the addition of folic acid at levels as low as 50 nM (data not shown), consistent with uptake mediated exclusively via FR-β. Lower concentrations of folic acid did not inhibit MTX transport completely in F2-MTX^{r}A at the cell densities studied due to incomplete saturation of the very high levels of FR-β expressed in these cells.

While L1210 and F2-MTX^{r}A cells are cultured in very different levels of folic acid (2.2 μM vs 0.5 nM, respectively), differences in the intracellular folate status of these cells did not influence the rates of transport; when grown for 1 week in the absence of folate in medium supplemented with glycine, adenosine and thymidine, MTX influx in L1210 and F2-MTX^{r}A cells was identical to that of cells grown in medium with the usual folic acid levels (data not shown).

In MA104 cells, the FR recycles between the cell surface and an acid-insensitive intracellular compartment [19]. To confirm that MTX sequestered by FR-β in F2-MTX^{r}A cells is internalized within the cytosol and not simply recycled within the cell membrane, these cells were incubated with 0.2 μM [^{3}H]MTX at 37° for 10 min, acid washed, resuspended in MTX-free medium, and the amount of acid-resistant [^{3}H]MTX in the cell was measured over 90 min. Over this interval, all of the [^{3}H]MTX was retained in the cell, confirming its presence in the cytoplasm and not in a membrane compartment that can recycle to the cell surface (data not shown).

RFC- and FR-β-mediated MTX influx kinetics

MTX influx mediated by the RFC in L1210 cells was compared with FR-mediated transport in F2-MTX^{r}A cells over a broad concentration range. A Lineweaver–Burk analysis of data from a representative experiment is shown in Fig. 2. The results indicate that the influx K_t and V_{max} for FR-β-mediated MTX influx are one-third and one-fourth those of the RFC, respectively (Table 1). The F2-MTX^{r}A influx K_t was comparable to the K_i value for MTX inhibition of folic acid binding to the FR-β of 1.7 μM [22]. To further characterize the nature of the RFC transport defect in F2-MTX^{r}A cells, influx kinetics were determined in presence of 1 μM folic acid over a broad range of MTX concentrations. The V_{max} for RFC-mediated influx in F2-MTX^{r}A cells was 4% that of the RFC in the L1210 cells, whereas the K_t for influx via the RFC in F2-MTX^{r}A was the same as in L1210 cells (Table 1). These and the above data indicate that essentially all MTX influx in F2-MTX^{r}A cells is mediated via the FR-β.

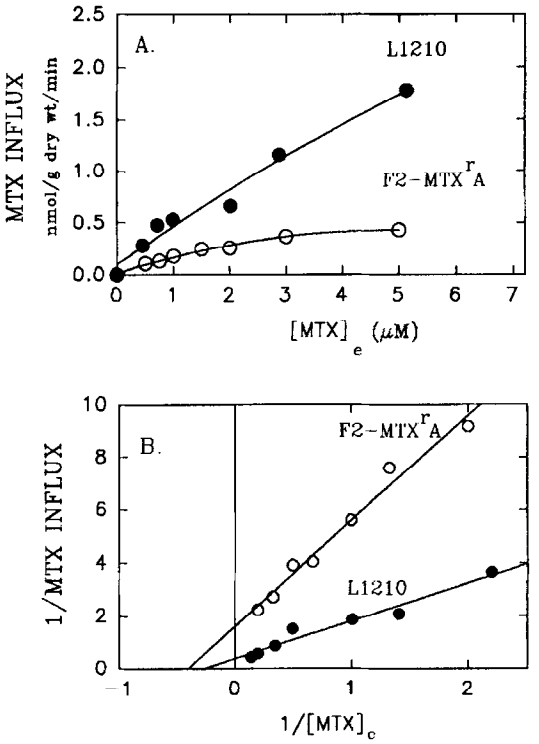

Fig. 2. MTX influx as a function of [MTX]$_e$. (A) L1210 and F2-MTX^{r}A cells were harvested, washed as described, and resuspended in HBS at 37°. [^{3}H]MTX was added over the concentration range of 0.5 to 5 μM, and samples were taken over an interval during which intracellular MTX accumulation did not exceed the dihydrofolate reductase binding capacity. Data are from a representative experiment. (B) Lineweaver–Burk plot of the data.

Energy dependence of [^{3}H]MTX influx

While the energy requirement of FR-mediated influx has not been defined, MTX influx via the RFC has been shown, depending upon the conditions, to be either unchanged or increased by metabolic inhibitors [4, 7, 29]. Sodium azide (10 mM) did not affect RFC-mediated [^{3}H]MTX influx but strongly inhibited (87%) FR-β-me-

Table 1. Kinetic parameters of RFC and FR-β-mediated influx of [^{3}H]MTX

Cell line	Transport route	Influx K_t (μM)	Influx V_{max} (nmol/g dry wt/min)
L1210	(RFC)	4.20 ± 0.52	2.90 ± 0.32
F2-MTX^{r}A	(RFC)	4.34 ± 0.06	0.06 ± 0.02
F2-MTX^{r}A	(FR-β)	1.25 ± 0.54	0.71 ± 0.37

[^{3}H]MTX influx was determined over a broad range of concentrations and over an interval in which the dihydrofolate reductase binding capacity was not exceeded to assure that accurate unidirectional measurements were obtained, as described in Materials and Methods. Results are the means ± SD of three experiments (L1210 RFC), eight experiments (F2-MTX^{r}A FR-β) or five experiments (F2-MTX^{r}A RFC). MTX influx mediated by the RFC in MTX^{r}A cells was assessed in the presence of 1 μM folic acid to abolish the transport component attributed to FR-β.

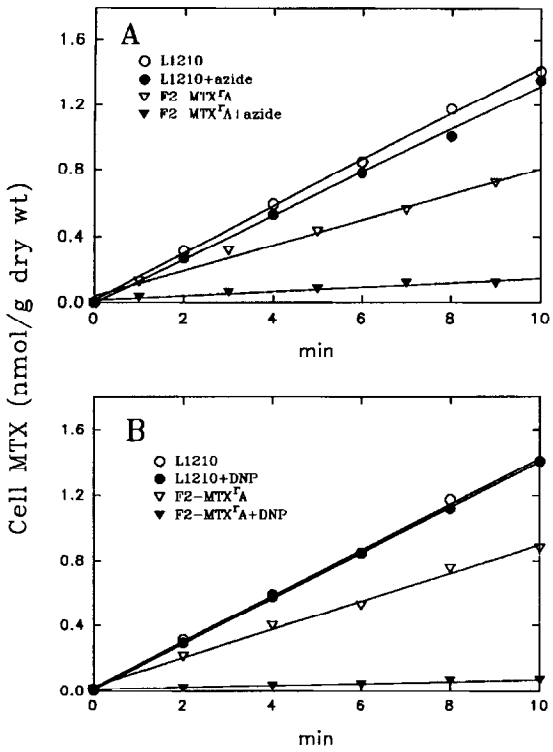

Fig. 3. Effects of azide or dinitrophenol on [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells. Cells were harvested, washed, and resuspended in HBS without glucose as described. Five minutes prior to the addition of [^{3}H]MTX to achieve a concentration of 0.2 μM, the cells were exposed to either 10 mM sodium azide or 0.5 mM dinitrophenol (DNP). The data represent the averages of two experiments.

diated [^{3}H]MTX influx in F2-MTX^{r}A cells (Fig. 3A). Similarly, 0.5 mM dinitrophenol did not alter RFC-mediated [^{3}H]MTX influx in L1210 cells, but virtually abolished (>93%) FR-β-mediated MTX influx in F2-MTX^{r}A cells (Fig. 3B). These data indicate that, unlike influx via the RFC, influx mediated by FR-β is highly dependent upon energy metabolism.

Effects of inhibitors of the RFC on MTX binding to, and influx mediated by, FR-β in F2-MTX^{r}A cells

MTX transport via the RFC has been shown to be sensitive to structurally unrelated organic anions such as probenecid [30–32] and BSP [8]. The sensitivity of FR-mediated transport to probenecid has been interpreted as suggesting a link between the two transport pathways [21, 33]. As depicted in Table 2, probenecid and BSP inhibited MTX influx by both the RFC and FR-β, but the degree of inhibition was markedly different. One millimolar probenecid inhibited RFC-mediated [^{3}H]MTX influx by 72% in L1210 cells, yet had no effect on FR-β-mediated uptake in F2-MTX^{r}A cells. At 10 mM probenecid, inhibition of FR-β-mediated transport was observed, but at a level far less than in L1210 cells. At 150 μM BSP, RFC-mediated [^{3}H]MTX influx in L1210 cells was inhibited by >95%, whereas there was only 55% inhibition of FR-β-mediated influx in F2-MTX^{r}A cells. CI-920 is an antibiotic transported by the RFC and inhibits MTX influx via this process [34]. At a concentration of 150 μM, CI-920 inhibited [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells by 83 and 24%, respectively. In general, these data suggest that, compared with FR-β-mediated transport, influx via the RFC is much more sensitive to inhibition by organic anions. As seen in Table 2, this inhibition of FR-β-mediated influx correlates with inhibition of MTX binding to FR-β. While 1 mM probenecid inhibited neither [^{3}H]MTX influx nor binding to FR-β, 10 mM probenecid resulted in 41 and 56% inhibition of binding and influx, respectively. BSP inhibited [^{3}H]MTX binding to FR-β by 83% and inhibited influx by 55%. Likewise, 150 μM CI-920 inhibited both [^{3}H]MTX binding and influx by similar values.

RFC-mediated influx of MTX has also been shown to be sensitive to anion exchange inhibitors such as substituted stilbenedisulfonates [35]. As indicated in Table 2, 200 mM SITS abolished RFC-mediated [^{3}H]MTX influx in L1210 cells, but inhibited FR-β-mediated uptake in F2-MTX^{r}A cells by only 55%. This inhibitory effect on MTX transport in F2-MTX^{r}A cells could be accounted for, to a large extent, by inhibition of binding to FR-β. These results indicate that organic anions have distinct and independent effects on each of the transporters and provide no evidence to support a linkage between the two systems.

Ion dependence of MTX influx

While MTX influx via the RFC is a sodium-independent process, it is highly sensitive to the anionic composition of the extracellular compartment and is inhibited by inorganic as well as organic anions [3, 8]. In the present study, when buffer NaCl was replaced isosmotically with HEPES, [^{3}H]MTX influx increased more than 2-fold in L1210 cells but decreased by 65% in F2-MTX^{r}A cells (Table 3). In both cell lines, influx was unchanged by substituting Na^+ with Li^+ and neither showed sensitivity to 1 μM ouabain (data not shown). Hence, neither uptake process is sensitive to the intra- or extracellular Na^+ concentrations, suggesting that FR-β-mediated influx may have an anion requirement.

As also shown in Table 3, the two transport systems responded differently to alterations in the anionic composition of the buffer. Anions inhibited RFC-mediated [^{3}H]MTX influx in L1210 cells (SO_4^{2-} > NO_3^- > isethionate > Cl^-) but generally stimulated FR-β-mediated influx in F2-MTX^{r}A cells (Cl^- > isethionate > NO_3^-). Only SO_4^{2-} was inhibitory to FR-β-mediated influx. Unlike the effects noted above for the organic anions, however, the changes in [^{3}H]MTX influx induced by these inorganic anions were not associated with a consistent parallel change in [^{3}H]MTX binding to FR-β; isethionate > SO_4^{2-} > Cl^- increased [^{3}H]MTX binding to FR-β, whereas NO_3^- slightly decreased binding (Table 3). Thus, while anions are required for transport by FR-β, Cl^- being the most effective, they are inhibitory to RFC-mediated influx.

pH dependence of MTX influx

Henderson and Strauss [27] previously described an energy-dependent, BSP-insensitive folate transport system in L1210 cells that is activated at low pH. Therefore, MTX influx was studied at pH 6.2 to determine whether such a system might play a role in MTX transport in F2-MTX^{r}A cells. As shown in Fig. 4, influx in L1210 cells was decreased by 69% when buffer pH was lowered from 7.4 to 6.2. In F2-MTX^{r}A cells, however, this pH change resulted in a 2.6-fold increase in MTX influx.

Table 2. Effects of probenecid, BSP, CI-920 or SITS on [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells and on specific binding of [^{3}H]MTX to FR-β in F2-MTX^{r}A cells

	L1210 (RFC) (% inhibition of influx)	F2-MTX^{r}A (FR-β) (% inhibition of influx)	F2-MTX^{r}A (FR-β) (% inhibition of binding to FR-β)
1 mM Probenecid	72	0	1
10 mM Probenecid	98	56	41
150 μM BSP	98	55	83
150 μM CI-920	83	24	20
50 μM SITS	81	34	14
200 μM SITS	97	55	38

The extracellular [^{3}H]MTX concentration was 0.2 μM. The data are the means of two experiments, each performed in duplicate.

Table 3. Effects of extracellular anion composition on [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells and on specific binding of [^{3}H]MTX to FR-β in F2-MTX^{r}A cells

Salt	L1210 (RFC) (% control influx)	F2-MTX^{r}A (FR-β) (% control influx)	F2-MTX^{r}A (FR-β) (% control binding)
NaCl (HBS)	100	100	100
HEPES	267	35	76
Sodium isethionate	66	57	123
$NaNO_3$	21	52	68
Na_2SO_4	12	15	121

The extracellular [^{3}H]MTX concentration was 0.2 μM. The data are the means of two experiments, each performed in duplicate, and are expressed as the percentage of the level of [^{3}H]MTX influx (0.135 and 0.046 nmol/g dry wt/min in L1210 and F2-MTX^{r}A, respectively) or binding (7.81 nmol/g dry wt in F2-MTX^{r}A) obtained in HBS buffer.

Similarly, binding of [^{3}H]MTX to FR-β at pH 6.2 increased approximately 2-fold relative to binding at pH 7.4 (15.3 vs 7.8 nmol/g dry wt, respectively, N = 2). This augmented [^{3}H]MTX influx at low pH could be inhibited only 70% by the addition of 100 nM folic acid, indicating that it is not mediated entirely by FR-β. Further, the residual 30% influx is not mediated by the RFC as it was abolished by 10 mM sodium azide and only partially inhibited (~50%) by 100 μM BSP. Therefore, at pH 6.2 the RFC functions with lowered efficiency and, while MTX influx via FR-β increases due to increased binding to the receptor, this cannot account entirely for the augmented influx observed in F2-MTX^{r}A cells at this pH.

DISCUSSION

The utilization of an L1210 leukemia cell line with high level expression of FR-β and a nonfunctional RFC permitted the characterization of FR-β-mediated transport distinct from transport mediated by the RFC. The properties of MTX influx in L1210 cells (low affinity for

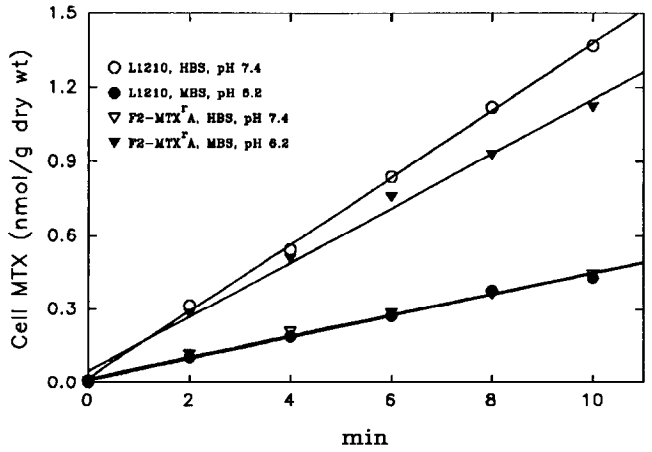

Fig. 4. Effects of pH on [^{3}H]MTX influx in L1210 and F2-MTX^{r}A cells. Cells were harvested, washed, and resuspended in either HBS buffer, pH 7.4, or in MBS buffer, pH 6.2, as described. At time zero, [^{3}H]MTX was added to achieve a concentration of 0.2 μM. The results are the averages of two experiments.

folic acid, high sensitivity to BSP and probenecid, inhibition by anions) have been studied extensively and are characteristic of transport via the RFC [3–6]. In contrast, virtually all MTX influx in F2-MTX^{r}A cells is mediated by FR-β. At 0.2 μM MTX, the concentration used for most of the studies described in this paper, less than 3% of total MTX influx in F2-MTX^{r}A cells could be accounted for by the RFC. The studies reported here indicate that FR-β-mediated transport is qualitatively different from RFC-mediated influx in many fundamental respects including ion, pH, and energy dependencies.

Uptake of folates mediated by FRs has been described as a low capacity, slow process [12, 24, 36], and influx via FR-β is slower than that mediated by the RFC. However, transport via the FR relative to influx mediated by the RFC will depend upon the level and specific isoform of FR expressed and the concentration at which transport is studied relative to the influx K_t. For FR-β-mediated influx in F2-MTX^{r}A, the V_{max} is one-quarter that of RFC-mediated transport in L1210 cells. Based upon this V_{max} difference and the K_t value for FR-β-mediated MTX influx relative to that of RFC-mediated MTX influx (Table 1), this difference in transport rates is maintained over a broad concentration range. FR-α-mediated MTX influx in an RFC-defective L1210 cell subline has been described recently [37]. At low MTX concentrations (≤0.1 μM), FR-α-mediated MTX influx proceeds at a rate equal to, or higher than, influx mediated by the RFC. However, because the influx V_{max} for FR-α-mediated transport is 1/16, and the K_t is 1/20 that of the RFC, at high MTX concentrations FR-α-mediated influx falls far below that mediated by the RFC [37].

The relative cycling rate, defined as influx V_{max} (nmol/g dry wt/min)/FR level (nmol/g dry wt) can be estimated for FR-β in F2-MTX^{r}A cells. Based upon the maximum influx velocity (0.71 nmol/g dry wt/min) and the level of expression of FR-β (15.7 nmol/g dry wt), the calculated cycling rate for FR-β in F2-MTX^{r}A cells is 45 nmol/μmol FR-β/min. This cycling rate is within 50% of the rate reported for FR-α in an L1210 subline (20 nmol/μmol FR-α/min; [37]).

The data presented here are consistent with an endocytotic mechanism for FR-mediated transport. It has been proposed that folate substrates bind to the FR and become internalized within a membrane vesicle. According to the model, upon acidification of the vesicle, folate is released from its receptor and gains entry into the cell by an organic anion carrier, after which the receptor is recycled back to the cell surface [19–21]. In the present study, the observed inhibitory effects of metabolic poisons, ion substitutions, and pH on FR-β-mediated influx in F2-MTX^{r}A cells could be accounted for by perturbations in several steps of this putative pathway.

FR-β-mediated influx was sensitive to metabolic poisons. Likewise, receptor-mediated endocytosis is highly energy dependent, requiring ATP for coated vesicle budding, fusion of endocytic vesicles, and *de novo* coated pit formation [38, 39]. Although FRs do not appear to cluster specifically in clathrin-coated vesicles [40], ATP may be required for sealing the vesicles during the process of internalization or for their recycling to the cell surface. Alternatively, metabolic poisons may prevent vesicular acidification that is required to release folates from the receptor [19, 21]. Endosomes are acidified by ATP-driven proton pumps, and a V-type proton pump has been implicated in FR-mediated uptake of folates in JAR human placental choriocarcinoma cells [33].

The ionic composition of the extracellular milieu can have complex effects on endocytic processes, affecting ligand affinity for the receptor, vesicular internalization, and ligand release. Endocytosis of the transferrin receptor in reticulocytes is considered to be chloride dependent and is diminished by replacement of chloride in the buffer with other anions [41]. Anion substitution does not affect binding to the transferrin receptor; rather the effects were attributed to either a requirement for a penetrating anion in the endocytotic process or to destabilization of the cell membrane by the chaotropic nature of some anions [41]. Chloride replacement could also affect acidification of the endocytic vesicle, since chloride is required for proton-translocation in clathrin-coated vesicles [42]. Consistent with the apparent chloride-dependent effects in other endocytotic processes, chloride is required for optimal transport mediated by FR-β. Isethionate and NO_3^- could replace chloride only partially and SO_4^{2-} was inhibitory. Distinct from transport, receptor binding is also affected by the anionic composition of the medium. Inorganic anions have been shown to enhance binding of 5-methyltetrahydrofolate to FR isolated from brush-border membranes of rat kidney, presumably due to the presence of an anion binding site that stabilizes a folate-anion-FR ternary complex [43]. Similarly, with the exception of NO_3^-, the anions tested in the present study enhanced MTX binding to FR-β (Table 3). The different effects of anion substitutions on MTX binding and influx in F2-MTX^{r}A suggest that chloride is required for FR-β internalization, the subsequent release of bound MTX in the cell, or both processes.

The effects of chloride on FR-β-mediated transport contrast sharply with effects on transport mediated by the RFC. For the RFC, anions appear to compete with folates for binding and influx at a single carrier site [4, 8]. Furthermore, the presence of extracellular anions appears to diminish or dissipate the transmembrane ionic gradient, which is considered to be the driving force that permits uphill transport of some folates and antifolates into cells [4, 8, 44].

In the present study, FR-β-mediated MTX influx was enhanced at pH 6.2 in contrast to the decrease in influx mediated by the RFC. The data suggest that this can be accounted for by increased binding to FR-β since the magnitude of stimulation of both parameters was comparable. While it is recognized that binding of folates to FRs is decreased at low pH, this occurs at pH levels below 5.0 [45]. In fact, binding by kidney proximal tubule FR shows a pH optimum of 5.5 [46]. A folate transport route that functions optimally at pH 6.2 has been described in MTX-resistant L1210 cells [27]. This system is energy dependent, insensitive to BSP and has a K_t value for folic acid in the micromolar range. A small component (30%) of MTX influx in F2-MTX^{r}A cells at pH 6.2 was not inhibited by low levels of folic acid, was inhibited partially by BSP, and was inhibited completely by azide. Hence, this transport flux has characteristics that are distinct from transport mediated by FR-β, the RFC, or the low pH route reported by Henderson and Strauss [27]. The nature of this influx component is under further study.

Kamen *et al.* [21] proposed that FR-mediated transport is linked to an RFC-like organic anion carrier. This

was based upon the observation that probenecid, a nonspecific anionic inhibitor of transport via the RFC, also inhibits FR-α-mediated transport in MA104 cells. However, the results reported here do not support a role for the RFC in FR-β-mediated uptake. First, the impaired mobility of RFC in F2-MTX^{r}A cells excludes its role in facilitating folate transport. Second, while probenecid does indeed inhibit MTX influx mediated by FR-β, this effect can be accounted for on the basis of direct inhibition of MTX binding to the FR. There are similar effects of BSP and CI-920 (RFC inhibitors) on MTX binding to, and influx mediated by, FR-β. Third, SITS was a potent inhibitor of RFC-mediated influx, but it was a very weak inhibitor of FR-β-mediated influx (Table 2). Taken together, these observations provide strong evidence that these two transport processes are not linked.

Recent studies have demonstrated extensive expression of FR-β in normal and malignant tissues of nonepithelial origin, and of FR-α in epithelial tissues and several carcinomas [47]. Isoform-specific differences in binding affinities [22, 48] and the level of FR as well as RFC expression must be considered as transport pathways are analyzed for their role in the delivery of folates and antifolates to specific tissues.

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