Enhancement of Folate Analogue Transport Inward in L1210 Cells during Methotrexate Therapy of Leukemic Mice: Evidence of the Nature of the Effect, Possible Host Mediation, and Pharmacokinetic Significance

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ABSTRACT

Studies are described that sought the basis for a discrepancy in values for a key kinetic parameter of methotrexate transport (influx \(V_{\text{max}}\)) in L1210 cells derived alternately from biochemical or pharmacokinetic measurements. Our results show that, within a short period of time following administration of a therapeutic dose of methotrexate to leukemic mice, influx of this folate analogue measured in L1210 cells removed from these mice was markedly increased. Enhancement of \(^{3}\text{H}\)methotrexate influx in these cells was observed within 15 min of drug administration, was maximum (up to 3-fold) within 2 to 3 h, then decreased with time until 24 h when influx was at the control level. Measurements of \(^{3}\text{H}\)methotrexate influx in cells removed from drug-treated mice were made after a period of incubation in drug-free medium to allow for efflux of exchangeable drug. Enhanced influx of \(^{3}\text{H}\)methotrexate was accounted for by an increase in influx \(V_{\text{max}}\) (influx \(K_{m}\) was unchanged) and was further enhanced (to a total of 5-fold) by coadministration of leucovorin. Also, enhancement of influx of \(^{3}\text{H}\)methotrexate in L1210 cells abrogated by the following administration of 1-ß-D-arabinofuranosylcytidine at a therapeutically equivalent dose to leukemic mice or following exposure of these cells to methotrexate or methotrexate with leucovorin during growth in culture. Methotrexate therapy did not affect all transport systems, since the same therapy of leukemic mice had no effect on influx of the purine nucleoside analogue, 9-ß-D-arabinofuranosyl-2-fluoroadenine, in these same L1210 cells. These findings suggest that stimulation of \(^{3}\text{H}\)methotrexate influx in L1210 cells during therapy with this folate analogue was not due to transstimulation during exchange between folate compounds and was not related to the antiproliferative effect of methotrexate on these tumor cells. The coadministration of cycloheximide with methotrexate to leukemic mice at a dose which markedly inhibited \(^{3}\text{H}\)-leucine incorporation into L1210 cell protein severely diminished the stimulation of \(^{3}\text{H}\)methotrexate influx. However, in L1210 cells removed from leukemic mice treated with methotrexate, there was no increase compared to control cells in affinity labeling with the \(N\)-hydroxysuccinimide ester of \(^{3}\text{H}\)methotrexate. This suggested that the effect of cycloheximide was not on increased synthesis of folate transporter and that increased rate of translocation of folate transporter, rather than increased amount of transporter, accounted for the increase in \(^{3}\text{H}\)methotrexate influx.

Although the stimulation of \(^{3}\text{H}\)methotrexate influx observed in L1210 cells removed from mice pretreated with this antifolate did not seem to result from the increased content of folate transporter, the nullification of this effect by cycloheximide does suggest that there is a requirement for new protein synthesis elsewhere. Since the stimulation of \(^{3}\text{H}\)methotrexate influx could not be demonstrated by preexposure of growing L1210 cells in culture to methotrexate with or without leucovorin, a role for the host is indicated in the mediation of this effect. Pharmacokinetic implications of these results are discussed.

INTRODUCTION

Notions as to the pharmacological behavior of various antineoplastic agents in animal tumor models or in patients, particularly, in proliferative tissue compartments, have often relied upon (see Refs. 1 to 4 pertaining to antifolates) measurements of specific pharmacokinetic parameters in isolated cell systems in vitro. However, to what extent such measurements have significant predictive value has rarely been addressed. Our earlier studies of folate analogue transport in L1210 cells have, in fact, revealed marked discrepancies in values obtained for certain kinetic parameters for transport when derived either in vitro (reviewed in Ref. 4) or from pharmacokinetic measurements in vivo (5). Specifically, the value for \(V_{\text{max}}\) of methotrexate influx in these tumor cells when derived from the latter studies was substantially greater than values obtained by direct measurement of this kinetic parameter in vitro. Pharmacokinetic parameters for methotrexate and related folate analogues have useful applications (6–10) in the development of new, more efficacious analogues or improved clinical protocols. For this reason, we have examined the basis for the observed discrepancy in this parameter for methotrexate influx in these tumor cells. Surprisingly, our results show that, within a short period of time following administration of a therapeutic dose of methotrexate to L1210 leukemic mice, the transport of this folate analogue inward (influx) in L1210 cells was appreciably increased. This enhancement of influx (up to 3-fold) was quantitatively accounted for by an increase in influx \(V_{\text{max}}\). Also, further enhancement of \(^{3}\text{H}\)methotrexate influx (an additional 2–3-fold) occurred when a molar equivalent dose of leucovorin was coadministered with methotrexate. In contrast, the same effect was not obtained on another system mediating the influx of \(^{3}\text{H}\)F-Ara A in these LI210 cells. Other studies showed that the stimulation of \(^{3}\text{H}\)methotrexate influx did not occur following administration of a therapeutically equivalent dose of Ara C, thus suggesting that the stimulation seen with methotrexate was unrelated to the antiproliferative effect of this antifolate. The stimulation in methotrexate influx seen following methotrexate administration might be host mediated, since no effect of pretreatment could be demonstrated in L1210 cells growing in culture. We also found that the stimulation of influx did not involve an increase in the amount of an affinity-labeled surface protein but could be abrogated by the coadministration of cycloheximide.

MATERIALS AND METHODS

General. Harvesting of tumor cells from the peritoneal cavity and maintenance of L1210/V cells in (female C57BL/6 × DBA/2 F1) (hereafter called B6D2F1) mice (purchased from the National Cancer Institute) have been described (4). All folate compounds were prepared

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4 The abbreviations used are: F-Ara-A, 9-ß-D-arabinofuranosyl-2-fluoroadenine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NHS, N-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Ara C, 1-ß-D-arabinofuranosylcytidine; \(V_{\text{max}}\), kinetic parameter of maximum influx at 37°C; \(K_{e}\), efflux rate constant at 37°C; TCA, trichloroacetic acid.
as the sodium salt in neutral aqueous solution. Samples of methotrexate and calcium leucovorin were obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. [3',5',9-3H]-Methotrexate (specific activity, 10 to 20 Ci/mol) and [3H]F-Ara A (specific activity, 14 Ci/mmole) were purchased from Moravek Biochemicals, City of Industry, CA. This material was purified before use by reverse-phase high-performance liquid chromatography (11). Final purity was >99%. Cycloheximide was purchased from Sigma Biochemicals, St. Louis, MO. All other chemicals were reagent grade.

Transport Experiments. These experiments were carried out by incubating cells with radioactive methotrexate at 37°C in a buffered saline solution 5 with 7 mM D-glucose (4). As described earlier (12), a rapid sampling procedure modified from that of Wohlebner et al. (13) was used during these studies. Similar procedures were used for measurements of influx of [3H]-F-Ara A. In each experiment, cell suspensions were made in [carboxy-'4C]inulin (New England Nuclear, Boston, MA) and centrifuged in order to determine (14, 15) the amount of aqueous medium carried down with the cell pellet. The value was used to determine a correction of the total radioactivity in the pellet to yield intracellular radioactivity. Total water in the cell pellet (intracellular and extracellular) was determined by centrifugation with H2O (3, 15) added to the cell suspension. Subtraction of extracellular water (determined from the labeled inulin value) from the total water gave the intracellular volume. Values for intracellular water obtained in this manner were in agreement with values obtained from wet- and dry-weight determinations (4). Average values for intracellular water were 3.6 ± 0.4 (SE) ml per g dry weight. Methods used for the measurement of intracellular dihydrofolate reductase content have been provided in earlier reports (4).

Affinity Labeling of L1210 Cells. Adopting methods described previously (16, 17), [3H]methotrexate was acidified by the addition of HCl (10 μmol per 4 nmol of [3H]methotrexate), dried under vacuum, and converted to N-hydroxysuccinimide-[3H]methotrexate by dissolving in 1 ml of anhydrous dimethyl sulfoxide containing 20 mM EDC and 20 mM NHS and incubating the mixture for 2 h at room temperature. If not used immediately, the mixture was stored for as long as 1 h at 0-4°C. L1210 cells were washed with buffer containing 20 mM HEPES-225 mM sucrose, pH 6.8, with MgO and resuspended in the same buffer to a density of 5 x 10⁷ cells/ml. NHS-[3H]methotrexate was then added to a final concentration of 2 to 100 nM in the presence or absence of 1000 μM methotrexate and incubated for 5 min at 37°C. These cells were then centrifuged at 2000 x g for 5 min at 0-4°C and washed twice by resuspending twice in HEPES-buffered saline to a final density of 1 to 2 x 10⁷ cells/ml. Cells were then extracted with 1% Triton X-100 at 0-4°C, the extract was precipitated with cold acetone (1 volume), and the precipitate was solubilized in 2% sodium dodecyl sulfate for scintillation counting.

Other Procedures. [3H]Leucine incorporation into L1210 cell protein was determined by administering 8 μCi (2 μg) of this radioactive amino acid i.p. to leukemic mice and harvesting L1210 cells 10 min later in cold 0.17 M NH₄Cl. After standing for 5 min in ice, the cells were centrifuged at 2000 x g for 5 min and washed twice by resuspension and centrifugation with cold 0.14 M NaCl plus 0.01 M potassium phosphate. Cells resuspended in the same solution were treated with an equal volume of cold 20% TCA. The precipitate was washed 3 times by resuspension and centrifugation with cold 10% TCA. Final suspension was in 0.14 M NaCl plus 0.01 M potassium phosphate before scintillation counting.

RESULTS

Some Preliminary Considerations. Our own derivation of various kinetic parameters for [3H]methotrexate transport in tumor cells in vitro relied (4) upon measurements made in a modified Krebs-Ringer solution plus 7 mM D-glucose. Although the composition of this buffered solution was physiological, we felt that it was necessary for the purposes of this study to evaluate [3H]methotrexate transport in L1210 cells under these conditions vis-a-vis transport of the same perinate in a relevant biological fluid. For this purpose, we used undiluted ascites fluid obtained from the peritoneal cavity of leukemic mice. Values for the various kinetic parameters of [3H]methotrexate transport in L1210 cells derived in this fluid were essentially the same as those derived in our transport buffer. For influx Kₘ and Vₘ₃₀ and the Kᵦ, these values were 3.83 ± 0.5 μM, 7.34 ± 0.9 nmol/min/g dry weight, and 0.23 ± 0.03 min⁻¹ in murine ascitic fluid and 3.71 ± 0.4 μM, 8.09 ± 1.2 nmol/min/g dry weight, and 0.21 ± 0.02 min⁻¹ in transport buffer. Thus, it would seem that the higher level of [3H]methotrexate influx in L1210 cells derived from pharmacokinetic data in vivo does not merely reflect a difference in the composition of the environment external to the cell.

Folata Analogue Transport in L1210 Cells Obtained from Mice Treated with Methotrexate. Measurements of initial [3H]-methotrexate influx were made with L1210 cells obtained from leukemic mice before and at various times after the administration of 12 mg/kg methotrexate s.c. This dosage is within the therapeutic range for methotrexate used in mice (10). Cells were washed, resuspended in transport buffer, and then incubated at 37°C for 30 min to allow for efflux of intracellular exchangeable methotrexate. Cells from untreated mice were handled in the same manner. Both control and untreated L1210 cells were then washed, resuspended in transport buffer, and incubated at 37°C with 2 μM [3H]methotrexate. From data given in Fig. 1, it can be seen that the velocity of influx of [3H]methotrexate in cells from mice treated with this antifolate increased rapidly with time. An increase in influx was seen within 15 min after administration of drug. Maximum enhancement of influx (approximately 3-fold) occurred in cells removed from mice 2 to 3 h after treatment with methotrexate. In cells removed at times later than 3 h after treatment, influx of [3H]methotrexate gradually declined with time so that, at 24 h after treatment, influx in these cells was reduced to that level found in control cells. Results similar to these were also obtained when mice were treated with different doses (3 or 48 mg/kg s.c.) of methotrexate.

Time courses for the intracellular accumulation of [3H]methotrexate in control L1210 cells and in L1210 cells removed 2.5 h after treatment of leukemic mice with 12 mg/kg methotrexate.
are shown in Fig. 2. In these experiments, both the initial influx and steady-state level of exchangeable \([^{3}H]\)methotrexate were increased approximately 2.5- to 3-fold compared to control. Influx shown in the inset of Fig. 2 also documented different rates of unidirectional entry of \([^{3}H]\)methotrexate initially into both treated and control cells. Otherwise, the characteristics of the time course for accumulation of this folate analogue in each case were essentially the same. The lack of any effect on \([^{3}H]\)methotrexate efflux from L1210 cells following treatment of leukemic mice with this antifolate could be assumed from the equivalence of the increase in both parameters that was obtained in these cells. This was confirmed by the data shown in Fig. 3. Cells were removed from untreated mice and from mice 2.5 h after treatment with 12 mg/kg methotrexate and preloaded with \([^{3}H]\)methotrexate by a 10-min period of incubation at 37°C with 5 \(\mu\)mol of this radiolabeled folate analogue. It can be seen from the figure (Fig. 3) that the time course for efflux and the nonexchangeable level of intracellular drug were similar with control and treated cells. Also from the decay time analysis shown in the inset, essentially the same \(t_{1/2}\) can be derived for intracellular drug loss from each group of cells. Other data presented in Fig. 4 illustrate the dose dependency of the effect seen on \([^{3}H]\)methotrexate influx in L1210 cells removed from mice 3 h after treatment with this antifolate. The effect on influx observed after treatment was detected at doses well below 1 mg/kg and was maximum at a methotrexate dose of 2 to 5 mg/kg s.c.

Folate Analogue Influx in L1210 Cells from Mice Treated with Methotrexate and Leucovorin. The similar effect on \([^{3}H]\)methotrexate influx in L1210 cells from mice receiving widely different doses of methotrexate appeared to suggest that the effect on influx of this folate analogue was unrelated to the antiproliferative effect of this drug on these cells. We pursued this question further by simultaneously administering s.c. leucovorin with methotrexate at a dose of 12 mg/kg to leukemic mice. Based upon prior studies (6-8), this amount of administered leucovorin would be expected to completely nullify the antiproliferative effect of methotrexate on these leukemia cells. From data given in Fig. 5 it can be seen that, rather than abrogating the effect of administered methotrexate, coadministration of leucovorin resulted in further enhancement of \([^{3}H]\)methotrexate influx in cells obtained from antifolate-treated mice. The data in this figure (Fig. 5) also show that the administration of leucovorin, itself, had no effect on \([^{3}H]\)methotrexate influx in these cells. We also sought to examine the generality of this effect on methotrexate influx for other transport systems in L1210 cells. For this purpose we selected a system mediating influx of adenosine and I Ara A that we had (12) characterized in L1210 cells. In contrast to our results on \([^{3}H]\)methotrexate influx, it was shown that influx of the purine nucleoside analogue, \([^{3}H]\)F-Ara A (data not shown), was not stimulated in

**Fig. 2.** Time courses for accumulation of \([^{3}H]\)methotrexate by L1210 cells removed from leukemic mice before and 3 h after treatment with 12 mg/kg methotrexate s.c. See text and legend of Fig. 1 for experimental details. Inset, initial time course. Points, average of 3 experiments (SE <±1%). **MTX**, methotrexate; **FAH\(_2\)**, reductase, folate-H\(_2\) reductase.

**Fig. 3.** Time courses for efflux of intracellular \([^{3}H]\)methotrexate by L1210 cells removed from leukemic mice before and 3 h after treatment with 12 mg/kg methotrexate s.c. Cells treated as described in the legend of Fig. 1 were preloaded with \([^{3}H]\)methotrexate at a concentration of 5 \(\mu\)mol by a 20-min incubation in transport buffer. Cells were then washed (0-4°C), resuspended in transport buffer, and incubated at 37°C. Additional details are provided in the text. Inset, decay time analysis of efflux data. Points, average of 3 experiments (SE <±13%). **MTX**, methotrexate.

**Fig. 4.** Methotrexate dose dependence for enhancement of \([^{3}H]\)methotrexate influx in L1210 cells obtained from leukemic mice. Experimental details as given in the text and legend of Fig. 1. Points, average of 3 experiments (SE <±19%). **MTX**, methotrexate.

**Fig. 5.** Time courses for intracellular accumulation of \([^{3}H]\)methotrexate by L1210 cells removed from leukemic mice before and 3 h after treatment with 12 mg/kg methotrexate and 12 mg/kg leucovorin s.c. Experimental details are provided in the text and legend of Fig. 1. Points, average of 4 experiments (SE <±14%). **MTX**, methotrexate; **CF**, leucovorin; **FAH\(_2\)**, reductase, folate-H\(_2\) reductase.
L1210 cells removed from mice after treatment with methotrexate and leucovorin.

Kinetic Analysis of Effects of Methotrexate, Leucovorin, or Ara C on [3H]Methotrexate Influx in Vivo. A comparison of data for kinetic parameters of [3H]methotrexate influx in L1210 cells obtained from mice before and after treatment with methotrexate and/or leucovorin is summarized in Table 1. Prior treatment with methotrexate had no effect on the K_m for [3H]-methotrexate influx. However, there was an increase in influx V_max proportional to the effect seen on initial influx of [3H]-methotrexate. Also, the sole effect of coadministration of leucovorin was a further increase in influx V_max. The results already described above (and Figs. 4 and 5) strongly argued against the notion that the effect seen on [3H]methotrexate influx in L1210 cells was in some way related to a suppression of proliferation described above (and Figs. 4 and 5) strongly argued against the notion that the effect seen on [3H]methotrexate influx in L1210 cells was in some way related to a suppression of proliferation described above (and Figs. 4 and 5) strongly argued against the notion that the effect seen on [3H]methotrexate influx in L1210 cells was in some way related to a suppression of proliferation. We obtained additional support for this conclusion by examining [3H]methotrexate influx in L1210 cells removed from mice treated with another cytotoxic agent. Influx of this analogue was measured in cells removed from mice 2.5 h after Ara-C was given at a dose (30 mg/kg) therapeutically equivalent to 12 mg/kg methotrexate. From data also given in Table 1, it can be seen that both parameters of [3H]methotrexate influx were unaltered in these cells when compared to untreated controls.

Folate Analogue Influx in L1210 Cells Exposed to Methotrexate in Vitro. We sought to reproduce in vitro the effect of prior treatment of leukemic mice with methotrexate on the influx of [3H]methotrexate in L1210 cells. Suspensions of proliferating L1210 cells in cell culture were exposed for as long as 3 h to 10 μM leucovorin. As in the in vivo experiments, treated and control cells were harvested, washed, and resuspended in transport buffer for a 30-min period of incubation at 37°C to allow for efflux of exchangeable drug from treated cells. Following resuspension in transport buffer with 2 μM [3H]methotrexate, there was no difference found (data not shown) in the initial velocity of influx between treated and control cells. This experiment was repeated several times with different concentrations of methotrexate and leucovorin in the range of 2 to 10 μM with the same result.

Effect of Cycloheximide on Antifolate Enhancement of [3H]-Methotrexate Influx in in Vivo-derived L1210 Cells. A possible basis for the increase in influx V_max associated with the enhancement of [3H]methotrexate influx in cells obtained from animals pretreated with this antifolate is an increase in synthesis of the reduced folate/methotrexate transporter in these cells. For this reason, we examined the effect of coadministered cycloheximide on the stimulation of [3H]methotrexate influx by this antifolate and leucovorin given 2.5 h earlier. We used a dose of 35 mg/kg cycloheximide i.p. given simultaneously with 12 mg/kg methotrexate and leucovorin. This dose of cycloheximide reduced [3H]leucine incorporation (data not shown) into acid-insoluble components of L1210 cells by 90 to 95% 30 min later. Two and one-half h after cycloheximide administration [3H]-leucine incorporation was still suppressed by 85%. From data given in Fig. 6, it can be seen that this dose of cycloheximide administered with the folate compounds markedly reduced the level of enhancement of [3H]methotrexate influx in those cells obtained from these treated mice. The data also show that cycloheximide had no effect on [3H]methotrexate influx in L1210 cells obtained from control mice. We also observed that treatment of leukemic mice with 12 mg/kg methotrexate, itself, reduced [3H]leucine incorporation into L1210 cells 2.5 h later by about 35% (data not shown).

Affinity Labeling with N-Hydroxysuccinimide-[3H]Methotrexate of L1210 Cells from Animals before and after Treatment with Methotrexate/Leucovorin. In view of the results obtained (Fig. 6) showing that cycloheximide abrogated the folate analogue-induced enhancement of [3H]methotrexate influx, additional evidence was sought for the notion that increased folate transporter synthesis may explain the effect seen. In these experiments we used an affinity label, N-hydroxysuccinimide ester of [3H]methotrexate (16, 17), for specifically labeling the folate transporter of L1210 cells obtained from control mice and mice 2.5 h after treatment with 12 mg/kg methotrexate and leucovorin. Since freely exchangeable folate compounds entering the external compartment will interfere with affinity labeling by this ester, we allowed all exchangeable methotrexate to efflux first from L1210 cells obtained from treated animals and control animals by resuspending cells twice in drug-free medium for 20 min at 37°C followed by extensive washing of the cells. These cells were then exposed (16, 17) in a nonionic buffer (HEPES-Mg-sucrose) to varying concentrations of the affinity label for 10 min at 23°C in the presence and absence of a "flooding" concentration (1 mM) of methotrexate. The difference in affinity labeling obtained in the presence and absence of 1 mM methotrexate delineated (16, 17) the specific labeling of the folate transporter from nonspecific binding at the cell surface. Contrary to what we had expected, the data given in Fig. 7 show that a similar amount of specifically labeled protein was present on the surface of cells obtained from either treated or control animals.

Table 1  Kinetic properties of [3H]methotrexate influx in L1210 cells harvested from control mice and 3 h after treatment of mice with methotrexate and/or leucovorin or Ara C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Influx (K_m)</th>
<th>Influx (V_max)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μM)</td>
<td>(nmol/min/g dry wt)</td>
</tr>
<tr>
<td>Control</td>
<td>3.86 ± 0.8</td>
<td>7.12 ± 1.11</td>
</tr>
<tr>
<td>MTX (12 mg/kg)</td>
<td>3.98 ± 1.3</td>
<td>20.3 ± 3.3</td>
</tr>
<tr>
<td>Leucovorin (12 mg/kg)</td>
<td>3.52 ± 0.6</td>
<td>7.4 ± 1.2</td>
</tr>
<tr>
<td>MTX + leucovorin</td>
<td>3.94 ± 0.6</td>
<td>37.2 ± 5.4</td>
</tr>
<tr>
<td>Ara C (30 mg/kg)</td>
<td>3.46 ± 0.5</td>
<td>6.92 ± 1.1</td>
</tr>
</tbody>
</table>

* Mice were treated s.c. 5 days after i.p. implant of 10^6 L1210 cells.
* Mean ± SE of the average of 3 separate experiments.

Fig. 6. The effect of cycloheximide on the enhancement of [3H]methotrexate influx in L1210 cells removed from leukemic mice 2.5 h after treatment with 12 mg/kg methotrexate and leucovorin. Thirty-five mg/kg of cycloheximide (CHX) were administered i.p. simultaneously with methotrexate (MTX) and leucovorin. [3H]Methotrexate was used at 0.5 μM for transport measurements. Additional experimental details are given in the text and legend of Fig. 1. Points, average of 3 experiments (SE <14%).
cells after the efflux period that was used prior to measurements of metabolites inside these cells does not appear to explain the effect observed. In light of the results obtained with cycloheximide coadministered with methotrexate, the results of the affinity-labeling experiments were unexpected. These results (Fig. 7) suggest that the enhancement of influx of $[^3H]$methotrexate in L1210 cells obtained in treated mice was not a consequence, either by increased synthesis or reduced turnover, of increased amounts of folate transporter. The only remaining possibility is that enhanced influx of $[^3H]$methotrexate in these cells was a consequence of increased rate of carrier translocation.

The inability to obtain stimulation of $[^3H]$methotrexate influx in L1210 cells by preexposure to methotrexate plus leucovorin during growth in vitro was also unexpected. This finding indicated that the effect of methotrexate on these cells may in some way be dependent upon drug-related events taking place in some way elsewhere in the host.

The ability to obtain stimulation of $[^3H]$methotrexate influx in L1210 cells by preexposure to methotrexate plus leucovorin during growth in vitro was at least in part, host mediated. In other words a relevant effect of administered antifolate may occur at some site in the animal other than the tumor cells. This, in turn, may mediate the stimulation of $[^3H]$methotrexate influx in L1210 cells. Thus, the ability of cycloheximide to abrogate the stimulation of $[^3H]$methotrexate influx in L1210 cells implies a requirement for new protein synthesis, not of the transporter itself, but for the evidence suggesting that the effect on $[^3H]$methotrexate influx in L1210 cells may in some way be dependent upon drug-related events taking place in some way elsewhere in the host.

The data obtained in these studies showed that a marked enhancement of $[^3H]$methotrexate influx and a corresponding increase in the value for influx $V_{\text{max}}$ occurred in L1210 cells following the administration of a therapeutic dose of methotrexate to leukemic mice. The effect on influx in these cells was observed almost immediately after methotrexate administration but was not maximum until 2 to 3 h later. Findings documenting an effect on influx $V_{\text{max}}$ with no change on influx $K_m$ associate the stimulation observed on $[^3H]$methotrexate influx with some alteration of the classical system transporting reduced folate and folate analogues in these cells. Three lines of evidence appear to suggest that the effect observed in L1210 cells was not an indirect result of the antiproliferative effects of methotrexate on these cells. (a) The magnitude of the effect observed did not vary with dosage of methotrexate over a wide therapeutic range. (b) The effect on influx observed was not nullified by a simultaneously administered dose of leucovorin. The coadministration of leucovorin, which had no effect alone, actually enhanced the effect of methotrexate, thus suggesting that the antiproliferative action of methotrexate was really limiting to the magnitude of the enhancement. (c) An effect on $[^3H]$methotrexate influx was not observed in L1210 cells removed from mice following the administration of a therapeutically equivalent dose of Ara C. Enhanced influx of $[^3H]$methotrexate as a consequence of exchange with intracellular folate compound or the presence of methotrexate polyglutamates inside the cell can also be ruled out. Although residual amounts of administered methotrexate may remain in L1210 cells after the efflux period that was used prior to measurements of $[^3H]$methotrexate influx, it has been shown that methotrexate is ineffective (18) as a transstimulator of folate compound influx by this system. In addition, most or all of any residual methotrexate remaining after the prolonged efflux period would be bound to dihydrofolate reductase. Also, $[^3H]$methotrexate influx was not stimulated in L1210 cells preexposed to methotrexate in vitro or to leucovorin alone in vivo or in vitro. Since intracellular leucovorin is an effective transstimulator (18, 19) of folate compound transport, this would suggest that the efflux period utilized to eliminate exchangeable preloaded folate compound from these cells was adequate for this purpose. Moreover, both folate compounds would be expected to be polyglutamated in L1210 cells both in vitro and in vivo. Since only methotrexate exposure in vivo resulted in stimulation of $[^3H]$methotrexate influx in L1210 cells, the mere presence of these metabolites inside these cells does not appear to explain the effect observed.

The inability to obtain stimulation of $[^3H]$methotrexate influx in L1210 cells by preexposure to methotrexate plus leucovorin during growth in vitro was also unexpected. This finding indicated that the effect of methotrexate on these cells may in some way be dependent upon drug-related events taking place in some way elsewhere in the host.

The coadministration of leucovorin, which had no effect alone, actually enhanced the effect of methotrexate, thus suggesting that the antiproliferative effects of methotrexate on these tumor cells. Perhaps of greater interest was the evidence suggesting that the effect on $[^3H]$methotrexate influx in L1210 cells may in some way be dependent upon drug-related events taking place in some way elsewhere in the host.

The inability to obtain stimulation of $[^3H]$methotrexate influx in L1210 cells by preexposure to methotrexate plus leucovorin during growth in vitro was also unexpected. This finding indicated to us that stimulation of $[^3H]$methotrexate influx in vivo was, at least in part, host mediated. In other words a relevant effect of administered antifolate may occur at some site in the animal other than the tumor cells. This, in turn, may mediate the stimulation of $[^3H]$methotrexate influx in L1210 cells. Thus, the ability of cycloheximide to abrogate the stimulation of $[^3H]$methotrexate influx in L1210 cells implies a requirement for new protein synthesis, not of the transporter itself, but as part of the host mediation of this effect.

It was of some interest, as well, to note that treatment of leukemic mice with methotrexate had no effect on another transport system studied for the purpose of examining the generality of the phenomenon observed with $[^3H]$methotrexate transport. This second system mediates (12) the influx of adenosine and of the purine nucleoside analogue, F-Ara A. While it is still possible that other transport systems may be affected in a manner similar to that shown for the classical reduced folate/methotrexate system, it would seem that this effect in L1210 cells is not shared by all transport systems.

In conclusion, we noted in these studies that the administration of methotrexate to mice bearing the L1210 leukemia has a marked effect on $[^3H]$methotrexate influx mediated by the classical system transporting folate coenzyme and folate analogues in these tumor cells. The significance of these results in respect to the pharmacokinetics of folate analogues in this tissue compartment seems apparent. Rather than a condition of constancy for all parameters of $[^3H]$methotrexate transport in L1210 cells in vivo, there are therapeutically induced dynamics associated with a major property of $[^3H]$methotrexate influx ($V_{\text{max}}$). The value for influx $V_{\text{max}}$ in L1210 cells varied over a 3-fold range after methotrexate treatment and a 5-fold range after methotrexate and leucovorin treatment beginning immediately after their administration to leukemic mice and lasting for a period of 24 h. These effects almost certainly
contribute to the profile for accumulation and net loss of intracellular drug in these cells during this period of time.

REFERENCES

Enhancement of Folate Analogue Transport Inward in L1210 Cells during Methotrexate Therapy of Leukemic Mice: Evidence of the Nature of the Effect, Possible Host Mediation, and Pharmacokinetic Significance

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