

Expression of a Folate Binding Protein in L1210 Cells Grown in Low Folate Medium¹

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ABSTRACT

We have isolated variants of L1210 cells (L1210B) expressing, in addition to the "classical" high affinity/low capacity system for reduced folate uptake, high levels of a membrane-associated folate binding protein. This folate binding protein was expressed in L1210 cells grown at low physiological folate levels (<0.5 nM), but down-regulated after transfer in standard high folate (2 μM) medium.

The binding capacity of L1210B cells for [³H]folic acid and [³H]-methotrexate was identical (5–11 pmol/10⁶ cells) but affinities were different. The affinities relative to folic acid were 0.5 for 5-methyltetrahydrofolate, 0.25 for 5-formyltetrahydrofolate, 0.08 for 10-ethyl-10-deazaaminopterin, and 0.05 for methotrexate, respectively.

L1210B cells exposed to low extracellular concentrations of [³H]folic acid (25 nM) accumulated 15 pmol [³H]folic acid/10⁷ cells over a 5-h period. [³H]folic acid accumulation by wild-type L1210 cells could not be demonstrated under these conditions.

The folate binding protein in L1210B cells could be specifically and covalently labeled at 4°C with a *N*-hydroxysuccinimide ester of [³H]-methotrexate or [³H]folic acid. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of detergent-solubilized membrane proteins showed a major labeled band with *M_r* 42,000–44,000.

INTRODUCTION

It is generally accepted that reduced folates and the folate analogue MTX³ are transported into L1210 murine leukemia cells via a carrier mediated process (1–4). This transport system has a high affinity (*K_m*, 1–5 μM) for 5-methyltetrahydrofolate, 5-formyltetrahydrofolate or MTX, but a low maximal velocity (1–12 pmol/min/mg protein) (reviewed in Ref. 1). Folic acid, however, is poorly transported by this system, judging from a *K_m* value of 400 μM (1, 5). Recently, Sirotiak *et al.* (6) demonstrated the presence of a second folate entry system in L1210 cells mediating the uptake of folic acid and 5-methyltetrahydrofolate with a 20-fold higher capacity than the "classical" system but with rather low affinities (*K_m* > 350 μM). The relatively low affinity of both transport systems for folates suggests that they may play an important role in the uptake of folate and its analogues at pharmacological micromolar concentrations rather than at physiological nanomolar levels. Serum folate levels (5–50 nM) (7) are at least two to four orders of magnitude lower than the *K_m* values of both transport systems. More recently, better candidates for the transport of folates at phys-

iological levels were described. Folate binding proteins, found in the membranes of a variety of tissues and cell lines (8–13), showed high affinities (*K_d* < 2 nM) for the predominant serum folate, 5-methyltetrahydrofolate and uphill transport at physiological levels (8, 14). In the present study we describe the reversible induction of a high-affinity folate binding protein in murine L1210 cells by selection in low folate medium. Its properties appear to be similar to folate binding proteins from other sources.

MATERIALS AND METHODS

Chemicals. RPMI 1640 medium with and without 2.2 μM folic acid and dialyzed sera were obtained from GIBCO, Grand Island, NY. DL-5-Formyltetrahydrofolate, DL-5-methyltetrahydrofolate, CHAPS, *N*-hydroxysuccinimide, and prestained molecular weight markers for SDS-PAGE were purchased from Sigma Chemical Co., St. Louis. 10-EdAM was a gift from Ciba-Geigy, Basel, Switzerland. Methotrexate was a gift from Pharmachemie, Haarlem, The Netherlands. [³H]MTX (specific activity, 10–20 Ci/mmol) and [³H]folic acid (specific activity, 0.5 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA, and Amersham, UK, respectively. Radiochemicals were purified prior to use by thin-layer chromatography as described before (2). Radiochemical purity of the labeled compounds was >99% after rechromatography.

HPLC Analysis. HPLC analysis of [³H]folates was carried out by injecting 6,000–15,000 ³H cpm of sample (in 50 μl) on a reversed-phase Lichrosorb 5-RP18 column (4.6 mm x 15 cm, Chrompack Int., Middelburg, The Netherlands) using 0.1 M *P_i* (pH 6.8) plus 15% methanol as the solvent system (15). The absorbance detector (Perkin Elmer LC75) was set at 280 nm and 307 nm for the detection of folic acid and methotrexate, respectively.

Selection of L1210 Variants. L1210 cells grown in standard RPMI 1640 medium (containing 2.2 μM folic acid) supplemented with 10% horse serum were transferred to RPMI 1640 medium without folic acid and 10% dialyzed horse serum. DL-5-Formyltetrahydrofolate was added as the sole folate source in stepwise decreasing concentrations keeping the growth rate at about 10–20% of parental L1210 cells. The cell cultures were refreshed twice weekly. At a concentration of 0.2 nM DL-5-formyltetrahydrofolate repeated transfers were made until the doubling time was similar to parental L1210 cells. The cells were then cloned in RPMI 1640 medium (without folic acid) containing 0.85% methylcellulose, 10% dialyzed horse serum, and 0.2 nM DL-5-formyltetrahydrofolate. After 10–14 days of incubation at 37°C colonies were picked, grown up in the above-mentioned medium, and screened for [³H]folate binding/uptake.

[³H]MTX Uptake/Binding Studies. Parental L1210 cells and variant L1210 cells were harvested during the mid log phase of growth and washed with one of the two buffers used in the transport studies: Buffer A was a HBSS (16) containing: 107 mM NaCl, 20 mM HEPES, 26.2 mM NaHCO₃, 5.3 mM KCl, 1.9 mM CaCl₂, 1 mM MgCl₂, and 7 mM D-glucose, pH 7.4 with NaOH. Buffer B was an anion-deficient buffer: MHS (17) containing: 20 mM HEPES, 225 mM sucrose, pH 7.4 with MgO. Cell suspensions (15 × 10⁶ ml) were incubated for selected times with 1.5 μM [³H]MTX (specific activity, 0.5 Ci/mmol). Uptake experiments were terminated by the addition of 9 volumes of ice-cold transport buffer, centrifugation at 800 × *g* for 5 min and another wash with 10 ml ice-cold transport buffer. To distinguish cell surface-bound [³H]-

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³ The abbreviations used are: MTX, methotrexate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 10-EdAM, 10-ethyl-10-deazaaminopterin; CHAPS, 3-[(3-chloramidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; HPLC, high-performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)ene-1-piperazineethanesulfonic acid; HBSS, HEPES balanced salt solution; MHS, magnesium-HEPES-sucrose; NHS-MTX, *N*-hydroxysuccinimide ester of MTX.

MTX from intracellular [^3H]MTX, the final cell pellet was resuspended in 0.5 ml of acidic saline, pH 3.5 (135 mM NaCl, 20 mM HEPES, 5.3 mM KCl, 1.9 mM CaCl₂, 1 mM MgCl₂, 7 mM D-glucose, pH 3.5 with HAc) and kept for 2 min at 4°C. Cells were then centrifuged in an Eppendorf microcentrifuge for 1 min at 13,000 × g. The total cell-associated radioactivity was divided in the supernatant fraction (containing [^3H]MTX originally bound to the cell surface, but stripped off at pH 3.5) and the cell pellet fraction (containing the acid-resistant intracellular [^3H]MTX) (14). The fractions were analyzed for ^3H radioactivity in Optifluor scintillation fluid (United Technologies Packard, Brussels, Belgium) using an Isocap/300 (Searle, Nuclear Chicago) with a counting efficiency for ^3H of 51%.

Viability of the cells was checked microscopically by trypan blue exclusion and was usually greater than 95%.

Cell Labeling/Gel Electrophoresis. L1210B73 cells were washed and resuspended in HBSS, pH 7.4 to a density of 10–30 × 10⁶/ml and labeled at 4°C for 5 min with the *N*-hydroxysuccinimide ester of [^3H]MTX (specific activity, 14 Ci/mmol) or [^3H]folic acid (specific activity, 0.5 Ci/mmol). NHS- ^3H]MTX and NHS- ^3H]folic acid were prepared as described by Henderson *et al.* (18, 19). Labeling in the presence of excess MTX or folic acid served as a specificity control. Incorporation of [^3H]MTX and [^3H]folic acid was determined according to Henderson and Zevely (19), except that 2% CHAPS was used for membrane protein solubilization and methanol for protein precipitation. Plasma membrane proteins were solubilized with 2% CHAPS from crude membrane fractions of labeled cells (19) and analyzed by SDS-PAGE according to the method of Laemmli (20). Gels were sliced and gel slices were digested in 0.5 ml 30% H₂O₂ for 2 h at 50°C and subsequently counted for radioactivity.

RESULTS

Isolation of L1210 Cell Variants. After a selection procedure in low folate medium, which took about 9 months, L1210 cell variants were obtained expressing high levels of a membrane-associated folate binding protein. (Evidence that the folate binding is protein associated will be shown later in this section.) In wild-type L1210 cells, grown in standard folate medium, this folate binding protein was undetectable. The folate binding capacity of the variants ranged from 5 to 11 pmol folate/10⁶ cells, which corresponds to approximately 3–6 × 10⁶ copies/cell. Further characterization in this study was done with one of the clones obtained (L1210B73). As shown in Fig. 1 the binding protein has a very high affinity for [^3H]folic acid whereas the affinity for [^3H]MTX is substantially lower: half maximal binding was observed at <1 nM and 20–30 nM, re-

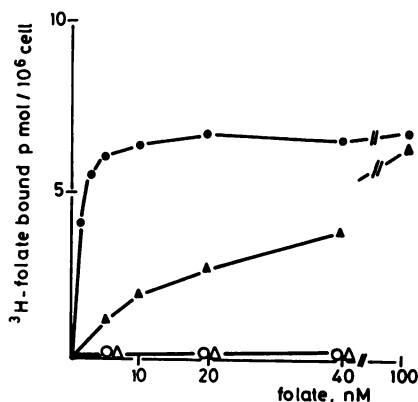


Fig. 1. [^3H]Folic acid (specific activity, 0.5 Ci/mmol) and [^3H]MTX (specific activity, 1.5 Ci/mmol) binding to L1210B73 cells as function of the extracellular folate concentration. L1210B73 cells (1 × 10⁶ in 15 ml HBSS, pH 7.4) were incubated at 4°C in the absence (closed symbols) or presence (open symbols) of a 500-fold excess folate. After 10 min incubation, cells were centrifuged at 1000 × g (5 min, 4°C), the supernatant removed by suction, and residual fluid removed by cotton tissues. Pellets were resuspended in water and counted for radioactivity. (▲) [^3H]MTX, (●) [^3H]folic acid.

spectively. The identity of cell surface-bound [^3H]folic acid and [^3H]MTX was confirmed by HPLC analysis (Fig. 2). More than 96% of the radioactivity that was stripped off by acidic saline treatment (see "Materials and Methods") eluted as single peaks with retention times identical to unlabeled folic acid or MTX. Relative affinities of the folate binding protein for a number of folates and folate analogues are given in Table 1. Folic acid has the highest affinity closely followed by 5-methyltetrahydrofolate and 5-formyltetrahydrofolate. Compared to folic acid, the folate analogues 10-EdAM and MTX have 13- and 20-fold lower affinities, respectively. The folate binding protein has a very low affinity for a molecular component of folic acid, *p*-aminobenzoylglutamate, indicating the specific nature of binding.

The expression of the folate binding protein in L1210B73 cells is dependent on the folate content of the growth medium. Transfer of L1210B73 cells from low folate medium to nonselective high folate medium for eight weeks diminished the expression of the folate binding protein to less than 10% of L1210B73 cells grown at low folate medium (Fig. 3).

Uptake/Binding Studies. Time courses for [^3H]MTX uptake in parental L1210 cells and L1210B73 cells, using a physiological buffer (HBSS, pH 7.4) and an anion-deficient buffer (MHS, pH 7.4), are shown in Fig. 4.

To distinguish the total cell-associated radioactivity in cell surface-bound [^3H]MTX (binding) and intracellular [^3H]MTX (uptake), cells were incubated in acidic saline, pH 3.5 (see

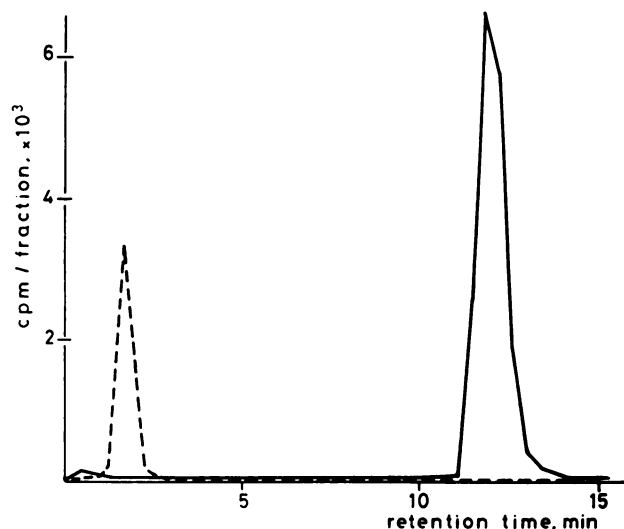


Fig. 2. Analysis of L1210B73 cell-associated radioactivity after [^3H]MTX and [^3H]folic acid binding studies described in Fig. 1. Cell surface-bound radioactivity was stripped off by acidic saline treatment (see "Materials and Methods") and analyzed by HPLC. ---, [^3H]folic acid; —, [^3H]MTX. The retention times for unlabeled folic acid and MTX standards were 1.5 min and 12.1 min, respectively. The recovery of [^3H]folic acid and [^3H]MTX radioactivity in the peak fractions was >96% of that applied to the column.

Table 1 Inhibition of [^3H]folic acid binding by folate derivatives

L1210B73 cells (5 × 10⁶/ml in HBSS, pH 7.4) were incubated for 10 min at 4°C with 100 nM [^3H]folic acid in the presence of increasing concentrations of folate derivatives. The relative concentration for 50% inhibition of [^3H]folic acid binding is depicted.

Binding inhibitor	Relative concentration for 50% inhibition
Folic acid	1
5-Methyltetrahydrofolate*	2
5-Formyltetrahydrofolate*	4
10-EdAM	13
MTX	20
<i>p</i> -Aminobenzoylglutamate	>500

* Expressed as the natural diastereoisomere in the racemic mixture employed.

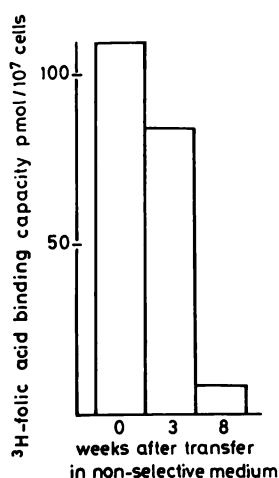


Fig. 3. ^3H Folic acid binding capacity of L1210B73 cells grown in nonselective RPMI 1640 medium (supplemented with $2.2 \mu\text{M}$ folic acid) and 10% horse serum. Prior to the ^3H folic acid binding assay, 5×10^6 cells were washed for 3 min in HBSS, HAc, pH 3.5 to remove cell surface bound folate. After centrifugation at $100 \times g$ (5 min, 4°C) cells were resuspended in 1 ml HBSS, pH 7.4 (4°C) containing $100 \text{ pmol } ^3\text{H}$ folic acid (specific activity, 0.5 Ci/mmol). Following incubation for 10 min at 4°C , cells were centrifuged for 1 min at $13,000 \times g$ in an Eppendorf centrifuge. The supernatant was removed by suction and residual fluid removed by cotton tissues. Pellets were resuspended in water and counted for radioactivity.

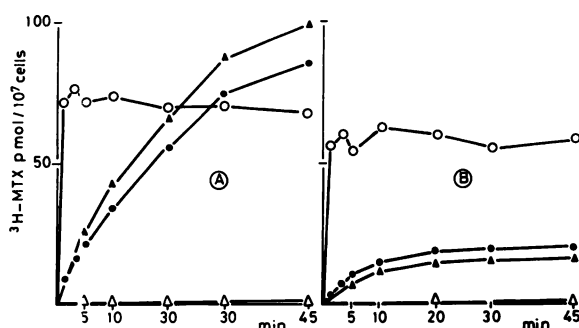


Fig. 4. Time course for accumulation of ^3H MTX (specific activity, 0.5 Ci/mmol) at 37°C by parental L1210 cells (triangles) and L1210B73 cells (circles) in anion-deficient MHS, pH 7.4 buffer (A) and physiological HBSS, pH 7.4 buffer (B). Extracellular ^3H MTX concentration was $1.5 \mu\text{M}$. Total cell-associated ^3H MTX was divided in cell surface-bound ^3H MTX (binding) and intracellular ^3H MTX (uptake) by treatment of cells with acidic saline as described in "Materials and Methods." ▲, ^3H MTX uptake parental L1210 cells; △, ^3H MTX binding L1210 cells; ●, ^3H MTX uptake L1210B73 cells; ○, ^3H MTX binding L1210B73 cells.

"Materials and Methods"). In both buffer systems, especially in the first part of the time course, most of the cell-associated ^3H MTX in L1210B73 cells is cell surface bound rather than intracellular. ^3H MTX binding was rapid in both buffer systems and reached a constant level of 60–75 pmol/ 10^7 cells. The pattern of ^3H MTX uptake in L1210B73 cells closely resembled that of parental L1210 cells. Influx rates and steady-state levels are higher in the MHS, pH 7.4 buffer than in the HBSS, pH 7.4 buffer. In the MHS, pH 7.4 buffer the ^3H MTX uptake reached the binding level, but in the HBSS, pH 7.4 buffer steady-state levels for uptake were 2.5-fold lower than the amount of cell surface-bound ^3H MTX.

Folate transport activity by the L1210B73 cell folate binding protein at a physiological folate level is shown in Fig. 5. Exposure of L1210B73 cells at 37°C to $25 \text{ nM } ^3\text{H}$ folic acid in folate-free RPMI 1640 medium resulted in the accumulation of $15 \text{ pmol } ^3\text{H}$ folic acid over a 5-h period. The amount of surface-bound ^3H folic acid reached a maximum after 1-h incubation, after which a slight decline in bound ^3H folic acid could be observed. In contrast to L1210B73 cells, wild-type

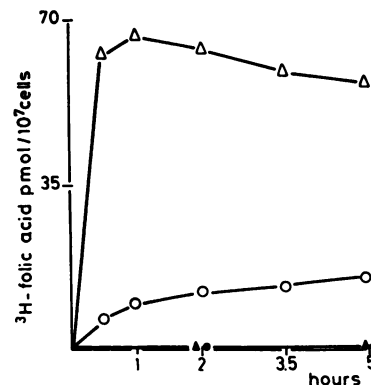


Fig. 5. ^3H Folic acid uptake by L1210B73 cells. L1210B73 cells ($6 \times 10^5/\text{ml}$) were maintained at 37°C in folate-free RPMI medium supplemented with 10% dialyzed horse serum, 0.1% ascorbic acid, and ^3H folic acid (specific activity, 0.5 Ci/mmol) at a final concentration of 25 nM . At the indicated time intervals aliquots were harvested by centrifugation and the cell pellet incubated for 2 min at 4°C with acidic saline, pH 3.5 to distinguish between ^3H folic acid binding and uptake (see "Materials and Methods"). △ and ▲, cell surface-bound ^3H folic acid; and ○ and ●, ^3H folic acid uptake in the absence (open symbols) or presence (closed symbols) of a 1000-fold molar excess of unlabeled folic acid.

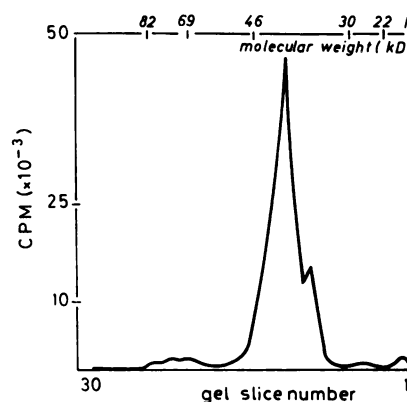


Fig. 6. SDS-PAGE of CHAPS-solubilized membrane proteins of NHS- ^3H MTX-labeled L1210B73 cells. Aliquots of $100 \mu\text{g}$ of membrane proteins were electrophoresed. The gel lanes were sliced, digested, and counted for radioactivity as described in "Materials and Methods."

L1210 cells neither bound nor accumulated ^3H folic acid under the conditions described in Fig. 5 (not shown).

Labeling of Folate Binding Protein of L1210B73 Cells. Intact L1210B73 (10^7) cells were labeled with either 100 nM NHS- ^3H MTX or NHS- ^3H folic acid at 4°C in the absence or presence of excess unlabeled MTX or folic acid, respectively. Preincubation for 3 min with unlabeled MTX/folic acid demonstrated that less than a 10-fold molar excess reduced the incorporation of ^3H MTX/ ^3H folic acid by 90% (not shown), suggesting specific labeling of the folate binding protein. Plasma membrane proteins of NHS- ^3H MTX-labeled L1210B73 cells separated by SDS-PAGE showed the incorporation of ^3H MTX in a protein of M_r 42,000–44,000 (Fig. 6). NHS- ^3H MTX labeling of L1210B73 cells in the presence of a 100-fold molar excess of MTX almost completely abolished the incorporation of ^3H MTX in this protein (not shown).

Using the labeling conditions described in Fig. 6, wild-type L1210 cells incorporated less than 3% of ^3H MTX compared to L1210B73 cells ($0.64 \text{ pmol/mg protein vs. } 22.1 \text{ pmol/mg protein}$, respectively). Furthermore, NHS- ^3H MTX labeling of wild-type L1210 cells required at least a 10,000-fold excess of unlabeled MTX for a 70% reduction of incorporated ^3H MTX (results not shown).

DISCUSSION

As the first regulatory step in the cytotoxic effects of antifolate drugs, (anti)folate transport systems have been extensively studied in a wide variety of tumor cells. These studies have shown that in a majority of tumor cells, including murine L1210 cells, a low capacity/high affinity transport system mediates the uptake of folate analogs MTX/10-EdAM and 5-substituted reduced folates, but not folic acid (1, 2, 6).

In the present study we described the isolation of a L1210 variant (L1210B73) expressing high levels of a membrane-associated folate binding protein, with very high affinities for folic acid and reduced folates, together with apparently normal levels, compared to parental L1210 cells, of the low capacity/high affinity folate transport system. Due to the presence of the folate binding protein in L1210B73 cells, the number of folate binding sites (5–11 pmol/10⁶ cells) is 30–70-fold higher than in parental L1210 cells (2, 16, 19). The selection procedure for the variants was in general the same as described by Sirotnak *et al.* (16) for the isolation of L1210 cell variants overproducing the reduced folate transport system. We have no explanation why our selection procedure resulted in variants overexpressing a folate binding protein rather than the reduced folate transport system. The only difference is that in our experiments dialyzed horse serum was used, instead of dialyzed fetal calf serum, but it seems unlikely that this is essential in obtaining the two different variants.

One important aspect of the expression of the folate binding protein in L1210B73 cells is that down-regulation occurred after prolonged cultivation in nonselective standard folate medium. This might be the reason that in standard L1210 cell cultures, grown for long times at high folic acid levels, no folate binding protein has ever been observed. The L1210B73 cell membrane binding capacity for [³H]folic acid is in the same range as for some other cultured cell lines, like KB cells (12) or HeLa cells (11), but about 10-fold higher than for monkey kidney MA104 cells (14). In accordance with folate binding proteins from other cell lines (9, 11, 12), the L1210B73 cell folate binding protein showed the highest affinity for folic acid and 5-methyltetrahydrofolate, but a substantially lower affinity for the folate analogues MTX/10-EdAM.

In addition to [³H]folic acid binding, L1210B73 cells could also take up [³H]folic acid when added in the medium at near physiological concentrations (Fig. 5). Although 5-methyltetrahydrofolate, and not folic acid, is the natural predominant serum folate, the almost identical affinities of the L1210B73 cell folate binding protein for 5-methyltetrahydrofolate and folic acid (Table 1) suggests that the uptake of 5-methyltetrahydrofolate could proceed analogous to folic acid. However, the exact mechanism of folic acid/5-methyltetrahydrofolate uptake by the folate binding protein in L1210B73 cells remains to be established.

Labeling of the L1210B73 cell folate binding protein by an *N*-hydroxysuccinimide ester of [³H]MTX, originally described as an affinity labeling reagent for the reduced folate transport system (19), showed great specificity. NHS-[³H]folic acid, which is not active as a probe for the reduced folate carrier (19), is equally potent for labeling the L1210B73 cell folate binding protein. The specificity of labeling the L1210B73 cell folate binding protein by NHS-[³H]MTX or NHS-[³H]folic acid was demonstrated by the fact that less than a 10-fold molar excess of unlabeled MTX/folic acid markedly reduced the incorporation of [³H]MTX/[³H]folic acid. This is substantially less than a 50,000-fold molar excess of MTX required for abolishment

of the specific labeling of the reduced folate transport system by NHS-[³H]MTX (19). The molecular weight of the folate binding protein from L1210B73 cells is well within the range of folate binding proteins from other sources, *M_r* 38,500 for human placenta (21), *M_r* 35,500 for human leukemia cells (10), and *M_r* 50,000 for the folate binding protein from KB cells (12).

The importance of both a folate binding protein and a reduced folate transport system within one L1210B73 cell is not clear. Sirotnak (1, 22) indicated that the low capacity/high affinity reduced folate/MTX transport system is normally expressed only in fetal tissues but a reexpression may occur in neoplastic tissues like the L1210 cell. In this way the tumor cell would be able to meet the increased needs of (folate-dependent) *de novo* synthesized nucleic acid precursors. Although the *in vivo* serum folate levels (5–20 nM) and the influx *K_m* by this transport system lie far apart, indicating that the transport system operates far below its maximal *in vitro* capacity, the level of influx seems to be more than sufficient for optimal L1210 cell growth (23). However, it is still unknown which mechanism regulates the entry of 5-substituted reduced folate compounds and how substantial concentrative uptake can be achieved at physiological serum levels of these folates. Recently, these issues were studied by Kamen and Capdevila (14, 24) for monkey kidney MA104 cells, which contain only a high affinity folate binding protein/receptor as uptake system for folic acid and 5-methyltetrahydrofolate. In these cells the intracellular folate content regulates the concentrative uptake of 5-methyltetrahydrofolate by cycling of the folate receptor. After internalization of receptor-bound 5-methyltetrahydrofolate, presumably by a process analogous to receptor-mediated endocytosis of other macromolecules, 5-methyltetrahydrofolate is released in an acidic compartment (lysosome). The externalization of the aporeceptor to the plasma membrane could be subject to feedback regulation because of preferential binding of intracellular folylpolyglutamates to the aporeceptor compared to the extracellular folylmonoglutamate forms (12).

Currently, investigations are underway to determine which of the two folate transport systems is the critical determinant in (anti)folate transport in L1210B73 cells at physiological and pharmacological concentrations.

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