Extent of the Requirement for Folate Transport by L1210 Cells for Growth and Leukemogenesis in Vivo

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ABSTRACT

In these studies the extent of the requirement for 5-methyltetrahydrofolate by L1210 cells for growth and leukemogenesis in vivo was addressed from the aspect of its cellular membrane transport. Growth characteristics and leukemogenesis in vivo were determined for parental and methotrexate-resistant L1210 cell variants with reduced capacity for folate coenzyme transport inward. These variants exhibited 6-, 16-, and 100-fold reductions as compared to parental cells in influx V_{max} for the high-affinity system transporting 5-substituted reduced folates and methotrexate. They also exhibited reduced saturated influx for methotrexate (3-fold higher K_{m}), but not for influx of 5-formyltetrahydrofolate or 5-methyltetrahydrofolate. The reduced influx capacity in these variants correlated with their increased requirement for reduced folates during growth in vitro and with the ability of the variants to proliferate and develop leukemia in vivo. Lack of growth potential in vivo for one variant appears to reflect the inability for net intracellular accumulation of reduced folate per se, since growth of this variant could be restored by treatment of mice with folic acid, but not with 5-methyltetrahydrofolate or 5-formyltetrahydrofolate, and following reversion to a more transport-proficient phenotype.

INTRODUCTION

Although it is tacitly assumed that some capacity for intracellular accumulation of 5-methyltetrahydrofolate via the high-affinity reduced folate-methotrexate transport system (1-4) is normally required by tumor cells for growth and survival in vivo, the extent of this requirement has never been documented. Moreover based upon the following considerations, it is likely that influx capacities for this transport system are expressed in many tumor cells to levels considerably beyond that normally required to maintain adequate intracellular levels of reduced folates during growth in the environment of the host. For instance influx capacities (V_{max}) for this system recorded in our own studies (5-10), or reported elsewhere in the literature (11-22), were found to vary substantially (10- to 12-fold) among a large variety of animal and human tumor cells with similar proliferative potential. Also we had derived (9, 10) methotrexate-resistant variants of the L1210 cell in vivo that exhibit normal proliferative potential, but have as much as a 5- to 7-fold reduction in values for influx V_{max} for 5-substituted reduced folates. Although many of the other tumor cells characterized in these earlier studies were in vitro derived, based upon other studies from our laboratory (5), it would appear that characteristics shown for this transport system in these cells most likely closely reflect those existing in the same cells from the tumor of origin.

Knowledge as to the extent of this requirement for folate coenzyme transport for tumor growth in vivo is of some significance. In the first place, inferentially it might provide some information as to the inherent ability in tumor cells for salvage of nucleosides or nucleobases, since this source of nucleic acid precursors would at least partially alleviate the need for folate-dependent macromolecular synthesis. Secondly it would provide some indication as to the maximum degree to which folate analogue resistance might occur in vivo by a reduction of mediated influx of these analogues by this system without compromising cell viability or growth potential.

We now describe a limited study addressing this issue made possible by the availability in our laboratory of a group of well characterized transport-altered, methotrexate-resistant variants of the L1210 cell. These variants exhibit varying reductions in influx V_{max} for the high-affinity system transporting reduced folates and methotrexate which correlate with their ability for growth in vitro and growth and leukemogenesis in vivo. Evidence is also provided which suggests that this variation in growth potential reflects the ability for net intracellular accumulation of reduced folates per se. Based upon these studies we estimated a minimum capacity for folate coenzyme influx in L1210 cells which will support maximum growth in vivo.

MATERIALS AND METHODS

[3',5',9',A-H]Methotrexate with a specific activity of 10–20 Ci/mmol, and d,L-[3',5',9',A-H]-5-formyltetrahydrofolate with a specific activity of 3 Ci/mmol were purchased from Moravek Biochemicals, City of Industry, CA. Samples of these materials were purified by high-performance liquid chromatography (23) prior to use. By rechromatography, radiolabeled purity was found to be greater than 98%. Folic acid was purchased from Sigma Chemical Co., St. Louis, MO, and d,L-[5-formyltetrahydrofolate, d,L-5-methyltetrahydrofolate, and methotrexate were provided by the Drug Synthesis and Procurement Branch, Division of Cancer Treatment, National Cancer Institute. Parental (L1210/V/Ci) and variant (L1210/R1, L1210/R24, and L1210/R25) L1210 cells were maintained in cell culture, and cells were harvested for experiments during log phase growth in RPM1 medium supplemented with 10% fetal calf serum containing 1% glutamine, 1% sodium pyruvate, and 0.05 mM mercaptopetanol.

Cancer Research. 1985, 45(20), 4732-4734.

*Supported in part by Grants CA 08748, CA 18856, and CA 22764 from the National Cancer Institute, and by Grant CH-26 from the American Cancer Society and the Elsa U. Pardee Foundation.

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Received 2/25/85; revised 5/20/85; accepted 6/13/85.
cells for efflux measurements, the absence of methotrexate polyglutama-
tes was verified by high-performance liquid chromatography (23). Cell
extracts were prepared by boiling cells in 0.01 m potassium phosphate
plus 0.14 m NaCl. Essentially all (>99%) of the radioactivity eluted as
authentic methotrexate. As described in detail earlier (5), a rapid sampling
procedure modified from that of Wohlheuter et al. (24) was used during
these studies. In each experiment cell suspensions were made in [car-
boxy-¹⁴C]inulin (New England Nuclear, Boston, MA) and then centrifuged
in order to determine (25, 26) the amount of aqueous medium which was
carried down and trapped in the cell pellet. With this measurement values
for intracellular water were derived (26) which approximated 3.6 ± 0.4
(SE) ml for all of the cell lines used in this study.

Maintenance, transplantation, and harvesting of tumor cells from the
peritoneal cavity of C57BL × DBA/2 F₁ (hereafter called BDF₂, mice
(Sprague-Dawley, Madison, WI) have been described (3, 10). Folate
compounds used for injection during in vivo experiments were prepared
as the sodium salt in neutral aqueous solution.

RESULTS AND DISCUSSION

Some properties of parental and variant L1210 cells are sum-
marized in Tables 1 and 2. The variant cells exhibit approximately
13-, 75-, and 250-fold increases (Table 2) in resistance to meth-
otrexate, respectively. This can be accounted for in part (Table
1) by a reduction in influx saturability (increased Kᵢₗ) for [³H]-
methotrexate as well as reduced influx capacity (Vₘₜ). The three
variations show 3-fold increases in Kᵢₗ for methotrexate compared
to parental cells, but not for [³H]-5-formyltetrahydrofolic acid. Also,
influx capacities for this system using either [³H]methotrexate or
[³H]-5-formyltetrahydrofolic acid as substrates are reduced 6-fold
(R1), 16-fold (R24), or 100-fold (R25). Efflux properties of these
variants are unaltered from that found in parental cells. Moreover,
levels of dihydrofolic reductase, as well as their inhibition by
antifolates (data not shown), are the same in variant and parental
cells.

From data presented in Table 2, it can be seen that the variant
cells during growth in vitro exhibit increased requirements for 5-
formyltetrahydrofolic acid as the sole folate source. This folate was
used as a model for the major circulating plasma folate, 5-
methyltetrahydrofolic acid. The increase in requirement for this re-
duced folate by two of the variants (R1 and R24) compared to
parental L1210 cells was inversely proportional to the relative
influx capacity needed for reduced folates exhibited by each variant
compared to parental cells. This proportionality was unexpected,
but was actually predicted from Michaelis-Menten theory
(see Ref. 3), since all of the concentrations of 5-formyltetrahy-
drofolic acid used are below the value for influx Kᵢₗ of this reduced
folate. Growth of R25 cells, however, did not occur at limiting
concentrations of this folate, and a value for E₀₅ (50% effective
concentration) could not be obtained. In contrast to these results
both the variant and parental cells exhibited similar requirements
for folic acid when used as the sole folate source.

During other studies it was found that not all of the variant
cells examined exhibited potential for growth and leukemogen-
esis in vivo. These results are shown in Table 3. When the same
number of R1, R24, or parental L1210 cells were transplanted
into mice, these grew rapidly and these animals developed
leukemia and died with similar survival times (7.3 ± 1.7). Examina-
tion of variant cells derived from these mice con-
irmed (data not shown) that they still exhibited transport prop-
erties typical of R1 and R24 variants. In contrast mice implanted
with the same number of R25 cells usually did not develop
leukemia. However, if mice transplanted with this variant were
also treated repeatedly with folic acid, growth and leukemogen-
esis did occur. At doses of 20 mg folic acid/kg, survival times
were the same as for mice implanted with parental cells. As the
dosage of folic acid was decreased, the survival time of treated
animals increased, so that, at a dose of 1 mg/kg no leukemia
developed. In contrast to these results mice given 5-methyltetra-
drofolic acid or 5-formyltetrahydrofolic acid (data not shown) at
1 or 10 mg/kg did not develop leukemia.

Among the animals implanted with large numbers of R25 cells
an occasional animal (1 of 15) eventually did develop leukemia
after approximately 3–4 weeks. Reimplantation of these cells
into mice resulted in rapid ascites development and death from
leukemia in approximately 8 days, as in the case of mice trans-
planted with the same number of parental cells. Biochemical
examination of these cells revealed (data not shown) influx
kinetics for [³H]methotrexate and [³H]-5-formyltetrahydrofolic
acid. Characteristics of [³H]-5-formyltetrahydrofolic acid and [³H]methotrexate transport in
parental and variant L1210 cells

Cells were harvested during late logarithmic phase of growth, washed once in
cold (0°C) 0.14 m NaCl plus 0.01 m potassium phosphate buffer (pH 7.4),
and resuspended in transport buffer (3) without serum. Initial influx measurements were
made at 37°C by varying substrate concentrations (3). Values for Kᵢₗ and Vₘₜ were
determined from reciprocal plots of the data (V₀/[drug]ᵢₗ), in which V₀ is exterior. Efflux
was measured by loading cells at 37°C for 60 min in the presence of 5 µM (L1210),
20 µM (R1), 100 µM (R24), or 200 µM (R25) [³H]methotrexate. The cells were then
washed with cold (0°C) phosphate-buffered saline and resuspended in transport
medium. Losses of intracellular drug were measured with time and values for the
rate constant were calculated from the slope of the decay-time plot for exchange-
able drug (3). Processing of samples and additional details are provided in earlier
reports (3, 5) and in the text. Data shown are an average of 4 to 5 separate
determinations ± SE.

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Kᵢₗ (³H]-5-HCO-THFₚ, b) (µM)</th>
<th>Kᵢₗ (³H]MTX (µM)</th>
<th>Vₘₜ (nmol/mg dry wt)</th>
<th>E₀₅ (³H]MTX (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>1.58 ± 0.3</td>
<td>3.98 ± 0.5</td>
<td>6.82 ± 0.6</td>
<td>0.192 ± 0.03</td>
</tr>
<tr>
<td>L1210/R1</td>
<td>1.39 ± 0.2</td>
<td>11.2 ± 2.3</td>
<td>1.09 ± 0.2</td>
<td>0.187 ± 0.04</td>
</tr>
<tr>
<td>L1210/R24</td>
<td>1.36 ± 0.2</td>
<td>13.3 ± 1.7</td>
<td>0.432 ± 0.03</td>
<td>0.187 ± 0.04</td>
</tr>
<tr>
<td>L1210/R25</td>
<td>1.45 ± 0.3</td>
<td>10.4 ± 2.1</td>
<td>0.073 ± 0.02</td>
<td>0.196 ± 0.04</td>
</tr>
</tbody>
</table>

* 5-HCO-THF, 5-formyltetrahydrofolic acid; MTX, methotrexate.

Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Relative influx capacity 5-HCO-THFₚ, b) (µM)</th>
<th>Growth inhibition, MTX (ng/ml)</th>
<th>Growth requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>100</td>
<td>2.8 ± 0.3</td>
<td>E₀₅ (ng/ml)</td>
</tr>
<tr>
<td>L1210/R1</td>
<td>15</td>
<td>41.3 ± 7.2</td>
<td>380 ± 33</td>
</tr>
<tr>
<td>L1210/R24</td>
<td>6</td>
<td>210 ± 32</td>
<td>490 ± 52</td>
</tr>
<tr>
<td>L1210/R25</td>
<td>1</td>
<td>684 ± 86</td>
<td>425 ± 63</td>
</tr>
</tbody>
</table>

* 5-HCO-THF, 5-formyltetrahydrofolic acid; MTX, methotrexate; E₀₅, 50% inhibitory concentration. Expressed as the natural diastereoisomer, L-5-formyltetrahydrofolic acid.

* No growth at limiting concentrations and values for E₀₅ can only be estimated to be between 200 and 500 nm.
which approximated those described (Table 1) for R1 cells. Thus it would appear that these cells originated from a revertant with a partially restored capacity for mediated accumulation of folate coenzymes. These results and the fact that proliferation in vivo could be restored in R25 cells by the administration of folic acid, but not of 5-methyltetrahydrofolate or 5-formyltetrahydrofolate, would suggest that the loss of proliferative potential and leukemogenesis in R25 cells was related only to an impairment of folate coenzyme transport inward.

The results of both in vitro and in vivo experiments showing differences among these variant cells in their requirement for folate coenzyme, but not folic acid, are in agreement with earlier notions (16, 19, 20, 26) of transport multiplicity for folate compounds. These earlier studies appeared to demonstrate two separate routes for folic acid entry, but not for entry of reduced folate coenzymes into L1210 cells.

A number of interesting conclusions are suggested by the results of this study. Although a "folate" requirement of tumor cells in vivo has already been inferred from the effects of antifolate drugs on these cells, this need is apparently satisfied by levels of specific folate transport inward much lower than hitherto imagined. For L1210 cells we estimate this to be no more than 0.3-0.4 nmol/min/g dry weight. If this value is applicable to other tumor cells, at least to those with similar growth and metabolic potential for folate interconversion, then it would appear that many tumor cells exhibit a substantial excess in capacity for mediated folate coenzyme accumulation. In these cases substantial reduction in influx capacity for folate coenzymes could occur in variant cells without compromising growth in vivo, and such cells would be resistant to classical folate analogues which share this mediated route of entry. Thus transport-altered resistance may be expected to be a common phenotype among folate analogue-resistant tumor cells, as has already been shown (9) for the L1210 leukemia during therapy in mice. Finally these results would clearly affirm the notion that L1210 cells, and perhaps other tumor cells, are unable to sustain in vivo any measure of growth and macromolecular synthesis by nucleoside or nucleobase salvage alone. This limitation may reflect inadequacies in the enzyme activities necessary for efficient salvage of specific nucleic acid precursors or, merely, insufficiencies in plasma concentrations of these precursors.

### Table 3

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Folic acid dose (mg/kg)</th>
<th>5-CH&lt;sub&gt;3&lt;/sub&gt;-THF dose&lt;sup&gt;a&lt;/sup&gt; (mg/kg)</th>
<th>Av. survival time (days ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>7.8 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/R1</td>
<td>7.4 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/R24</td>
<td>7.3 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/R25</td>
<td>&gt;45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/R26</td>
<td>2.5</td>
<td>13.4 ± 2</td>
<td></td>
</tr>
<tr>
<td>L1210/R27</td>
<td>5</td>
<td>11.3 ± 1</td>
<td></td>
</tr>
<tr>
<td>L1210/R28</td>
<td>10</td>
<td>9.2 ± 1</td>
<td></td>
</tr>
<tr>
<td>L1210/R29</td>
<td>20</td>
<td>7.8 ± 1</td>
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<tr>
<td>L1210/R30</td>
<td>&gt;45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210/R31</td>
<td>1</td>
<td>&gt;45</td>
<td></td>
</tr>
<tr>
<td>L1210/R32</td>
<td>10</td>
<td>&gt;45</td>
<td></td>
</tr>
<tr>
<td>L1210/R33</td>
<td>7.7 ± 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 5-CH<sub>3</sub>-THF, 5-methyltetrahydrofolate.

<sup>b</sup> Expressed as the natural diceresteromer, L-5-methyltetrahydrofolate.

### REFERENCES


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