Amethopterin Transport in Ehrlich Ascites Carcinoma and L1210 Cells

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SUMMARY

Accumulation of amethopterin by L1210 and Ehrlich ascites cells in vitro was mediated by a slow, temperature-sensitive, and partly saturable process. The structural specificity of the process could be measured by competition studies; differences were found between the two cell lines. At physiologic drug levels, i.e., those found in vivo during drug therapy, concentrative uptake of amethopterin was found in L1210 but not in Ehrlich cells. This may explain the sensitivity of L1210 to amethopterin and the lack of sensitivity of the Ehrlich cells. A correlation was found between affinity of several amethopterin derivatives for the L1210 transport process and the effectiveness of these compounds in inhibition of tumor growth in vivo.

INTRODUCTION

Amethopterin is a chemotherapeutic agent which inhibits growth of many animal leukemias, as shown by drug-promoted survival of tumor-bearing hosts. Presumably, the drug acts by inhibition of the enzyme dihydrofolate reductase, to which it is tightly bound (19, 24). Development of drug resistance was initially ascribed to elevated levels of the target enzyme (5, 10, 17), but such an elevation was not found in a variety of “naturally resistant” murine leukemias (20) nor in a murine leukemia cell line selected for drug resistance in culture (6). Considerable data has been reported which indicates that amethopterin transport is impaired in some drug-resistant cell lines. This was first reported by Fischer (6), who found a 14-fold decrease in drug uptake accompanied by development of drug-induced resistance in one cell line. A later report (25) also implicated a transport barrier in amethopterin-resistant sublines of a murine leukemia. A more extensive study involved examination of 10 transplantable mouse leukemias with a spectrum of response to amethopterin (15). In these lines, differing degrees of response to amethopterin in vivo associated with natural resistance, drug-induced resistance, and collateral sensitivity were all correlated with cellular capacity for drug uptake. Uptake was relatively high in drug-sensitive lines which responded to treatment with amethopterin, and correspondingly low in drug-resistant lines. Some drug-resistant human leukemias also demonstrated impaired uptake of the drug (11, 14).

The present report describes an investigation of the nature of amethopterin transport by Ehrlich and by L1210 cells. Jacquez (12) has recently reported the apparent absence of a transport system for amethopterin in Ehrlich ascites carcinoma cells and that the major portion of drug entry resulted from death of a fraction of the cells which then were freely permeable to amethopterin. The L1210 cell line is sensitive to the drug in vivo (7, 23), while the Ehrlich line is relatively insensitive (22). We measured the uptake of amethopterin, in both cell lines, as a function of time, temperature and extracellular drug level. The level generally employed corresponded to that found in vivo during standard drug therapy studies. The ability of several analogs to compete with amethopterin for transport was measured to define the specificity of the accumulation process.

MATERIALS AND METHODS

The L1210 ascitic tumor was obtained from DBA/2 or BDF1 mice on the 6th day following intraperitoneal inoculation with 10^6 tumor cells. Ehrlich cells were obtained from white male Swiss mice inoculated 6 days previously with 0.5 ml of undiluted ascitic fluid. Tumor-bearing mice were obtained from Mr. I. Wodinsky of Arthur D. Little, Inc., Cambridge, Massachusetts.

The methods used for isolation of animal leukemia cells have been described (15). Briefly, ascitic fluid was collected from animals, diluted with 2 volumes of an isotonic solution containing, per liter, 6 grams of NaCl, 6 grams of tris(hydroxymethyl) aminomethane, and 1 gram of Versene. The pH was adjusted to 7.6 at 0°C. The tumor cells were collected by centrifugation (150 × g for 5 minutes) and washed with isotonic saline. If enough red cells were present to impart a distinct pink color, cell preparations were freed from contaminating erythrocytes by brief osmotic shock. Some cell preparations were damaged by rapid freezing to −80°C followed by thawing, to allow measurements of transport by such cells. For drug transport studies, all cell preparations were resuspended in 12 volumes of 62 mM tris(hydroxymethyl) aminomethane (pH 7.2), 65 mM NaCl, 15 mM KCl, and 8 mM CaCl2, a mixture suggested by studies of Yunis et al. (26). Occasionally, Krebs-Ringer phosphate or bicarbonate buffers or tissue-culture preparations were substituted.

Aliquots containing 150 μl of cell suspensions were incubated
with amethopterin-3H, under the previously stated conditions, usually to a final level of 0.2 µM. In some experiments, nonradioactive amethopterin or amethopterin analogs were also added. Incubations were terminated by dilution of suspensions with 5 volumes of ice-cold incubation medium, and cells were collected by centrifugation at 1000 rpm for 30 seconds in a Misco model 5500 microcentrifuge (Microchemical Specialties, Berkeley, California). The resulting cell pellets were washed by resuspension in ice-cold medium, followed by centrifugation for 30 seconds. To measure efflux of labeled drug, some cell preparations were then reincubated in fresh medium and thereafter collected as before.

The intracellular radioactive drug was extracted by suspending the cells in 250 µl of 0.01 N acetic acid at 60°C for 10 minutes. Cellular debris, which contained negligible amounts of radioactivity, was removed, mixed with 10 ml of BBOT solution, and radioactivity determined with a Mark I Nuclear-Chicago scintillation counter. Chromatographic examination of cell extracts showed that no significant metabolism of amethopterin had occurred in either cell line during the incubations.

Capacity for concentrative uptake of cycloleucine and exclusion of inulin were used as measures of the metabolic state of cell preparations. Cells incubated for 15 minutes at 37°C in medium containing 0.1 mM of radioactive inulin or cycloleucine were washed as described above, and cellular radioactivity determined.

All data presented here are expressed in micromoles per kg of cell water, after correction for trapped extracellular fluid in cell pellets. For each cell preparation, wet weights were obtained after heating cell pellets at 100°C for 2 hours.

RESULTS

Measurement of Cellular Integrity by Inulin and Cycloleucine Transport

Under the conditions described, concentrative uptake of cycloleucine was found for both lines: approximately 3-fold for the Ehrlich cells and 3.5- to 4-fold for L1210. Addition of glucose (1 mg/ml) resulted in a 2- to 3-fold stimulation of concentration by both cell types. Cell preparations that failed to show concentrative uptake of cycloleucine were found to stain with trypan blue and consisted mainly of dead cells. After incubation in medium containing labeled inulin, followed by the washing procedure outlined above, both Ehrlich and L1210 cells contained less than 0.002 mM inulin. In contrast, cells previously damaged, by freeze-thawing, accumulated 0.06 mM inulin.

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Uptake of amethopterin by L1210 and Ehrlich cells was relatively slow compared with the almost instantaneous uptake we observed for labeled water, thiourea (Chart 1), purines, and pyrimidines. Maximum uptake of amethopterin required about 30 minutes (Chart 1), at which time an intracellular/extracellular drug distribution ratio of about 3 was found with L1210 cells, and a ratio of 0.6 with Ehrlich cells. Uptake of amethopterin was not affected by addition of glucose (0.1-1.0 mg/ml) and was inhibited 2- to 3-fold by use of phosphate-buffered medium. No advantage was gained by the use of bicarbonate-buffered medium, or by the use of any of several tissue-culture media.

The steady-state amethopterin levels reached (Chart 1) were not much altered by changes in the composition of the incubation medium. Omission of K+, increasing the K+ level to 60 mM (at the expense of Na+), or substituting TES buffer for all of the Na+ did not significantly affect the drug distribution ratio achieved by either cell type. Variation in the Ca++ level between 0 and 10 mM did not change the drug distribution level but increasing the Ca++ level to 20 mM, or substitution of 5 mM Versene for calcium chloride, resulted in a decrease of

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1 Amethopterin-3',5'-H (9170 mc/mnmole) was purchased from the Nuclear Chicago Corporation and purified by descending chromatography on Whatman No. 1 paper using 0.05 N sodium phosphate at pH 7 as the solvent. The principal UV-absorbing spot was cut out and the radioactivity was eluted with water and concentrated by lyophilization.

2 Drug analogs were supplied by Dr. J. W. Ruegsegger of the Lederle Laboratories, and purchased from Calbiochem Corporation.

3 Toluene, 600 ml, methyl Cellosolve, 400 ml, naphthalene, 60 gm, and BBOT (Packard Instrument Company), 4 gm.

4 Inulin and cycloleucine, both labeled with 14C in the carboxyl groups, were purchased from the New England Nuclear Corporation.

CHART 1. Uptake of labeled amethopterin by L1210 (O) and Ehrlich (•) cells, and uptake of thiourea (X) by either cell line as a function of incubation time. The medium contained 0.2 µM amethopterin or 0.5 µM thiourea, at 37°C.

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the drug distribution ratio, for both cell types, to 75% of control values. Addition of N-ethylmaleimide or 2,4-dinitrophenol at $10^{-3}$ M or of iodoacetate at $10^{-2}$ M decreased the amethopterin distribution ratio for both cell types to 30% of control values.

Uptake of amethopterin by both cell lines was equally temperature sensitive (Chart 2). As the external drug level was increased from 0.2 mM to 2 mM, uptake by both lines increased, but relative uptake fell (Table 1). Concentrative uptake by L1210 was found only at low external drug levels. The data of Chart 1 were obtained with 0.2 mM amethopterin in the medium. When drug levels of 0.2 mM or higher were used, uptake by both lines was temperature insensitive (Chart 2).

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug level in medium</th>
<th>Cellular distribution ratio*</th>
<th>Ratio L1210/Ehrlich</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 µM</td>
<td>2.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2 µM</td>
<td>1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>20 µM</td>
<td>0.32</td>
<td>0.13</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>0.11</td>
<td>0.065</td>
</tr>
<tr>
<td>2 mM</td>
<td>0.056</td>
<td>0.050</td>
</tr>
</tbody>
</table>

* Ratio of cellular level (reached after incubation for 30 minutes at 37°C) to extracellular level. Values represent averages of 3 measurements which differed by not more than ±10%.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound tested*</th>
<th>Leukemic cell line*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1210</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>12</td>
</tr>
<tr>
<td>3',5'-Dichloroamethopterin</td>
<td>3</td>
</tr>
<tr>
<td>N^10-Ethylaminopterin</td>
<td>6</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>14</td>
</tr>
<tr>
<td>Aspartyl aminopterin</td>
<td>15</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>18</td>
</tr>
<tr>
<td>9-Methyl amethopterin</td>
<td>10</td>
</tr>
<tr>
<td>Folic acid</td>
<td>80</td>
</tr>
<tr>
<td>N^5-Methyl folate</td>
<td>80</td>
</tr>
<tr>
<td>Pteroyl-glutamic acid</td>
<td>85</td>
</tr>
<tr>
<td>Pteric acid</td>
<td>85</td>
</tr>
<tr>
<td>Xanthopterin</td>
<td>85</td>
</tr>
</tbody>
</table>

* Cells were incubated for 15 minutes at 37°C in medium containing 0.2 mM labeled amethopterin + 20 µM specified test compounds.

**DISCUSSION**

The temperature sensitivity found for amethopterin uptake in L1210 and Ehrlich cells is generally associated with any slowly penetrating substrate, and does not, in itself, prove
that a specifically mediated process is involved, but the finding that temperature-sensitive uptake could only be demonstrated if the medium contained low drug levels strongly suggests the presence of a temperature-sensitive, saturable, mediated process capable of net drug concentration at low drug levels, and a temperature-insensitive diffusion process, which predominates at high drug levels.

The data of Table 1 suggest that amethopterin uptake in both Ehrlich cells and L1210 was partly saturable. Such a situation has also been described for amino acid uptake by diaphragm (1).

The structural requirements for inhibition of amethopterin uptake by L1210 and Ehrlich cells have been partly determined by competition studies. Uptake of labeled amethopterin was inhibited by several folate derivatives with an amino group in the 4 position of the pteridine ring, and by folic acid. This suggests affinity of these compounds for a common transport site (18, 21). Since 2,4-diaminofolate derivatives are tightly bound to dihydrofolic reductase (24), this enzyme might be thought to be the “common transport site.” The finding that folic acid competes with amethopterin-14C for the site argues against this since folic acid is not bound to dihydrofolic reductase (13). The competition between amethopterin and folic acid for entry into these cells might provide a partial explanation of the ability of the latter compound to reverse amethopterin toxicity (3, 16).

It is interesting to note that two amethopterin derivatives which strongly inhibited amethopterin entry into L1210 (dichloroamethopterin and N4-ethylaminopterin) were more effective than amethopterin against L1210 in vivo (23). This suggests to us that these analogs penetrate the cells more readily than amethopterin. Drug analogs showing poor competition for entry (aminopterin, aspartylaminopterin) were less effective against L1210 (23).

The relatively low capacity for amethopterin uptake by Ehrlich cells, as reported here, may be the basis for resistance of this line to amethopterin (22). A comparison of pertinent reports shows that levels of the drug-sensitive enzyme dihydrofolic reductase in Ehrlich cells (2) is essentially identical with the latter concentration, Hakala found optimal stimulation of amethopterin uptake in S-180. The lowering of steady-state cell amethopterin levels caused by N-ethylmaleimide, dinitrophenol, or iodoacetic acid suggest that uptake or retention of the drug may be energy-dependent.

Data presented here suggest to us that a specific mediation process is involved in cellular accumulation of amethopterin by many mammalian cell types, and that altered uptake of the drug is an important factor in drug resistance. Several lines of evidence support the concept of mediated amethopterin transport: amethopterin accumulation (a) was saturable, (b) showed structural specificity, (c) was impaired in some amethopterin-resistant mouse leukemias, and (d) was temperature-sensitive. Methods described here should be useful for evaluating the capacity for amethopterin accumulation by various cell lines. Competition studies as described here may also be useful in view of the apparent correlation between affinity of amethopterin analogs for the transport process and response of L1210 to the drug.

REFERENCES


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